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Role of the *gag* Polyprotein Precursor in Packaging and Maturation of Rous Sarcoma Virus Genomic RNA

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Rous sarcoma virus nucleocapsid protein (NC) has been shown by site-directed mutagenesis to be involved in viral RNA packaging and in the subsequent maturation of genomic RNA in the progeny viral particles. To investigate whether NC exerts these activities as a free protein or as a domain of the polyprotein precursor $Pr76^{gag}$, we have constructed several mutants unable to process $Pr76^{gag}$ and analyzed their properties in a transient-transfection assay of chicken embryo fibroblasts, the natural host of Rous sarcoma virus. A point mutation in the protease (PR) active site completely prevents $Pr76^{gag}$ processing. The full-length $Pr76^{gag}$ polyprotein is still able to package viral RNA, but cannot mature it. A shorter *gag* precursor polyprotein lacking the C-terminal PR domain, but retaining that of the NC protein, is, however, unable even to package viral RNA. This indicates that the NC protein can participate in packaging viral RNA only as part of a full-length $Pr76^{gag}$ and that the PR domain is, indirectly or directly, also involved in RNA packaging. These results also demonstrate that processing of $Pr76^{gag}$ is necessary for viral RNA dimerization.

All retroviruses express a gene encoding their structural proteins (*gag* gene). In Rous sarcoma virus (RSV) this gene is expressed by translation of the genomic 35S mRNA in the cytoplasm of the infected cells. The product is a polyprotein precursor of 76 kDa, $Pr76^{gag}$. This precursor gives rise, after processing, to five mature viral proteins: MA, the matrix-associated protein; p10, a protein of unknown function; CA, the major structural component of the capsid; NC, the nucleocapsid protein; and PR, the protease responsible for the stepwise proteolytic cleavage of the *gag* precursor (for reviews, see references 22 and 34). Another gene, *pol*, is expressed by translation of the genomic 35S mRNA, as a *gag-pol* polyprotein precursor $Pr180^{gag\text{-}pol}$, by a frameshift at the end of the *gag* gene.

The processing of the *gag* polyprotein precursor ($Pr76^{gag}$) requires activation of the protease PR and may occur either during (e.g., RSV) or after (e.g., murine leukemia virus) budding of the virus particle from the host cell membrane (reviewed in reference 4). It has been suggested that the activation of the proteolytic activity of PR is autocatalytic (2, 32), but how it is regulated, particularly with respect to its initiation, is unknown. Retroviral proteases, which are aspartic proteases, are found as dimers in their active form (32). Their active site is conserved between species; a mutation of Asp to Arg has been shown to abolish the activity of human immunodeficiency virus type 1 PR (13, 19).

The process of assembly of the retroviral particle in the infected cell begins with the formation, under the host cell membrane, of a core complex containing the *gag* and *gag-pol* polyprotein precursors, 35S viral RNA, and the tRNA primer of reverse transcriptase. Budding of the core through the membrane then takes place with either concomitant or subsequent maturation of the viral particle (3), a step that yields the diploid genome common to all retroviruses. Site-directed mutagenesis of the *gag* gene has shown that the retroviral NC protein is involved in packaging viral RNA into the particles (7, 9–12, 15, 28–30, 36). We have previously reported that this protein is also involved in the dimerization of the two 35S viral RNA molecules during the maturation step: the mutant Prc-1, which has a dipeptide (Val-Pro) insertion in the NC-proximal Cys-His box at position +7, is unable to completely mature the virion RNA (30).

According to the above model of virus assembly, packaging of viral RNA precedes $Pr76^{gag}$ processing, and therefore it appears that the NC protein functions as a domain of $Pr76^{gag}$ in packaging viral RNA. However, in the dimerization of the viral genomic RNA it could function in either the precursor or the mature form. To delineate the structural context of the functional domains of the NC protein involved in these two activities, we have constructed mutants unable to cleave the $Pr76^{gag}$. We did this by introducing a point mutation either in the active site of PR or at the cleavage site between NC and PR in $Pr76^{gag}$ (presumed to be the first point of proteolytic cleavage [36]). We have also deleted the PR protein, and, because this deletion inhibits the expression of the *gag-pol* polyprotein precursor, we constructed another mutant, defective only in *gag-pol* precursor production, to serve as a control.

All these mutants were characterized in a transient-transfection assay in the natural host for RSV, chicken embryo fibroblasts, for synthesis of $Pr76^{gag}$, release of viral particles, and their protein and RNA content; viruses produced in the transient-transfection assay were also characterized biologically for infectivity. The results indicate that a point mutation in the PR active site completely abolishes $Pr76^{gag}$ processing and that the unprocessed *gag* precursor polyprotein $Pr76^{gag}$ is capable of packaging viral RNA but not of maturing it. However, a shorter *gag* polyprotein, lacking the C-terminal PR domain but retaining the NC, is unable even to package viral RNA.

MATERIALS AND METHODS

Cell culture. Chicken embryo fibroblasts, prepared from CDI-EV-O eggs (Gs^- and Chf^-; Central Diergeneeskundig Institute, CDI Lelystad, The Netherlands) were grown in Dulbecco modified Eagle medium containing 5% fetal calf

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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 specified by the instructions accompanying the mutagenesis kit (Bio-Rad). *E. coli* DH5α was rendered competent as described previously (26). Plasmid DNAs were purified from either small or large cultures by the alkaline lysis method and, for transfection, were further purified by equilibrium density gradient centrifugation in cesium chloride-ethidium bromide (26).

Cloned DNAs. Plasmid pAPrc has already been described (30). It contains a nonpermuted copy of the provirus RSV Prague C strain. Plasmid pAsPrc is a *Sal*I-*Eco*RV subclone of pAPrc in pRB322 (30) containing the entire *gag* sequence. The mutations p15-1 and p3 were constructed in pBS*gag*, a 773-bp *Pst*I-*Eco*RI fragment cloned in the phagemid vector Bluescribe (+) (Stratagene, San Diego, Calif.). The two other mutations, U76 and CM5, were constructed in pBS*gag-p*, a 1,747-bp *Xba*I-*Bgl*II fragment cloned in Bluescribe (+).

Site-directed mutagenesis. The following oligonucleotides were synthesized on an Applied Biosystems 381A DNA synthesizer and purified as previously described (25): p15-1, 5′-GTCCGCTCCAGATCTCAACAGCGCGGT-3′; p3, 5′-GTCATCGCTCTAGAGACGCCAG-3′; CM5, 5′-CATCGC TACGAGAC-3′; and U76, 5′-AGTGAGAACAGTGGTTTA AACTATAGGTTTGTCAAGCGG-3′. For all mutants, the selection method described by Kunkel (20) was used, with some modification. Briefly, single-stranded uracil-containing template DNA was obtained by introducing the phagemid pBS*gag* or BS*gag-p* into *E. coli* CJ-236 (*dut ung*) and infecting the cells with helper phage M13K07, as described by Vieira and Messing (35). The mutagenic strand was synthesized as specified by the instructions accompanying the mutagenesis kit (Bio-Rad). The resulting double-stranded DNA was introduced into *E. coli* DH5α (*dut*$^+$ ung$^+$ RecA$^-$) by the $CaCl_2$ transformation method, and the bacteria were grown on LB-ampicillin plates (ampicillin was present at 100 μg/ml).

Introduction of the mutation was confirmed by the dideoxy-chain termination method of DNA sequencing by using avian myeloblastosis virus reverse transcriptase primed with a synthetic oligonucleotide complementary to the 3′ end of the RSV NC (38). The mutated fragments were cloned back into pAsPrc, and then the *Sal*I-*Eco*RV fragment of pAsPrc was introduced into pAPrc.

Transfection and infectivity. Chicken embryo fibroblasts, either freshly prepared or kept frozen in the presence of 15% glycerol, were used after two to seven passages. Transfection (30) and the infectivity test (29) were performed as described previously.

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 CA (p27) (27), as described previously (30).

Exogenous template reverse transcriptase activity. Exogenous template reverse transcriptase activity was measured as described previously (29).

Purification of viral RNA and Northern analysis. 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 were removed by centrifugation at 15,000 × *g* for 10 min at 4°C, and a portion of the sample was kept for immunoblotting analysis. Virus was pelleted through a cushion of 20% sucrose in NTE (0.1 M NaCl, 10 mM Tris hydrochloride [pH 8.0], 1 mM EDTA [pH 8.0]) by centrifugation for 1.5 h at 35,000 rpm (160,000 × *g*) at 4°C in a Beckman SW40 rotor. It was critical not to freeze the harvested medium before RNA extraction.

Medium was carefully removed by aspiration to avoid contamination of the viral pellet with culture medium. The virions were lysed in 100 mM NaCl–50 mM Tris (pH 7.5)–10 mM EDTA–1% sodium dodecyl sulfate–100 μg of proteinase K per ml–50 μg of yeast tRNA per ml at 37°C for 20 min. RNA was extracted twice with phenol-chloroform and once with chloroform and 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 DNaseI per ml at 37°C for 20 min to remove contaminating plasmid DNA. For Northern (RNA) analysis, viral RNA was analyzed by a nondenaturing Northern blot procedure as described previously (17, 18).

RESULTS

Construction of mutants. Figure 1 illustrates the construction of the four virus mutants. The aspartate residue of the active site of the protease was changed to an arginine (mutant p15-1), since such a change has been shown to inactivate human immunodeficiency virus type 1 PR (13, 19). To abolish cleavage at the NC-PR junction, we changed the first amino acid of the protease, a leucine, to an arginine (mutant p3). This change was expected to prevent recognition of the site by PR (31, 32). The protease was deleted from the *gag* precursor protein by replacing the first codon of the PR by a stop codon (mutant CM5). To prevent the expression of the *gag-pol* precursor protein, we disturbed the frameshift by mutating two nucleotides known to be important (14); a termination codon was also introduced to stop the expression of the *gag-pol* precursor protein should the frameshift still occur (mutant U76).

The mutant DNA clones were transfected into cultured chicken embryo fibroblasts and characterized in a transient-transfection assay for the following parameters: (i) synthesis of the *gag* gene precursor protein; (ii) release of viral particles and their protein content; (iii) viral RNA content of the virions; and (iv) infectivity of the virions produced in the transient-transfection assay.

Expression of the mutant *gag* precursor proteins in transfected cells. Viral mutant DNAs were transfected into chicken embryo fibroblasts, and after 60 h the cells from one dish were lysed, the proteins were immunoprecipitated with an anticapsid-specific polyclonal serum, and the recovered viral proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and detected by immunoblotting, all as described in Materials and Methods. All the mutants, except the deletion mutant of the protease, produced the stable viral *gag* precursor $Pr76^{gag}$ in approximately the same quantity as the wild type (Fig. 2). For the protease deletion mutant a smaller precursor was expected, but the background on the filter prevented the visualization of such a protein. The presence of mature CA is probably due to abortive and/or budding virus particles bound to the cell membrane.

Production of viral particles by mutants. Virions produced in the transfection were analyzed to determine whether maturation or packaging of viral *gag* proteins was affected by the mutations. The viral particles were purified as described in Materials and Methods and analyzed by immuno-

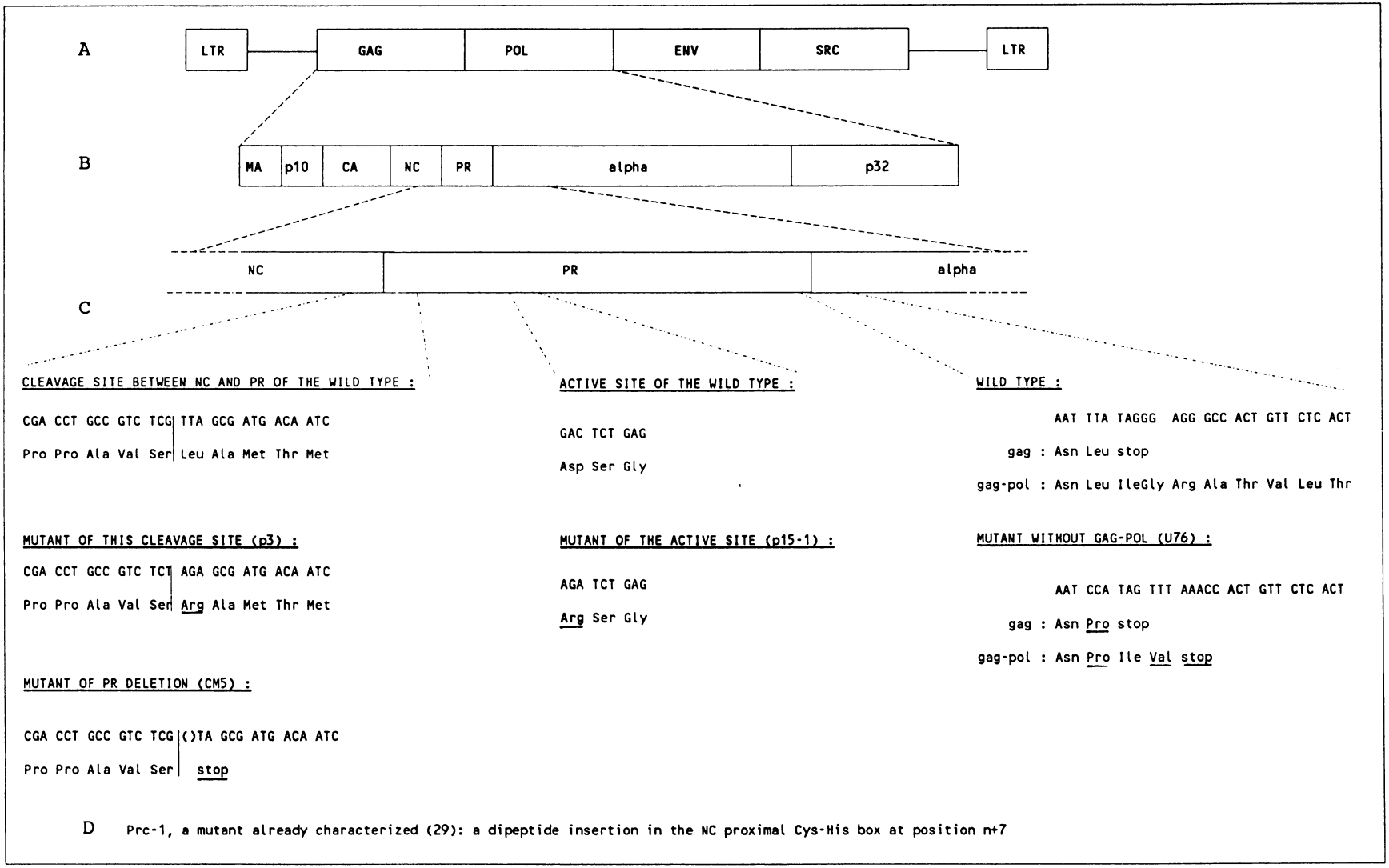

FIG. 1. Structure of the mutants constructed. (A) Complete genome of RSV Prc as a linear provirus. The regions encoding the *gag*, *pol*, *env*, and *src* genes are shown in boxes. (B) Enlargement of the *gag* gene. (C) Nucleic acid and amino acid sequences of the wild-type virus and the mutations introduced at the active site of the protease, at the NC-PR junction, and at the end of the protease. (D) Prc-1 mutant.

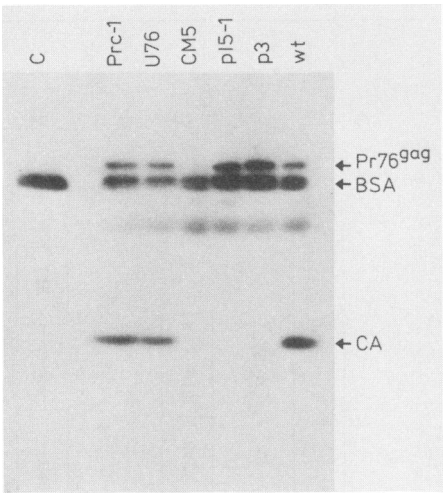

FIG. 2. Intracellular viral proteins of cells transfected with all the mutants. Cell lysates were immunoprecipitated with a polyclonal antibody against CA, following by protein A-Sepharose adsorption. The eluted proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (21) and detected by immunoblotting with anti-CA sera and ^{125}I-labeled protein A. Autoradiography was carried out for 40 h with an intensifying screen. Lanes: C, control transfection with no DNA; Prc-1, transfection with the mutant Prc-1 DNA (30); U76, CM5, p15-1, and p3, transfection with each of the mutant DNAs, respectively; wt, wild-type DNA transfection.

blotting with polyclonal anti-CA, anti-PR, or anti-MA sera. The results are shown in Fig. 3.

The mutant U76 produces viral particles with processed *gag* proteins, in the same quantity as the wild type. The mutant without protease (CM5) forms viral particles with lower efficiency (about half that of the wild type), but contains a smaller *gag* precursor protein of about 63 kDa, which corresponds to the predicted size for a *gag* precursor protein lacking the protease. Thus, deletion of the protease from the *gag* precursor protein does not abolish the formation of viral particles, but the precursor is not processed. The mutant with the Asp-to-Arg substitution in the active site of the protease (p15-1) forms viral particles with an unprocessed *gag* precursor protein. The yield is about the same as that of the wild type. Therefore, as expected, the protease is inactivated by this mutation. Furthermore, the lack of the *gag* precursor cleavage does not prevent the formation of viral particles, showing that processing of the precursor occurs after budding. The mutation in the cleavage site between NC and PR does not impair the formation of viral particles. These particles contain the full-length *gag* precursor protein and also a protein corresponding in size to the precursor of the protease deletion mutant. This smaller protein is not recognized by antibody against the protease, but is recognized by antibodies against the matrix or capsid proteins, showing that the C-terminal region of the *gag* precursor protein is missing. This suggests that this mutation does not fully abolish cleavage at a site between NC and PR. However, as no cleaved PR can be detected under these conditions, and as the smaller precursor does not undergo further processing, it is possible that the cleavage has not occurred at the same position as in the wild type.

Reverse transcriptase activity of the mutant viral particles. The active reverse transcriptase was quantitated in pelleted virions by the exogenous template assay as described in Materials and Methods.

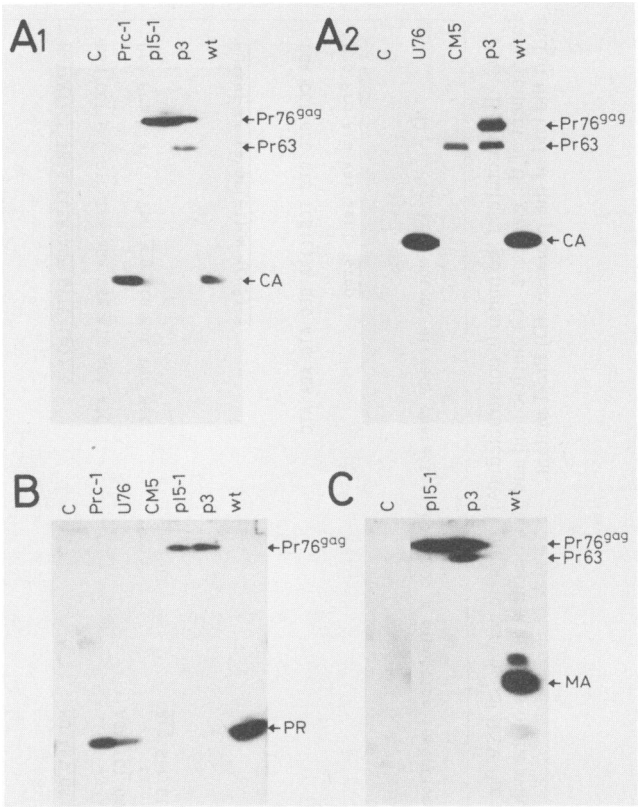

FIG. 3. Analysis of the mutant virion *gag*-encoding proteins. Virions produced by the transfected cells were purified as described in Materials and Methods. Viral proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by immunoblotting with polyclonal antibodies against RSV CA (A_1 and A_2 [duplicate experiments]), PR (B), or MA (C) and ^{125}I-labeled protein A. Autoradiography was carried out for 36 h (panel A) or 3 days (panels B and C). Lanes are the same as in Fig. 2.

Both the mutants that are unable to synthesize the *gag-pol* precursor (U76 and CM5) have, as expected, no reverse transcriptase activity. Additionally, no activity was detected in the two other mutants (p15-1 and p3) in which *gag* precursor cleavage is prevented (Fig. 4). This would also be expected if PR activity is necessary for the processing of the Pr180$^{gag\text{-}pol}$ precursor to yield active reverse transcriptase. Although the *gag-pol* precursor band is not visible in Fig. 2, in similar experiments its presence confirms the above interpretation (data not shown).

Viral RNA content of the mutant virions. To determine whether the mutations affected the packaging and/or the maturation of the viral RNA, we extracted nucleic acids from the virions released in the transient-transfection assay and analyzed them by Northern blotting under conditions which preserve the secondary and tertiary structure of the RNA (17). The blot was hybridized with a full-length RSV DNA probe (Fig. 5).

Mutant U76 is able to package the normal quantity of viral RNA and to dimerize it into 70S RNA. However, when the protease is deleted from the *gag* precursor protein (with the concomitant loss of the *gag-pol* precursor protein), no viral RNA is detected in the particles. Since the NC domain is still present in this mutant and since both protease mutants (p15-1 and p3) still package RNA, it would appear that it is not the lack of cleavage of NC from the precursor, but rather

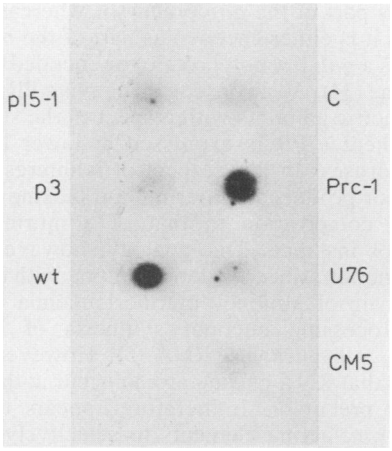

FIG. 4. Reverse transcriptase activity of mutant virions. Virions produced by the transfected cells were purified, and reverse transcriptase was quantitated by the exogenous template assay as described in Materials and Methods. Labels: C, control transfection with no DNA; Prc-1, transfection with the mutant Prc-1 DNA (30); U76, CM5, p15-1, and p3, transfection with each of the mutant DNAs, respectively; wt, wild-type DNA transfection.

a conformational effect, that is responsible for the observed phenotype.

When the *gag* precursor protein is not cleaved, owing to mutation either in the active site of the protease or at the cleavage site, the virus packages almost as much viral RNA as the wild type, but in the form of a monomer (Fig. 5), as shown by comparison with the 35S form of the Prc-1 mutant, which is unable to dimerize completely the viral RNA (30). These data suggest that processing of the *gag* precursor protein is not necessary for viral RNA packaging, but is necessary for the formation of the viral RNA dimer.

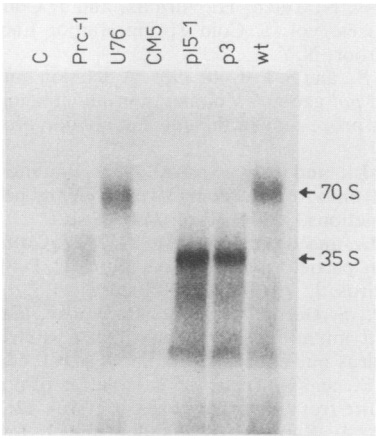

FIG. 5. Viral RNA content of the virions produced in a transient-transfection assay. The virions produced in a transient-transfection assay were purified and viral RNA was extracted, as described in Materials and Methods. After size fractionation on a nondenaturing 0.8% agarose gel, the RNA was electrotransferred to a nylon membrane and hybridized with a random-primed, ^{32}P-labeled probe specific for RSV RNA. The filter was exposed for 2 days with an intensifying screen. The amount of virions used was normalized with respect to their CA protein content. Lanes are the same as in Fig. 2. Arrows represent the mobility of 70S (virion) and 35S (intracellular viral) RNA.

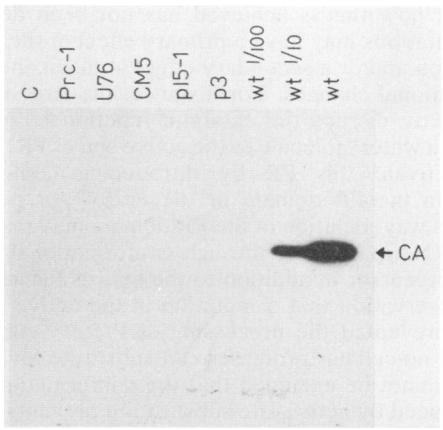

FIG. 6. Infectivity of the mutants. Chicken embryo fibroblasts were transfected with the mutant plasmids as for a transient-transfection assay. The culture medium was collected after 60 h and was used to infect fresh cells. After 8 days, the medium was analyzed by immunoblotting for the presence of viral proteins. Autoradiography was carried out for 20 h with an intensifying screen. Lanes: C, infection with 3 ml of medium harvested from the mock-transfected cells; Prc-1, U76, CM5, p15-1, and p3, infection with 3 ml of medium harvested from cells transfected with each of the mutants; wt, infection with 3 ml of medium harvested from cells transfected with the wild-type plasmid; wt 1/10, infection with 0.3 ml of the same medium; wt 1/100; infection with 0.03 ml of the same medium.

All the mutants are unable to replicate. To determine the infectivity of the mutants, we transfected chicken embryo fibroblasts with the mutant plasmids as for a transient-transfection assay. The culture medium was collected after 60 h and used to infect fresh chicken embryo fibroblasts. After 8 days, the culture medium was analyzed for viral particles by immunoblotting (Fig. 6). The results indicate that all the mutants are unable to replicate. These results were predicted, since the only mutant to have a mature genomic RNA dimer has no reverse transcriptase.

DISCUSSION

To study the role of processing of the *gag* precursor polyprotein Pr76^{gag} during RSV virion assembly and maturation, we have constructed a series of mutations.

First, we attempted to prevent processing of Pr76^{gag} through mutation of either the enzyme protease or the substrate. Replacement of the aspartate residue of the active site of the avian PR by an arginine completely prevented the proteolytic processing of Pr76^{gag}, as was the case with the PR of human immunodeficiency virus type 1 (13, 19). Changing the leucine residue at the cleavage site between NC and PR in Pr76^{gag}, the substrate of PR, to an arginine, did not entirely abolish this cleavage. The partial cleavage (occurring in about one-third of the molecules) was different from that of the wild type in that only one product was detected, whose size and protein composition corresponded to that of Pr76^{gag} lacking PR. Since neither PR nor any other proteolytic products were detected, we suspect that this cleavage of the mutant Pr76^{gag} was aberrant, occurring at the incorrect position. The phenotype of this mutant suggests that correct release of PR from the precursor is required for the further cleavages.

The protease mutations at the active site of the enzyme or at the substrate site block processing of precursor polypep-

tides, but how this is achieved has not been determined. Those mutations may have a primary effect at the site of the substitution and/or a secondary effect at distal sites through conformational changes. For instance, the Arg substitution may directly disrupt the catalytic function by preventing access of a water molecule to the active site of PR, but it may also inactivate this PR by introducing conformational changes in the PR domain or the $Pr76^{gag}$ or both. In an analogous way, deletion of the PR domain may result in lack of viral RNA packaging through conformational change in the *gag* precursor in addition to the loss of PR activity.

The observation that a mutation in the active site of PR entirely prevented the processing of $Pr76^{gag}$ strongly suggests that no cellular proteases can substitute for PR. However, it cannot be excluded that the conformational distortion produced by active-site substitution prevents both viral and cellular enzymes from acting. Irrespective of whether the mutations in PR resulted in complete or partial inhibition of $Pr76^{gag}$ processing, their effects on viral RNA were the same: packaging was unaffected, but dimerization of viral RNA subunits was absent. This directly demonstrates that uncleaved *gag* precursor protein is able to capture viral RNA but that cleavage of the precursor must occur before or during viral RNA maturation. These two properties have previously been inferred but not demonstrated. The first finding was predicted, because processing is known to occur during or after virus particle formation. Therefore, our results do not support the concept that encapsidation and dimerization are two linked events occurring during the course of virus assembly, as proposed from in vitro studies with NC protein (1).

Site-directed mutagenesis experiments have shown that the NC is involved in the packaging and maturation of retroviral RNA (8, 12), and the data presented here indicate that it is $Pr76^{gag}$ that recognizes viral RNA for packaging. Consequently we investigated whether a *gag* precursor polyprotein lacking the PR domain, but retaining all the others, in particular that of NC, would still be able to capture viral RNA. Such a deletion of the PR domain was achieved through inhibition of its translation. We found that this mutant is unable even to package viral RNA. The lack of *gag-pol* polyprotein in this mutant is unlikely to be the cause of this phenotype, since our mutant, which expresses the protease without synthesizing *gag-pol* precursor, is normal in packaging and dimerization of the viral RNA. Altogether, this indicates that the PR domain is involved, directly or indirectly, in the capture of RSV RNA. On the other hand, free NC is found associated with the genomic RNA in the virion (6, 11, 23, 24, 33). Thus, NC appears to have a dual function depending on whether it exists as a domain of $Pr76^{gag}$ or free after cleavage during the maturation process, an example of how retroviruses can dispose of the same linear information to achieve multiple functions. It should also be pointed out that, in agreement with other results (36), the lack of PR does not prevent particle formation, as has been described for murine retroviruses (16).

It might be argued that the point mutation CM5 described here mediated its effect by altering the viral RNA sequence rather than by changing the amino acid sequence of the protein. This is unlikely because no known packaging signal is localized in the region of the mutation (24a) and because two mutations (CM5 and p3) in the same region of the genome, but with very different effects on the translated proteins, gave rise to different phenotypes.

One peculiarity of avian retroviruses (with one exception, spleen necrosis virus [37]) is the fact that the protease is expressed as part of the *gag* precursor whereas in all other retroviruses it is either encoded as part of the *pol* gene (and expressed as a *gag-pol* polyprotein) or encoded in a different reading frame (34). As a consequence, avian PR is expressed in stoichiometric amounts with respect to the structural *gag* proteins, whereas PR is expressed at lower levels in the other retroviruses. In this respect, it is interesting that the *gag* precursor produced by our mutant lacking PR is analogous in its composition to that of a murine retrovirus ($Pr65^{gag}$), for instance. This analogy, however, is not reflected in function, since a deletion mutant with a mutation in the PR domain of Moloney murine leukemia virus, which prevents processing but not synthesis of $Pr65^{gag}$ and $Pr200^{gag\text{-}pol}$, still packages RNA (5). However, it has not been shown that RNA packaging can occur in the absence of the *gag-pol* precursor. It therefore appears that the role played by *trans*-acting elements to selectively capture the retroviral genomic RNA instead of subgenomic viral RNA or cellular RNAs is more complex than was previously thought.

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