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Properties of Avian Retrovirus Particles Defective in Viral Protease

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The structural and enzymatic components of retroviral cores are formed by proteolytic cleavage of precursor polypeptides, mediated by the viral protease (PR). We constructed an active-site mutation, D37I, in the PR of avian leukosis virus. The D37I mutation was introduced into an infectious DNA clone, and quail cell lines expressing the mutant virus were established. These cell lines produce normal amounts of virus particles, the major internal protein components of which are the uncleaved *gag* and *gag-pol* precursors. As in other retroviral systems, the protease-defective virions are noninfectious and retain the "immature" type A morphology as determined by thin-section transmission electron microscopy. The virion cores are stable at nonionic detergent concentrations that completely disrupt wild-type cores. Digestion of mutant virions with exogenous PR in the presence of detergent leads to complete and correct cleavage of the *gag* precursor but incomplete cleavage of the *gag-pol* precursor. The protease-defective virions encapsidate normal amounts of genomic RNA and $tRNA^{Trp}$ that is properly annealed to the primer-binding site, but some of the genomic RNA remains monomeric. Results from UV cross-linking experiments show that the *gag* polyprotein of mutant virions interacts with viral RNA and that this interaction occurs through the nucleocapsid (NC) domain. However, within mutant virions the interaction of the NC domain with RNA differs from that of mature NC with RNA in wild-type virions. Reverse transcriptase (RT) activity associated with mutant virions is diminished but still detectable. Digestion of the virions with PR leads to a fivefold increase in activity, but this PR-mediated activation of RT is incomplete. Since in vitro cleavage of the *gag-pol* precursor is also incomplete, we hypothesize that amino acid sequences N terminal to the reverse transcriptase domain inhibit RT activity.

Although it involves only three gene products, retroviral assembly is a complex process. The major structural and enzymatic protein components of the virion core, encoded by the *gag* and *pol* genes, are initially translated as large polyprotein precursors (9). For type C retroviruses, the polyproteins are targeted to the plasma membrane, where they initiate viral assembly through interactions with the genomic RNA and the glycoproteins encoded by the *env* gene. In the first stage of assembly, as revealed by thin-section transmission electron microscopy, type C virions appear as a crescent-shaped outward pucker directly under the plasma membrane. The nascent virions are enlarged into a spherical "bud" surrounded by the lipid bilayer of the plasma membrane. Budding virions have an "immature" or type A morphology characterized by a centrally positioned electron-dense ring-shaped core with a lucent center (3, 65). During or shortly after release of the virion, the core structure undergoes a morphological change to the "mature" type C morphology (3, 27, 68). Mature virions have a centrally positioned sphere-shaped core of uniform electron density. During these final stages of morphogenesis, the polyprotein precursors are cleaved by the viral protease (PR) to liberate the mature core components. Core maturation is a direct consequence of polyprotein processing, as evidenced by two important observations. First, maturation is coincident with processing (36, 37, 67, 68); second, protease-defective retrovirus particles fail to undergo maturation and are composed of unprocessed polyproteins (8, 14, 23, 26, 48, 64, 66).

In all retroviruses, the *gag* gene encodes a polyprotein precursor that provides at least three major structural components to the virion core, MA, CA, and NC. The membrane-associated or matrix protein (MA) interacts with the inner face of the viral lipid envelope and in all retroviruses is derived from the N terminus of precursor polyproteins. The capsid protein (CA) is believed to form a regular shell around the innermost nucleocapsid structure. The nucleocapsid is composed of genomic viral RNA that is tightly complexed with the nucleocapsid protein (NC). In the avian sarcoma-leukosis virus (ASLV) system, the *gag* gene product is a 76-kilodalton (kDa) polyprotein, $Pr76^{gag}$, which is composed of five major protein domains that are released by proteolytic processing. From amino terminus to carboxy terminus, these domains are: MA-p10-CA-NC-PR. The ASLV system is the only well-studied retroviral system in which PR is encoded in *gag*. In the mammalian retroviruses PR is encoded either within the 5′end of *pol* (and thus translated only as a *gag-pol* polyprotein) or in a separate reading frame located between *gag* and *pol* (translated as a *gag*-PR and a *gag*-PR-*pol* polyprotein). Translation of PR in mammalian retroviruses relies on either frameshifting (bovine leukemia virus, human immunodeficiency virus [HIV], human T-cell leukemia virus type I, and mouse mammary tumor virus) or termination suppression (murine leukemia virus [MuLV]), and as a consequence, mammalian retroviruses synthesize and package much less PR than avian retroviruses do. The *pol* gene of ASLV encodes the other enzymatic components of a virion core, reverse transcriptase (RT) and integrase (IN), which function to synthesize and integrate viral DNA, respectively. The ASLV *pol* gene is translated as a 180-kDa *gag-pol* polyprotein ($Pr180^{gag\text{-}pol}$) produced by ribosomal frameshifting before the *gag* termination codon (20).

The proteases (PR) encoded by retroviruses, pararetroviruses, and retrotransposons all have a highly conserved sequence at the active site, Asp-Ser/Thr-Gly, shared by all aspartic proteases (1, 35, 59, 60; for reviews of retroviral proteases, see references 30 and 57). Recently, X-ray crys-

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tallography has demonstrated that the proteases of HIV type 1 and Rous sarcoma virus (RSV) have very similar three-dimensional structures which resemble those of well-known cellular aspartic proteases (31, 42). While the viral enzymes are homodimers composed of two subunits, the cellular enzymes are composed of single polypeptides with two homologous tandem domains (59). The results of gel filtration and cross-linking experiments imply that the proteases of RSV, HIV, and bovine leukemia virus are active as dimers in solution (15, 22, 28).

In every system tested to date, altering the active-site aspartate residue in PR completely destroys protease activity (14, 26, 29, 33, 35, 45, 60, 66, 70). Mammalian viruses with PR deletions or active-site mutations direct the formation of virions that are composed of unprocessed polyproteins, are noninfectious, and when observed have immature type A morphology (8, 14, 22, 26, 48). Changes in the virion core that are likely to result from polyprotein cleavages include destabilization of the virion core (37, 49, 68) and organization or dimerization of genomic RNA. That maturation of virion cores may be necessary for dimerization of genomic RNA is suggested by analyses of the RNA structure of rapidly harvested virions. Rapidly harvested RSV is composed largely of immature particles, which have been found to contain either incompletely dimerized RNA (5, 7, 27) or dimerized RNA that is stable only in high salt concentrations (58).

Evidence from most but not all retrovirus systems suggests that during virion assembly reverse transcriptase is activated by proteolytic maturation of the initially inactive *gag-pol* polyprotein (8, 11, 23, 32–34, 36, 43, 46, 47, 67). This concept was originally formulated from the observations that (i) activation of RSV and MuLV reverse transcriptase is coincident with virion maturation and proteolysis of the *gag-pol* polyprotein (36, 43, 67) and (ii) *pol*-related protein (presumably $Pr180^{gag\text{-}pol}$) isolated from RSV-infected cells demonstrates low RT activity (47). However, in 1985 Crawford and Goff described an MuLV mutant (*dl*2905) containing a partially deleted PR that exhibits nearly normal levels of virion-associated RT activity (8), suggesting that proteolytic processing of the *gag-pol* polyprotein is not always necessary for activation of RT activity. Though there has been some controversy surrounding this conclusion (46), it has been confirmed by in situ (gel) RT assays of *dl*2905 viral proteins fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (S. Goff, personal communication). In HIV, results from bacterial expression indicate that proteolytic processing is necessary for activation of HIV reverse transcriptase (11, 32–34). Furthermore, protease-defective HIV virions exhibit a level of RT activity reduced by a factor of 6 relative to wild-type virions (14). The mechanisms by which PR-mediated cleavages lead to activation of RT are not understood.

To gain a better understanding of the role that PR plays in the formation of infectious avian retrovirus particles, we engineered an active-site mutation into the PR-coding sequence of full length avian leukosis virus (ALV) DNA constructs. As expected, cells that express these protease-mutant constructs release noninfectious virions that are biochemically and morphologically immature. The immature virions produced are likely to preserve transient interactions that occur during the early stages of assembly. Thus, a structural analysis of protease-mutant virions may provide insight into the assembly process and facilitate a better definition of the steps that lead to an infectious virion. In this report we describe several structural and enzymatic properties of protease-mutant ALV virions.

MATERIALS AND METHODS

DNA constructs. The RCAS*neo* vector used in our experiments contains a full-length Schmidt-Ruppin A (SR-A) viral DNA originally described by Hughes et al. (18) and a neomycin phosphotransferase gene (*neo*) which was subsequently engineered into the *Cla*I restriction site of the parental RCAS vector (gift of A. M. Skalka). The *neo* gene replaces the v-*src* gene and is translated from a subgenomic viral mRNA that would otherwise encode $pp60^{v\text{-}src}$.

All plasmids were constructed by common subcloning techniques and propagated in the DH5-α strain of *Escherichia coli*. Nucleotides of the RSV genome are numbered (with superscript numbers) according to the method of Schwartz et al. (56). A mutation (D37I) in the PR-coding domain was introduced into RCAS*neo* in the following manner. Site-directed mutagenesis of the plasmid pSR*gag* was performed as described previously (44) with a mutagenic oligonucleotide that changes the GAC codon for the active-site aspartate at residue 37 to ATA, which encodes isoleucine, and also creates an *Eco*RV site. This plasmid contains the leader, *gag*, and part of the *pol* gene to nucleotide 2731, flanked by two long terminal repeats. To recreate a complete viral DNA, a trimolecular ligation was carried out with a *Sac*I^{255}-*Bss*HII^{2724} fragment from pSR*gag*D37I, a *Bss*HII^{2724}-*Sna*BI^{3202} fragment, and the complementary backbone from RCAS*neo*. In order to construct the PrC*neo* D37 and PrC*neo* D37I plasmids, we initially subcloned *Xho*I^{630}-*Kpn*I^{4995} fragments (containing most of the *gag* and *pol* coding sequences) from the pSV.GP.myr_1D37 and pSV.GP.myr_1D37I plasmids (provided by J. Wills and R. Craven) into the multiple-cloning site of the Bluescript KSII+ vector (Stratagene Inc.). The resulting plasmids, named pXK.D37 and pXK.D37I, respectively, are composed of genomic segments from both the PrC and SR-A strains of RSV. The SR-A sequences, *Bgl*II^{1630}-*Eco*RI^{2319}, were obtained from either pPR.*gag*D37 or pPR.*gag*D37I. The PrC sequences, *Xho*I^{630}-*Bgl*II^{1630} and *Eco*RI^{2319}-*Kpn*I^{4995}, were originally derived from the pATV-8 plasmid (J. Wills, personal communication). In the second step of construction, the *Sac*I^{255}-*Xho*I^{630} fragment from pATV-8 was subcloned into pXK.D37 and pXK.D37I. The resulting plasmids are named pSXK.D37 and pSXK.D37I. In the final cloning step, the PrC*neo* D37 and D37I plasmids were constructed by replacing the *Sac*I^{255}-*Kpn*I^{4995} sequences of RCAS*neo* with a *Sac*I^{255}-*Kpn*I^{4995} fragment from either pSXK.D37 or pSXK.D37I, respectively.

Cells and virus isolation. QT35 quail cells were propagated in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum (GIBCO), 2.0% vitamins, 1.0% dimethyl sulfoxide, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 10% tryptose phosphate broth. Transfection was done by the method of Chen and Okayama (6). G418-resistant transformants were selected as mixed populations of cells and maintained in 25 μg of active G418 per ml. Culture medium was collected every 24 h from confluent 100-mm-diameter plates of cells. After removal of debris by low-speed centrifugation, the virus was collected by sedimentation through a cushion of 10% sucrose–STE (100 mM NaCl, 10 mM Tris hydrochloride [pH 7.5], 1 mM EDTA), at 30,000 rpm for 90 min in a Beckman 50.2 Ti rotor. The virus pellets were softened at 4°C in 30 μl of STE for 1 h before being suspended by pipetting. For some experiments, the

virus was purified further by equilibrium density sedimentation in 20 to 60% (wt/vol) sucrose–STE step gradients spun at 40,000 rpm for 12 h at 4°C in a Beckman SW-60 rotor. [^{35}S]methionine-labeled virus was prepared from confluent 100-mm-diameter plates. The cells were washed in phosphate-buffered saline (PBS) and pulse-labeled for 1 h with 100 μCi of [^{35}S]Met in 2.5 ml of methionine-free medium. Then 5 ml of complete medium was added, and the cells were allowed to grow for an additional 15 h. The resulting virus was purified by sucrose density gradient sedimentation.

Electron microscopy. Sucrose density gradient-purified virions were sedimented through a 20% sucrose–STE cushion (Beckman TLA 100.3 rotor; 60,000 rpm for 10 min at 4°C). Protease mutant cores were pelleted similarly except that mutant virions were adjusted to 0.1% Triton X-100 in 5% sucrose–STE and incubated at 37°C for 15 min prior to centrifugation. The pelleted virions or cores were fixed with 4% glutaraldehyde in 0.1 M cacodylate (pH 7.0). After primary fixation, the samples were washed with buffer alone, postfixed in 1% OsO_4 in 0.1 M cacodylate (pH 7.0), and dehydrated through ascending concentrations of ethanol. Following dehydration, the samples were removed from the centrifuge tubes with propylene oxide and embedded in Epon 812. For infected cells, confluent plates were washed in PBS, fixed with 2% glutaraldehyde in 0.1 M cacodylate (pH 7.4), washed with buffer, and then postfixed with 1% OsO_4 in 0.1 M cacodylate (pH 7.4). After postfixation, the cells were washed in 0.1 M sodium hydrogen maleate (pH 5.15) and stained with 2% uranyl acetate in 0.1 M sodium hydrogen maleate (pH 6.0). The samples were then dehydrated in increasing concentrations of ethanol, removed from the plates with propylene oxide, and embedded in Spurr resin. Thin sections of all samples were stained with lead citrate and examined with a Phillips 301 transmission electron microscope at 80 kV.

Antisera and immunoblotting. Anti-MA and anti-CA antisera were raised in rabbits that had been injected with SDS-PAGE-purified avian myeloblastosis virus (AMV) antigens MA (p19) or CA (p27). Anti-NC (p12) antiserum was raised in a rabbit that had been injected with AMV p12 purified by gel filtration in 6 M guanidine. Goat anti-reverse transcriptase (RT) antiserum was obtained from the National Cancer Institute. Polyclonal rabbit anti-4-kDa^{pol} antiserum was a gift from R. Katz and A. M. Skalka (2). Proteins were electrophoretically transferred from SDS-polyacrylamide gels to Immobilon-P membranes (Millipore Corp.) in 20% methanol–25 mM Tris–190 mM glycine at 0.8 A for 2 h in a Bio-Rad Transblot apparatus. Alternatively, for Western slot blot analyses, virus particles were serially diluted (1:3) in STE and then boiled for 5 min in 10% SDS-PAGE sample buffer before being transferred to the same membrane with a slot blot manifold. Following transfer, the membranes were blocked with 10% low-fat milk in PBS for 1 h at room temperature and then probed for 12 h at 4°C with 1:500 dilutions of antisera in blotting buffer (PBS with 1% bovine serum albumin plus 0.05% Tween 20 [Sigma Chemical Co.]). After three washes in 300 ml of RB (15 mM NaCl, 10 mM Tris hydrochloride [pH 7.5], 1 mM EDTA), the membranes were incubated at 4°C for at least 6 h with 20 to 50 μCi of ^{125}I-labeled protein A (for rabbit antisera) or protein G (for goat antisera) in 100 ml of PBS containing 10% low-fat milk. The membranes were then washed three times in 300 ml of RB plus 1 M NaCl and finally wrapped while wet and exposed to Kodak X-AR-5 X-ray film at −70°C with an intensifying screen for 10 to 15 h. The same blots were reblocked in PBS containing 10% low-fat milk before being probed with subsequent antisera.

Protease digestion. AMV protease (PR) was isolated from disrupted AMV either by 6 M guanidine hydrochloride gel filtration, or by chloroform-methanol extraction (21). PR digestion was performed by adjusting virus particles (5 to 15 plate-days [see legend to Fig. 4] in 100 μl of STE) to PR digestion conditions (730 mM NaCl, 70 mM Tris acetate [pH 6.8], 0.7 mM EDTA, 0.1% Triton X-100, 5.3% glycerol, 1 mM dithiothreitol with or without 60 μg of PR per ml) and incubating the particles at 39°C for 30 min. Digestions were stopped either by boiling the particles in SDS-PAGE load dye or by further adjusting the sample to reverse transcriptase assay conditions.

Preparation and analysis of viral RNA. Except as stated otherwise, virions in STE were lysed and digested in 100 μg of proteinase K per ml–2.5 mM EDTA–0.5% SDS for 30 min at 37°C. This procedure was followed by extraction once with TE (10 mM Tris hydrochloride [pH 7.5], 1 mM EDTA)-equilibrated phenol and once with chloroform-isoamyl alcohol (24:1). The nucleic acids were then precipitated with 2.5 volumes of ethanol in the presence of 30 mM sodium acetate (pH 4.8). Air-dried RNA pellets were suspended in sterile glass-distilled water that had been pretreated with 0.1% diethylpyrocarbonate. Cellular RNA was isolated as previously described (25). For Northern (RNA) blotting, native RNA was electrophoresed in a 10-cm horizontal 0.8% agarose gel for 3 h at 45 V (25 mA) as described by Khandjian and Méric (25). To permit visualization of the RNA molecular weight markers, the gel was stained in 1 μg of ethidium bromide per ml for 30 min. The agarose gel was soaked in 50 mM NaOH for 1 h at room temperature prior to electrophoretic transfer of the RNA to a Zeta-Probe membrane (Bio-Rad Laboratories) for 4 h at 30 V (1 A) in 25 mM sodium phosphate (pH 6.5) with a Transblot apparatus (25). For Northern slot blot analysis, freshly isolated virus particles (no older than 48 h) were lysed and serially diluted (1:3) in ice-cold 10 mM NaOH–2 mM EDTA before being passed through a Zeta-Probe membrane. All membranes were probed with ^{32}P-antisense RNA complementary to sequences from *Kpn*I^{4995} to *Eco*RI^{2319} by standard procedures.

To analyze the tRNA annealed to the viral PBS sequence, viral RNA was extracted from sucrose gradient-purified virions in the presence of 0.1 M NaCl as described above, and its concentration was determined by Northern slot blot analysis. Approximately 30 ng of viral RNA was placed into a 15-μl volume of RT buffer (20 mm Tris hydrochloride [pH 8.0], 100 mM NaCl, 30 mM dithiothreitol, 6 mM magnesium acetate) containing 5 units of AMV RT (United States Biochemical Corp.). End-labeling reactions were initiated by the addition of either 10 μCi of [α-^{32}P]dATP alone or 10 μCi each of [α-^{32}P]dATP and [α-^{32}P]dTTP (3,000 Ci/mmol). The reaction mixtures were incubated for 30 min at 39°C, and then the products were analyzed by sequencing gel electrophoresis and autoradiography.

UV cross-linking analysis. The following protocol was initially obtained from Claude Méric, and subsequently modified in our laboratory. Freshly isolated virus was UV irradiated for 4 min at 4°C under a short-wave UV Chromato-Vue Transilluminator (model C-61; Ultraviolet Products Inc., San Gabriel, Calif.) with the top cover removed (emission wavelength maximum at 254 nm). After UV irradiation, the virions were lysed by incubation in 1.0% sodium deoxycholate (DOC) at 37°C for 30 min and then immunoprecipitated in at least 10 volumes of immunoprecipitation buffer (IPB) (150 mM NaCl, 50 mM Tris hydrochloride [pH

8.5], 1% Triton X-100, 1% DOC, 20 mM EDTA), with 15 μl of polyclonal rabbit anti-NC antisera and 150 μl of 50% (vol/vol) protein A–Sepharose CL-4B (Pharmacia). Alternatively, the lysed mutant virions were digested with PR prior to immunoprecipitation. The resulting immunoprecipitates were washed three times with 1 ml of IPB and two times with 1 ml of TE before being subjected to RNase A digestion with 500 μg of RNase A per ml at 39°C for 30 min. After RNase A digestion, the immunoprecipitates were washed twice in 1 ml of TE and twice in 1 ml of kinase buffer (1.5 mM spermidine, 50 mM Tris hydrochloride [pH 8.0], 5 mM $MgCl_2$, 1 mM dithiothreitol, 5% glycerol). The immunoprecipitates were then divided in half and either (i) incubated together with 5 μCi of [γ-^{32}P]ATP (3000 Ci/mmol) and 30 units of T4 polynucleotide kinase (United States Biochemical) for 1 h at 39°C or (ii) incubated for 1 h at 39°C in kinase buffer with no additional substances (control). After incubation, the immunoprecipitates were washed three times with 1 ml of IPB before disruption by boiling in 30 μl of SDS-PAGE load dye. The liberated proteins and ^{32}P-labeled protein-RNA adducts were then fractionated by SDS-PAGE (13% polyacrylamide) and subsequently electrotransferred to an Immobilon-P membrane. The membrane was then submitted to autoradiography with Kodak X-AR-5 X-ray film for 12 h at −70°C with an intensifying screen. Subsequently, the membrane was immunoblotted with rabbit anti-NC antiserum.

Reverse transcriptase assays. The RNA-dependent DNA polymerase activity of virion-associated reverse transcriptase was measured as the incorporation of [α-^{32}P]dTTP into high-molecular-weight DNA through utilization of an exogenously provided homopolymeric RNA template, poly(rA), and a DNA primer, oligo(dT). Reactions were initiated by placing the samples into premixed RT assay ingredients which were designed to be compatible with either virus in STE or PR-digested virus. The final RT reaction mixtures consisted of 50 mM Tris hydrochloride (pH 8.0), 170 mM NaCl, 20 mM $MgCl_2$, 5 mM dithiothreitol, 0.5 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid], 0.05% Triton X-100, 8 μg of oligo$(dT)_{12-18}$ per ml, 16 μg of poly(rA) per ml, 10 μCi of [α-^{32}P]dTTP (3000 Ci/mmol; Amersham Corp.) per ml, and 1 μM dTTP (final specific activity, 10 Ci of [α-^{32}P]dTTP per mmol). The final reaction volumes were either 50 μl for serially diluted virus or 150 μl for PR-digested virus. The large RT assay volume for the PR-digested virus is required to dilute sufficiently the high salt concentration and low pH of the digestion mixture. Reactions were carried out for 60 min at 37°C and stopped by the addition of an equal volume of TE-equilibrated phenol followed by mixing. For each of the serially diluted samples, a 10-μl sample of the aqueous phase was spotted onto DE-81 anion-exchange paper (Whatman, Inc.). For the PR digestion experiments, the aqueous phases were subjected to ethanol precipitation together with 10 μg of carrier tRNA (*E. coli*) and then suspended in 30 μl of sterile distilled water before being spotted in 10-μl amounts onto DE-81 paper. The DE-81 paper was washed and subjected to autoradiography (13). After autoradiography, the radioactive spots were quantified by Cerenkov counting.

RESULTS

Generation of cell lines and preliminary characterization of mutant virus. We engineered the D37I protease mutation into a DNA clone of the SR-A strain of Rous sarcoma virus, in which the neomycin phosphotransferase gene (*neo*) replaces the v-*src* gene (RCAS*neo*D37I; Fig. 1A) (18). The D37I mutation was also built into a viral DNA with the same backbone, but with the *gag* and *pol* genes primarily derived from the PrC strain of RSV (PrC*neo*D37I; Fig. 1B). Except where noted, all of the results described below were obtained with the RCAS*neo* constructs. The wild-type (D37) or protease-defective constructs were transfected into the QT35 quail cell line, and G418-resistant transformants were either batch selected or picked as single clones. We assessed the protein content of virus particles by pulse-labeling cells with [^{35}S]methionine. Virus particles shed into the culture medium were sucrose gradient purified and then analyzed by SDS-PAGE and fluorography (Fig. 2A). The major ^{35}S-labeled proteins found in wild-type virions were the mature CA, MA, and PR (NC is not visible as a separate band because it is poorly resolved from PR in this gel system and because it has only one methionine residue) (Fig. 2A, lane 2). In contrast, protease-mutant virions were found to be composed of the two unprocessed polyproteins Pr76^{gag} and Pr180$^{gag\text{-}pol}$ (designated Pr76^{D37I} and Pr180^{D37I}) (lane 1). Variable amounts of host-derived polypeptides of unknown identity were also found associated with both wild-type and mutant particles. Pulse-chase labeling experiments demonstrated that protease-mutant and wild-type virions were assembled and released with similar kinetics (data not shown). Thus, as in other retrovirus systems, PR activity is required for all proteolytic cleavages of the *gag* and *gag-pol* polyproteins but is not required for virus assembly. In all retroviral and pararetroviral systems tested to date, protease-defective virions are noninfectious (8, 14, 23, 26, 48, 60). To determine if this rule also applies to the ALV system, we analyzed the infectivity of protease-defective SR-A virions by using the ability of virus particles to confer G418 resistance to QT35 cells. The mutant virus particles were at least 3 orders of magnitude less infectious than wild-type virions, which produced approximately 100 colonies per ng of CA protein (data not shown). Since the mutant virions yielded no G418-resistant colonies, they are likely to be completely noninfectious.

Stability of immature D37I virion cores when treated with detergent. Yoshinaka and Luftig previously demonstrated that morphologically immature MuLV cores enriched in unprocessed polyprotein can be isolated by sucrose gradient sedimentation of virions that have been pretreated with nonionic detergents (i.e., 1% Nonidet P-40) (68). Furthermore, the protease-deficient Gazdar strain of murine sarcoma virus produces immature virus particles with cores that are stable in 0.5% Triton X-100 (49). To define the detergent stability of protease-defective ALV cores, we incubated both wild-type and mutant SR-A virions under various conditions and then measured the fraction of viral protein that could be recovered as a pellet after centrifugation. To prevent potential artifacts from detergent titration, the detergents were present in at least a 15-fold weight excess over viral proteins. The proteins present in the supernatants and pellets were fractionated by SDS-PAGE and subjected to immunoblot analysis with polyclonal anti-CA and anti-MA antisera (Fig. 2B). Intact wild-type and protease mutant virions both were recovered in the pellets of the detergent-free controls (D37, lanes 15 and 16; D37I, lanes 9 and 10). We found that the wild-type virus was completely disrupted in either 0.1% DOC (lanes 11 and 12) or 0.5% Triton X-100 (lanes 13 and 14). In contrast, mutant cores were stable in 0.5% Triton X-100 (lanes 5 and 6) and partially stable in 0.1% DOC (lanes 7 and 8). The mutant virions were disrupted only in 0.1% SDS (lanes 3 and 4) or 1.0% DOC

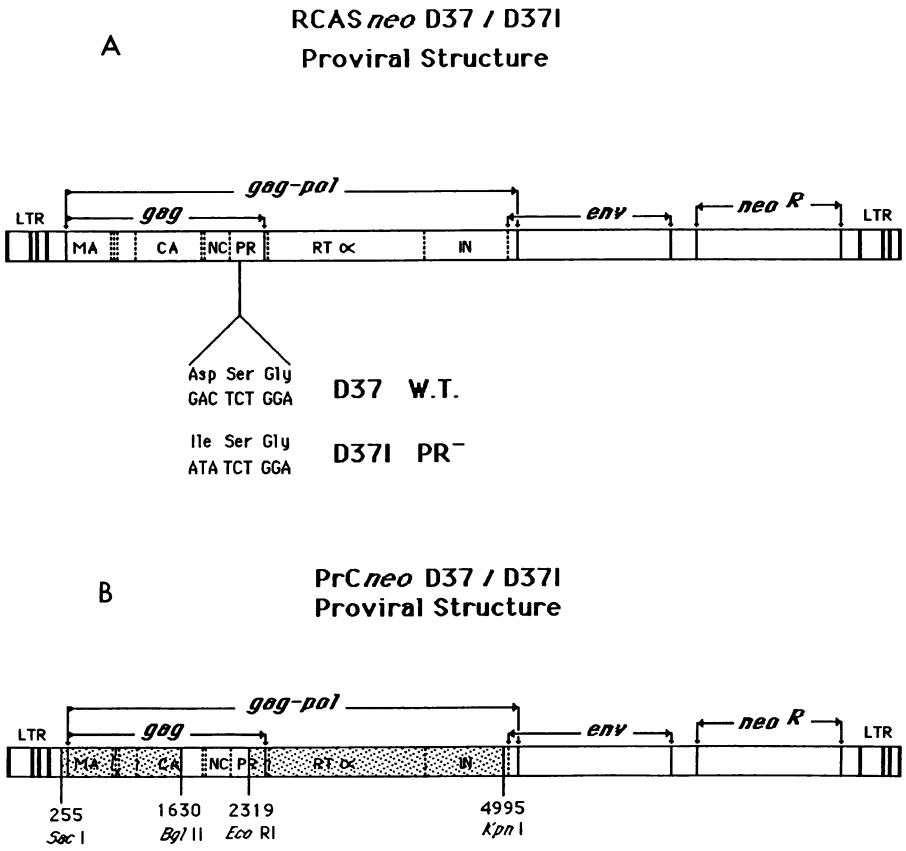

FIG. 1. Structure of wild-type (D37 W.T.) and protease-mutant (D37I PR$^-$) ALV constructs. (A) RCAS*neo* plasmids. Site-directed mutagenesis was performed to create an Asp → Ile mutation at position 37 (D37I) in the PR-coding domain of *gag*, and this mutation was moved into the RCAS*neo* vector. (B) The PrC*neo* plasmids were constructed by replacing most of the *gag* and *pol* sequences of the RCAS*neo* constructs with PrC sequences (stippled areas; *Sac*I^{255}-*Bgl*II^{1630} and *Eco*RI^{2319}-*Kpn*I^{4995}) originally derived from pATV-8. The *gag* gene encodes the major structural components of a virion core, MA, CA, NC, and PR. The *pol* gene encodes reverse transcriptase (63 kDa; RTα), IN, and a C-terminal 4-kDa^{pol} peptide. The *gag* and *gag-pol* translation products (Pr76^{gag} and Pr180$^{gag\text{-}pol}$) are indicated with arrows above the coding sequences for the *gag* and *pol* genes. Dotted vertical lines indicate sites of proteolytic cleavage mediated by PR, while the heavy vertical lines mark the three parts of each long terminal repeat (LTR). The envelope protein gene (*env*) and the neomycin phosphotransferase gene (*neo* ^{R}) are also indicated.

(lanes 1 and 2). Electron microscopy confirmed that the immature viral core remained intact in 0.5% Triton X-100 (Fig. 3M), ruling out the possibility that nonspecific aggregation is responsible for the appearance of *gag* protein in the pellet. Taken together, these results indicate that protease-mutant ALV virion cores are stable under nonionic detergent conditions that lead to complete disruption of wild-type virus particles.

Ultrastructural analysis. Proteolytic maturation of retroviral polyproteins during virion assembly results in morphological maturation of the virion core. In all cases examined to date, protease deficiencies block retroviral maturation (14, 23, 48, 64). We examined purified wild-type and mutant ALV virions by thin-section transmission electron microscopy (Fig. 3). As expected, the protease-defective virions showed an electron-dense ring-shaped core characteristic of the immature type A morphology (Fig. 3B), which was also noted by Voynow and Coffin for a naturally occurring ALV mutant (64). By comparison, wild-type virions have centrally positioned, uniformly electron-dense cores characteristic of mature type C morphology (Fig. 3A). Since less than 0.1% of purified wild-type virus particles have immature morphology, we were able to perform thin-section electron microscopy on mixed viral pellets to obtain size measurements for both D37 and D37I virus particles in the same sample (Fig. 3L). The average diameters for protease mutant virions (mean ± standard deviation, 83 ± 11 nm; n = 81) and wild-type virions (77 ± 12 nm; n = 83) are in agreement with the average diameters previously reported for ASLV virus particles in thin section (80 nm) (3). These dimensions were calculated by using an electron micrograph of a standard reference grid pattern taken at the same time and magnification as the virus electron micrographs.

Cells expressing virus particles were also prepared for thin-section electron microscopy. As expected, while in the budding stage of virion morphogenesis at the plasma membrane, both wild-type and mutant viruses showed immature morphology (D37 [Fig. 3C and D] and D37I [Fig. 3E and F]) (3). In addition, some wild-type and mutant virus particles were localized within multivesicular vacuoles of host cells (D37 [Fig. 3G, H, and K] and D37I [Fig. 3I and J]). Wild-type particles with either mature (Fig. 3G and H) or immature (Fig. 3K) morphology could be seen within the vacuoles. In every cell observed, at least one multivesicular vacuole was found to contain a virus particle, though more than one virion was rarely seen in any given vacuole. The presence of virions in cytoplasmic vacuoles was unexpected because it is generally believed that type C virion assembly occurs at the

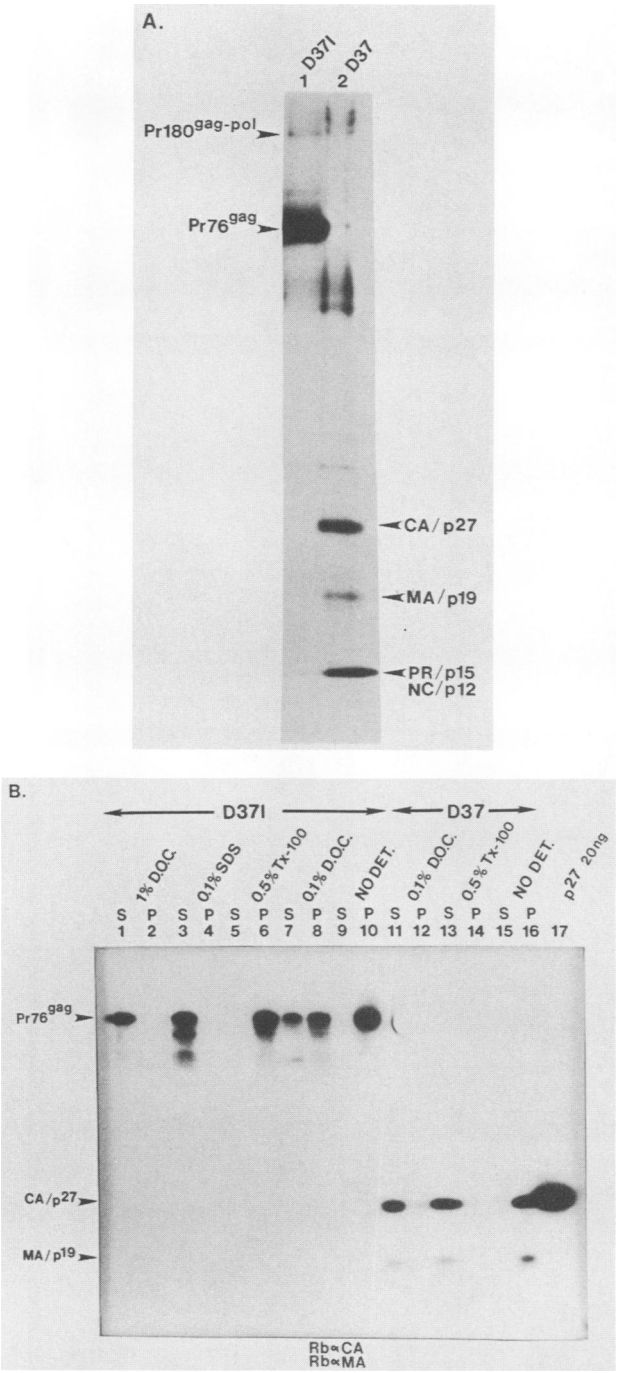

FIG. 2. Effects of the protease mutation on polyprotein processing and virus stability. (A) Protein composition. Cells expressing mutant or wild-type virus were pulse-labeled with [^{35}S]methionine for 1 h, followed by a 14-h chase. The resulting ^{35}S-labeled mutant (D37I; lane 1) and wild-type (D37; lane 2) viruses were purified by sucrose density gradient sedimentation and analyzed by SDS-PAGE and fluorography. (B) Stability in the presence of detergent. Sucrose density gradient-purified mutant (D37I; lanes 1 to 10) and wild-type (D37; lanes 11 to 16) viruses were incubated at 37°C for 30 min in 30-μl volumes of 5% (wt/vol) sucrose–STE together with detergents as indicated. The samples then were layered onto 20% (wt/vol) sucrose–STE cushions containing the same detergent conditions present during incubation and centrifuged for 20 min at 60,000 rpm in a Beckman TLA-100 centrifuge at 18°C. The proteins present in the supernatants (S) and pellets (P) were fractionated by SDS-PAGE and analyzed by immunoblotting with a mixture of rabbit anti-CA (RbαCA) and rabbit anti-MA (RbαMA) antisera. Lanes 1 and 2, 1.0% DOC; lanes 3 and 4, 0.1% SDS; lanes 5, 6, 13, and 14, 0.5% Triton X-100 (Tx-100); lanes 7, 8, 11, and 12, 0.1% DOC; lanes 9, 10, 15, and 16, no detergent; lane 17, 20 ng of purified AMV CA (p27) protein standard.

plasma membrane (3). We have not attempted to investigate the mechanism leading to vacuolar localization. Perhaps the long half-life for assembly in QT35 cells ($t_{1/2}$ = 5 h) results in endocytosis of some virions prior to extracellular release. Recently, Peng et al. expressed two protease-deficient HIV type 1 proviral constructs in COS-M9 cells and observed that in contrast to wild-type virions, mutant virions often budded into cytoplasmic vacuoles (48). Our results differ from those of Peng et al. in that both wild-type and protease-mutant ALV virions were seen within vacuoles and only fully formed virions (no budding virions) were associated with such structures. In total, approximately 10 virus particles were observed for cells expressing each virus, mutant or wild type. In either case, at least half of the particles were observed to be localized within multivesicular vacuoles. On average, less than one virus particle was observed for any given thin section of a cell. However, both budding and vacuolar virions were, on occasion, seen in the same thin section.

The immature cores of the protease-defective ALV particles were stable in 0.5% Triton X-100 (Fig. 2B, lanes 5 and 6). Previous experiments in our laboratory had demonstrated that such a treatment completely removes the phospholipid component of the protease-defective Gazdar virions (L. E. Southard and V. M. Vogt, unpublished observations). Together, these observations suggested that immature D37I viral cores free of envelope membrane could be visualized readily by thin-section electron microscopy. We incubated mutant virions in 0.1% Triton X-100, centrifuged them through a sucrose cushion, and then prepared the resulting "stripped" cores for electron microscopy. The immature mutant cores appeared to have a double-ring electron density (Fig. 3M). Size measurements indicated that the inner electron-dense ring corresponds to the single ring observed in the core of intact mutant virions. The outer ring was easily observed only when the viral envelope was solubilized with Triton X-100. We suggest that the outer electron-dense ring of mutant cores is normally obscured by the tightly apposed lipid envelope of the intact D37I virions.

Processing of the *gag* and *gag-pol* polyproteins of mutant virions. Experiments based on in vitro digestion with PR have provided a large body of evidence that ASLV protease is responsible for proteolytic maturation of the avian retroviral *gag* and *gag-pol* polyproteins (2, 10, 43, 62–64). To extend these observations, we examined the ability of purified AMV PR to proteolytically process the *gag* and *gag-pol* polyproteins of freshly isolated protease-defective virions. All digestion mixtures included 0.1% Triton X-100 to permeabilize the viral envelope; without detergent, no digestion occurred (not shown). The digestion products were analyzed by sequentially probing a single immunoblot with a regimen of different anti-*gag* or anti-*pol* antisera. This technique yields a set of autoradiographs that reveal the location of each different polypeptide for which antibodies are available. In these experiments, the *gag* antigens were analyzed by probing initially with polyclonal antisera to CA and MA (Fig. 4A). This analysis revealed that exogenous PR digestion results in proper processing of $Pr76^{D37I}$ to produce

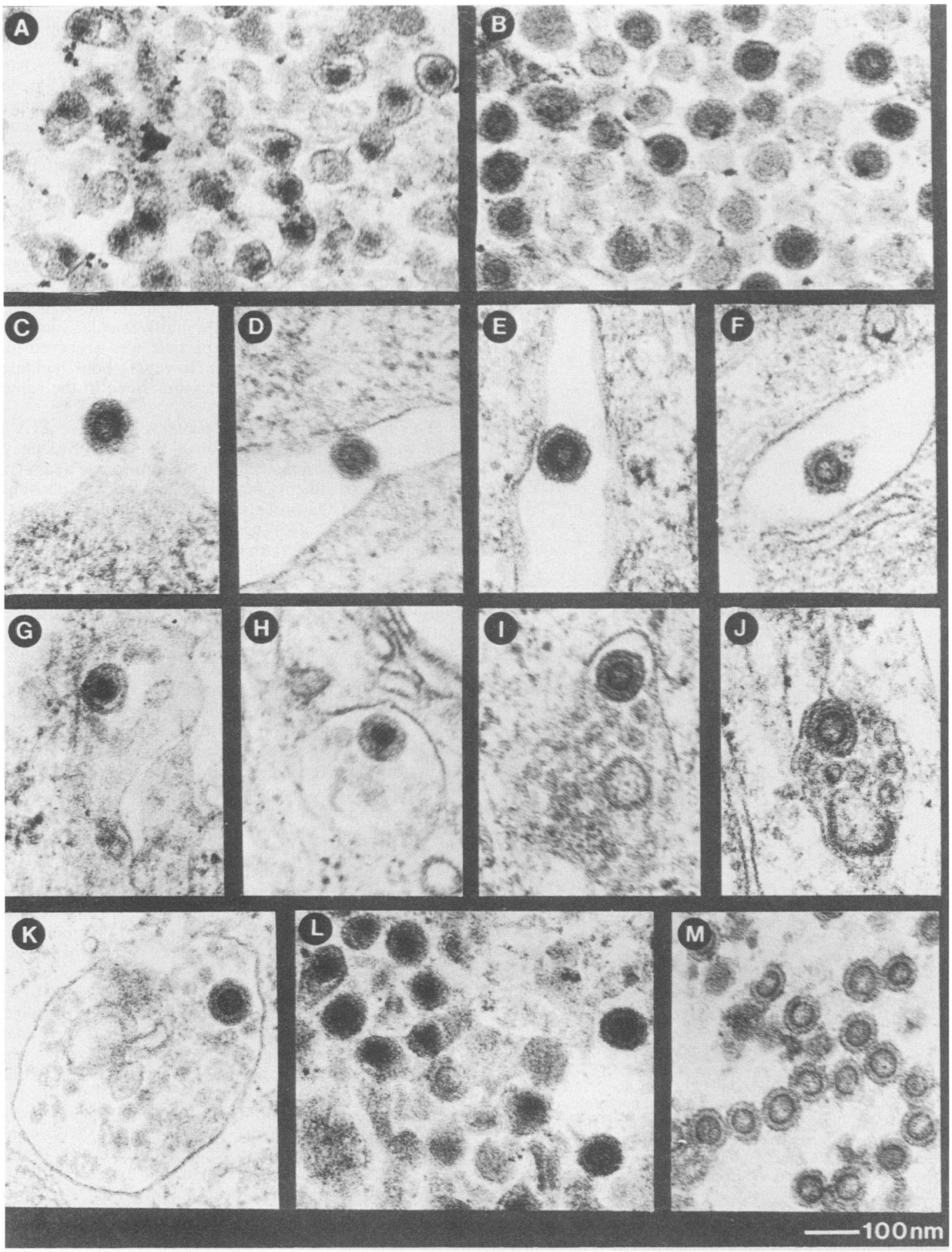

FIG. 3. Electron microscopy. Thin sections through pellets of purified virus particles or through infected cells were prepared. (A) Wild-type virus pellet; (B) protease-mutant virus pellet; (L) pellet of mixture of wild-type and mutant particles; (C, D, G, H, and K) cells expressing wild-type virus; (E, F, I, and J) cells expressing mutant virus. Budding wild-type (C and D) and mutant (E and F) virions have immature morphology. Multivesicular cytoplasmic vacuoles contain mature (G and H) and immature (K) wild-type virus particles. Protease-mutant virus particles are also found in multivesicular vacuoles (I and J). Panel M is a thin section through a pellet of mutant cores that resulted from pretreating D37I virions with 0.5% Triton X-100 before centrifugation through a 20% sucrose–STE cushion.

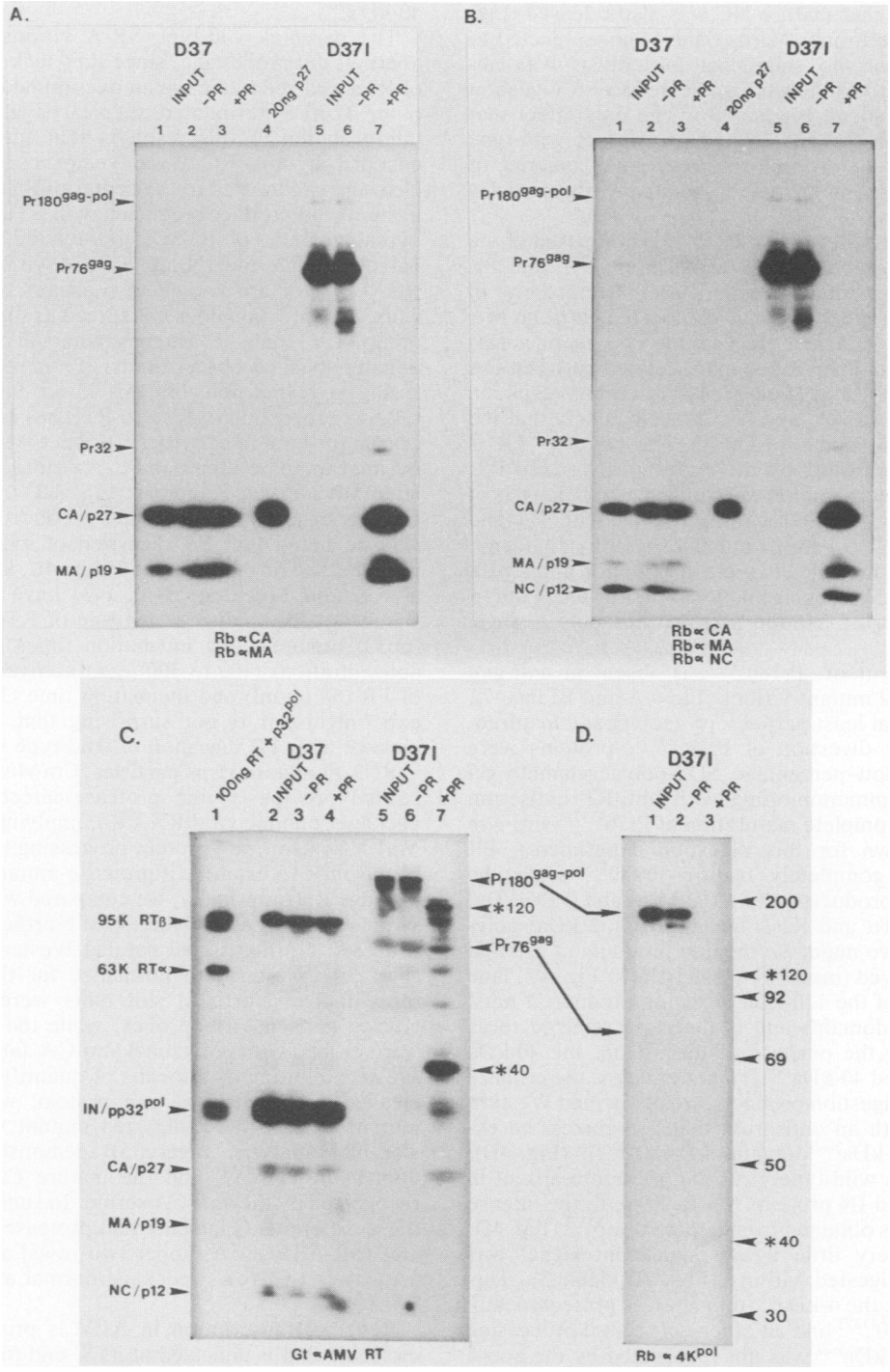

FIG. 4. Immunoblots of wild-type and mutant virus before and after digestion with PR. The virus was subjected to one of the following conditions prior to SDS-PAGE and immunoblot analysis: no treatment (INPUT), digestion conditions in the absence of protease (−PR), or digestion with viral protease (+PR). One plate-day is the amount of virus shed from a 10-cm-diameter plate of confluent cells in 24 h. The gel for panels A and B contained 13% polyacrylamide, and the gels for C and D contained 10% polyacrylamide. (A) Blot probed with a mixture of rabbit anti-CA (RbαCA) and anti-MA (RbαMA) antisera. Lanes 1 to 3, 0.5 plate-days of wild-type virus; lane 4, 20 ng of AMV CA (p27) protein standard; lanes 5 to 7, 0.5 plate-days of mutant virus. (B) Same blot shown in panel A, subsequently probed with rabbit anti-NC (RbαNC) antiserum. (C) Blot probed with goat anti-RT antiserum. Lane 1, 100 ng of AMV RT (α/β heterodimer) plus 100 ng of partially purified RT32^{pol} (IN) from *E. coli*; lanes 2 to 4, 4 plate-days of wild-type virus; lanes 5 to 7, 4 plate-days of mutant virus. (D) Blot probed with rabbit anti-4-kDa (Rbα4kDa^{pol}) antiserum. Lanes 1 to 3, 1 plate-day of mutant virus.

mature CA and MA (Fig. 4A, lane 7). The blot shown in Fig. 4A was subsequently probed with anti-NC antiserum (Fig. 4B) to demonstrate that mature NC was also released (Fig. 4B, lane 7). Relative to input virus (lane 5; not subjected to digestion conditions), no significant proteolysis was observed when mutant virions were subjected to PR digestion conditions without added PR (lane 6). The only effect that PR digestion had on the *gag*-related proteins of wild-type virus particles was to process the very small amount of residual $Pr76^{gag}$ present in freshly isolated virus particles (Fig. 4A, lane 3).

In order from N to C terminus, $Pr76^{gag}$ is composed of the following major *gag* domains: MA, p10, CA, NC, and PR (52, 56). Two other small domains are also present in $Pr76^{gag}$: (i) a stretch of 22 amino acids, located between MA and p10 (61), which is further cleaved into two peptides (51) and (ii) a 9-amino-acid peptide domain located between CA and NC (56). Since PR digestion of mutant virions results in proper release of CA, MA, and NC, it seems likely that the other cleavages also occur properly. The matured PR is obscured on Western blots by the exogenously added PR. However, proper release of PR was confirmed by analysis of [^{35}S]methionine-labeled D37I virions treated with PR and then analyzed by SDS-PAGE and fluorography (data not shown). This technique failed to yield information about p10, because this unusual protein is known to rapidly leach out of SDS-PAGE gels during fixation (52) and has only a single methionine residue.

The *gag-pol* precursor, $Pr180^{D37I}$, is absent in anti-*gag* blots of PR-digested mutant virions (Fig. 4A and B, lane 7), indicating that it is at least partially proteolytically matured. To further analyze digestion of $Pr180^{D37I}$, proteins were fractionated on a low-percentage SDS-polyacrylamide gel and analyzed by immunoblotting with anti-RT antiserum (Fig. 4C). Despite complete maturation of $Pr76^{D37I}$ (anti-*gag* blots are not shown for this digestion experiment), PR digestion did not completely mature $Pr180^{D37I}$ into the known *pol*-related products—the α (63-kDa) and β (95-kDa) subunits of RT (RTα and RTβ) and the IN (32 kDa) polypeptide. Instead, two major *pol*-related products of 120 and 40 kDa were observed (marked by asterisks in Fig. 4C, lane 7). Given the size of the 120-kDa digestion product, it must contain some *gag* domains and is therefore referred to as 120-kDa$^{gag\text{-}pol}$. For the purpose of discussion, the 40-kDa product is designated 40-kDa^{pol}. To better define the primary structure of these digestion products, we performed Western immunoblotting with an antiserum that recognizes the extreme C-terminal 4-kDa^{pol} domain of $Pr180^{gag\text{-}pol}$ (Fig. 4D), which is cleaved in wild-type virus and therefore absent in the mature α, β, and IN proteins (2). Relative to the intense anti-4-kDa^{pol} signals obtained from intact $Pr180^{D37I}$ (Fig. 4D, lanes 1 and 2), very little if any significant signal was observed for PR-digested virions (Fig. 4D, lane 3). This result indicates that the 4-kDa^{pol} domain was proteolytically removed from $Pr180^{D37I}$ and all other *pol*-related processing products. Since 40-kDa^{pol} was not recognized by the anti-4 kDa^{pol} antiserum this peptide is not equivalent to $p36^{IN}$ (IN-4-kDa^{pol}) (2). The blot in Fig. 4D was subsequently probed with polyclonal anti-RT serum to confirm the presence and location of 120-kDa$^{gag\text{-}pol}$ and 40-kDa^{pol} (marked by asterisks in Fig. 4D). Eisenman et al. previously described a 130-kDa *gag-pol* processing intermediate that is composed of PR, RTα, and IN domains and is produced upon exogenous PR digestion of rapidly harvested immature PrC virions (10). We speculate that 120-kDa$^{gag\text{-}pol}$ may be similar or identical to this 130-kDa protein. We are currently in the process of utilizing other domain-specific anti-*pol* antisera to better define the structure of 120-kDa$^{gag\text{-}pol}$ and 40-kDa^{pol}.

The parental wild-type SR-A virions appear to exhibit aberrant *pol* processing, since they lack significant amounts of RTα and seem to have an overabundance of IN (or some other 32-kDa *pol*-related species) (Fig. 4C, lane 2). One interpretation of this result is that the *pol* gene product encoded by the RCAS*neo* vector contains an extra PR cleavage site located in the center of RTα. Cleavage at such a site would produce a deficiency in RTα and a concomitant overabundance of 32-kDa *pol*-related proteins (the two halves of RTα and IN). The SR-A virions encoded by the RCAS vector are known to replicate very poorly in quail cells, and infection does not spread as rapidly as it does with other ALV strains (S. Hughes, personal communication, and our unpublished observations). Perhaps aberrant *pol* processing is responsible for this lower infectivity, since the relative proportion of RTα to RTβ has been suggested to be crucial for viral infectivity (17). Since wild-type *pol* processing appears to be altered in SR-A virions, we constructed the alternative PrC*neo* vectors (Fig. 1B) and are now in the process of analyzing their *pol* products. Finally, it should also be noted that PR digestion of wild type SR-A virus particles had no effect on RTβ (Fig. 4C, lane 4). Alexander et al. (2) and Moelling et al. (43) have demonstrated that significant PR-mediated cleavage of RTβ into RTα and IN (pp32) requires long incubation times (5 h) and high PR concentrations (100 to 300 μg/ml). Given the concentration of PR (60 μg/ml) and incubation time (30 min) used in our experiments, it is not surprising that RTβ remained uncleaved after PR digestion of wild-type virus.

RNA in mutant virus particles. Crawford and Goff demonstrated previously that protease-defective MuLV virions package genomic viral RNA (8), implying that at least in the MuLV system, polyprotein processing is not necessary for packaging. To examine if protease-mutant ALV virions also package RNA normally, we compared wild-type and mutant virions by using a combination of Northern slot blot analysis (Fig. 5A, Northern) and parallel Western slot blot analysis (Fig. 5A, Western) to normalize for the amount of viral protein. The Northern slot blots were probed with ^{32}P-labeled antisense riboprobes, while the Western slot blots were probed with polyclonal anti-CA antiserum. To validate Western blotting as a means of quantifying the amounts of cleaved and uncleaved *gag* protein, we compared intact mutant virions to PR-digested mutant virions by Western slot blot analysis. Figure 5B demonstrates that the CA domain in $Pr76^{D37I}$ and the mature CA are equally well recognized by the anti-CA serum. In summary, the results of these experiments indicate that protease-mutant ALV particles (SR-A [Fig. 5A, upper two rows] as well as PrC [Fig. 5A, lower two rows) package normal amounts of genomic RNA.

Reverse transcription in ALV is primed by a $tRNA^{Trp}$ molecule that is annealed at its 3′ end to the primer-binding site of genomic RNA. On the basis of the sequence, the first three deoxyribonucleotides added to the tRNA primer are predicted to be AAT. To determine if $tRNA^{Trp}$ is properly annealed to the primer-binding site in mutant virions, we performed end-labeling experiments with purified viral RNA extracted in 0.1 M NaCl. Equal amounts of viral RNA were incubated with AMV reverse transcriptase and [α-^{32}P]dATP, with or without additional [α-^{32}P]dTTP. The products were fractionated by denaturing (8 M urea) PAGE and visualized by autoradiography (Fig. 6). In the presence of [α-^{32}P]dATP

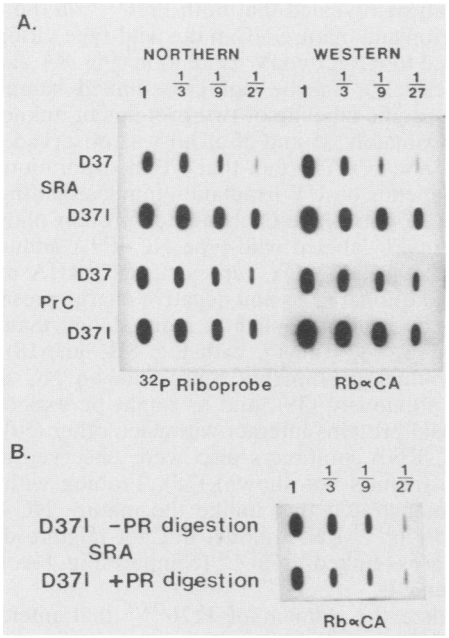

FIG. 5. Analysis of the genomic RNA content of mutant virions. (A) Northern slot blot analysis of protease mutant viral RNA. Virus (2 plate-days) was serially diluted (1:3) in STE. Each sample from the dilution series was divided into two equal parts, one of which was diluted 10-fold in Northern lysis buffer at 4°C and blotted to a Zeta-Probe membrane (NORTHERN). The other half was diluted 10-fold in 2% SDS and blotted to an Immobilon-P membrane (WESTERN). Northern blots were probed with ^{32}P-labeled antisense riboprobes. Western blots were probed with rabbit anti-CA antiserum (RbαCA) followed by ^{125}I-labeled protein A. Blots were exppsed to X-ray film for 12 h. SR-A, Virions from cells expressing the RCAS*neo* constructs; PrC, virions from cells expressing the PrC*neo* constructs. (B) Quantitative comparison of the CA epitopes of intact and PR-digested Pr76^{gag}. Equal amounts of D37I virus were subjected to digestion conditions with (+PR) or without (−PR) added protease. After incubation, the two samples were adjusted to 2% SDS and placed on ice. The protein content of the −PR sample was corrected with the addition of an amount of PR equal to that used for digestion of the other sample. Both samples were then immediately boiled in SDS, serially diluted, and slot blotted to a membrane. The blot was probed with rabbit anti-CA antiserum (RbαCA). To confirm that Pr76^{D37I} remained intact in the −PR sample and was completely digested in the +PR sample, samples of each were analyzed by SDS-PAGE and immunoblotting (data not shown).

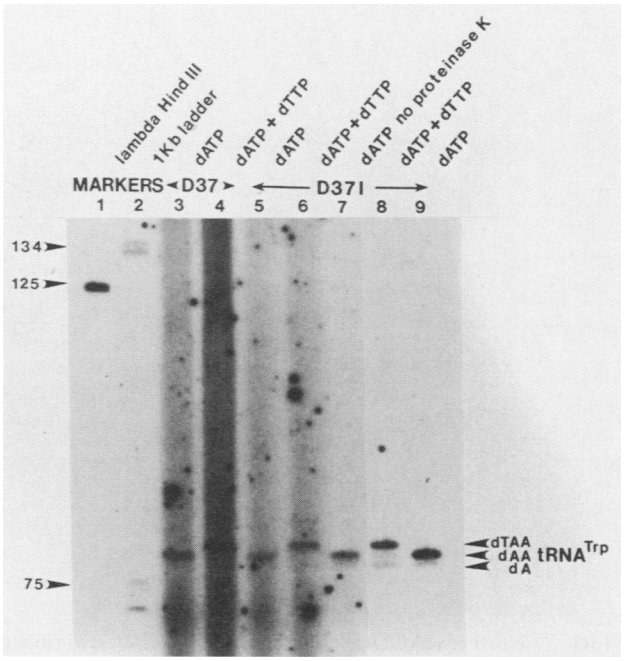

FIG. 6. End labeling of the tRNA^{Trp} primer. Viral RNA was extracted from sucrose density gradient-purified virus and quantified by Northern slot blot analyses. Equal amounts of RNA (about 30 ng) were used in each 15-μl reaction mixture, which included 5 U of AMV RT and 10 uCi of [α-^{32}P]dATP (lanes 3, 5, 7, and 9), or 10 uCi of both [α-^{32}P]dATP and [α-^{32}P]dTTP (lanes 4, 6, and 8). Lane 1, ^{32}P-labeled *Hin*dIII-digested lambda bacteriophage DNA; lane 2, ^{32}P-labeled 1-kil.-base-pair DNA ladder from Bethesda Research Laboratories; lanes 3 and 4, reactions with wild-type viral RNA; lanes 5 and 6, reactions with mutant viral RNA; lane 7, a reaction with mutant viral RNA that was extracted directly with phenol without prior proteinase K digestion; lanes 8 and 9, reactions with a different preparation of mutant viral RNA. Numbers at the left indicate the positions and sizes (in bases) of marker DNAs. dA, dAA, and dTAA tRNA^{Trp} mark the species of tRNA that are elongated by one, two, or three bases. Nucleic acids were electrophoretically separated on a 7 M urea–8% polyacrylamide gel which was then submitted to autoradiography for 6 h.

alone, reverse transcription of wild-type (lane 3) or mutant (lanes 5, 7, and 9) RNA produced radiolabeled species 77 nucleotides in length, 2 nucleotides longer than tRNA^{Trp} itself. These products were extended by a single dTMP residue when [α-^{32}P]dTTP was included in the reaction mixtures (lanes 4, 6, and 8), as predicted for tRNA^{Trp} annealed at the primer-binding site. The same result was obtained when proteinase K was omitted and protease-mutant viral RNA was prepared directly by phenol extraction in 0.1 M NaCl (lane 7). Since the labeled products were qualitatively and quantitatively similar for mutant and wild-type virions, we conclude that the protease mutation does not affect tRNA^{Trp} packaging or annealing to the primer-binding site.

Several early publications described that rapidly harvested (3- to 5-min collections) RSV particles are immature in morphology and contain significant amounts of 35S monomeric genomic RNA, which is aggregated or dimerized to a 70S form when the virus is allowed to mature at 37°C (5, 7, 27). However, Stoltzfus and Snyder have suggested that this observation results from phenol extracting rapidly harvested RSV in a low-salt medium (0.1 M NaCl), a condition in which the dimeric structure of immature RNA is not stable (58). We were prompted by this information to examine the structure of genomic RNA from protease mutant virions extracted in the presence of 0.1 or 0.5 M NaCl. Purified viral RNA was subjected to native agarose gel electrophoresis, electrophoretically transferred to a membrane, and subsequently probed with a ^{32}P-labeled antisense riboprobe (Fig. 7). Although some experiments showed variations, typically about one-half of the genomic RNA extracted from mutant virions migrated as 35S monomers (lane 4). This result was obtained when either 0.1 M NaCl (not shown) or 0.5 M NaCl (lane 4) was present during extraction. The other half of the RNA of mutant virions appeared to be a species of higher-order structure (lane 4). This species usually migrated slightly more slowly and more heterogeneously than the wild-type 70S RNA (compare lanes 3 and 4), suggesting that it may not be identical to the wild-type dimer. Denaturation

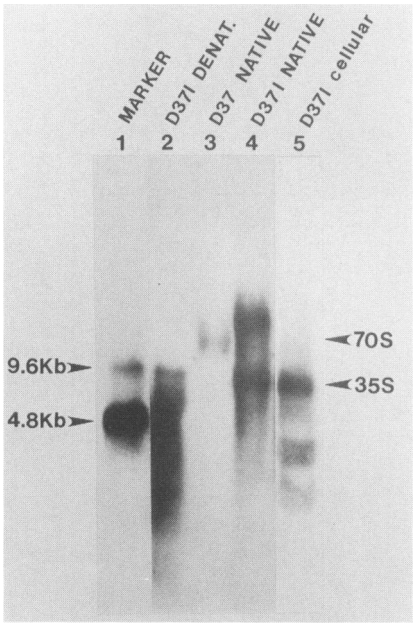

FIG. 7. Northern blot analysis of native RNA. RNA was isolated from mutant and wild-type virus, except that mutant virus was lysed and extracted in 0.5 M NaCl, while wild-type virus was lysed and extracted in 0.1 M NaCl. RNAs were electrophoresed through a 0.8% agarose gel and then blotted to a membrane. The membrane was probed with a ^{32}P-labeled antisense RNA complementary to sequences from nucleotide positions 2319 to 4995. This probe does not hybridize to subgenomic mRNAs. Lane 1, 4.8-kilobase T7 marker RNA transcript synthesized in vitro, including viral sequences from nucleotides 255 to 4995. The marker RNA spontaneously forms a 9.6-kilobase dimer, as previously described (4). Lane 2, Mutant viral RNA denatured for 1 min at 100°C prior to electrophoresis; lane 3, native wild-type RNA; lane 4, native mutant RNA; lane 5, RNA isolated from cells expressing the mutant virus.

of mutant RNA by boiling for 1 min resulted in the release of fragments that appear as a smear below the small amount of residual intact 35S monomer (lane 2), as might be expected since the virus in this experiment was collected at 24-h intervals. Denatured wild-type RNA showed a similar result (data not shown). Taken together, our data suggest that in the ALV system, RNA packaging and dimerization are partially separable events, as has been noted previously (41). One interpretation of the difference between wild-type and protease-mutant RNA is that the mutant RNA fails to form complete or stable dimers like the wild-type RNA.

Viral RNA-protein interactions. UV cross-linking has been used to identify associations between viral RNA and NC in RSV and MuLV virions (38, 53). We have adopted a UV cross-linking protocol, provided by Claude Méric, to probe protein-RNA interactions in protease-mutant virus particles. Freshly isolated virions were either UV irradiated or maintained on ice before being disrupted with 1.0% DOC and immunoprecipitated with anti-NC antiserum. The resulting immune complexes were digested with RNase A to remove excess RNA from the protein-RNA adducts and to produce free 5′ OH groups. The protein-RNA adducts were subsequently radiolabeled with T4 polynucleotide kinase and [α-^{32}P]ATP, fractionated by SDS-PAGE, and transferred to a membrane matrix. The membrane was submitted to autoradiography (Fig. 8A) and then probed with anti-NC antiserum and ^{125}I-labeled protein A before a second autoradiography (Fig. 8B).

This analysis revealed that both Pr76^{D37I} in the protease-mutant virion and mature NC in the wild-type virion became cross-linked to RNA by UV irradiation (Fig. 8A, lanes 1 and 15, respectively). In the non-cross-linked samples, only weak nonspecific labeling of two proteins of unknown identity (approximately 35 and 50 kDa) was observed (Fig. 8A, lanes 3, 7, and 17). The fact that ^{32}P incorporation into *gag* proteins depends on UV irradiation implies that the labeling is not due to phosphate-exchange or protein phosphorylation. The major labeled wild-type NC-RNA adduct, which had a size of ca. 13 kDa, represents NC-RNA monomers (38). The additional mass and negative charge present in this species made it migrate slightly more slowly than free NC (compare Fig. 8A, lane 15, with Fig. 8B, lane 18). The ca. 25-kDa product presumably represents an NC dimer, as observed previously (38), and as might be expected since nucleocapsid proteins interact with each other (50). Higher-order NC$_n$-RNA multimers also were observed on longer exposures (results not shown) (38). Probing with anti-NC serum demonstrated that unlike the mature NC-RNA adduct, the Pr76^{D37I}-RNA adduct did not migrate differently from non-cross-linked Pr76^{D37I} (compare Fig. 8A, lane 1, to Fig. 8B, lane 4).

To localize the domain of Pr76^{D37I} that interacts with RNA, the UV cross-linking protocol was modified to include PR digestion of mutant virions after DOC disruption. When anti-NC antibodies were used for immunoprecipitation, a 13-kDa ^{32}P-labeled NC-RNA adduct was observed (Fig. 8A, lane 5). In contrast, when anti-MA or anti-CA antisera were used, no significant ^{32}P signals were observed (not shown). These results indicate that Pr76^{D37I} interacts with viral RNA predominantly or entirely through its NC domain. Two important aspects of exogenous PR digestion should be noted. First, predisruption of mutant virions in 1.0% DOC did not affect subsequent exogenous proteolytic maturation of Pr76^{D37I} (Fig. 8B, lane 12). Second, protease digestion of Pr76^{D37I} was complete and unaffected by UV cross-linking, as monitored by SDS-PAGE and anti-NC immunoblotting (Fig. 8B, compare lanes 11 and 12).

Quantitative analysis of the cross-linking data suggests that the NC domain in the precursor interacts with the RNA in a different manner than does mature NC of wild-type virions. In our early experiments, approximately equal quantities of protease-mutant and wild-type virus particles were UV cross-linked. Under these conditions, no significant ^{32}P labeling of Pr76^{D37I} was observed, even though wild-type NC always yielded a strong signal. The same result was obtained regardless of the antibody used to immunoprecipitate the uncleaved *gag* precursor. We discovered subsequently that very large quantities of mutant virus particles are needed to produce ^{32}P labeling of Pr76^{D37I}. For example, in the experiment shown in Fig. 8, immunoblotting with anti-NC showed a signal for D37I that was at least 50 times greater than the barely detectable signals from wild-type NC (Fig. 8B; compare D37I [lanes 2 to 8] to D37 [lanes 16 to 18]). We estimate from the ratio of ^{32}P to ^{125}I that the apparent cross-linking efficiency is reduced by a factor of at least 1,000 for Pr76^{D37I} compared with mature NC of wild-type virions. This can be seen, for example, by comparing the ^{32}P signals from the NC-RNA adducts (Fig. 8A; D37I [lane 5] and D37 [lane 15]) with the ^{125}I signals from the total NC (Fig. 8B; D37I [lane 8] and D37 [lane 18]). We interpret this result to mean that the points of contact between RNA and the NC domain of Pr76^{D37I} are different from the points of contact between RNA and mature NC.

Reverse transcriptase activity. ALV reverse transcriptase

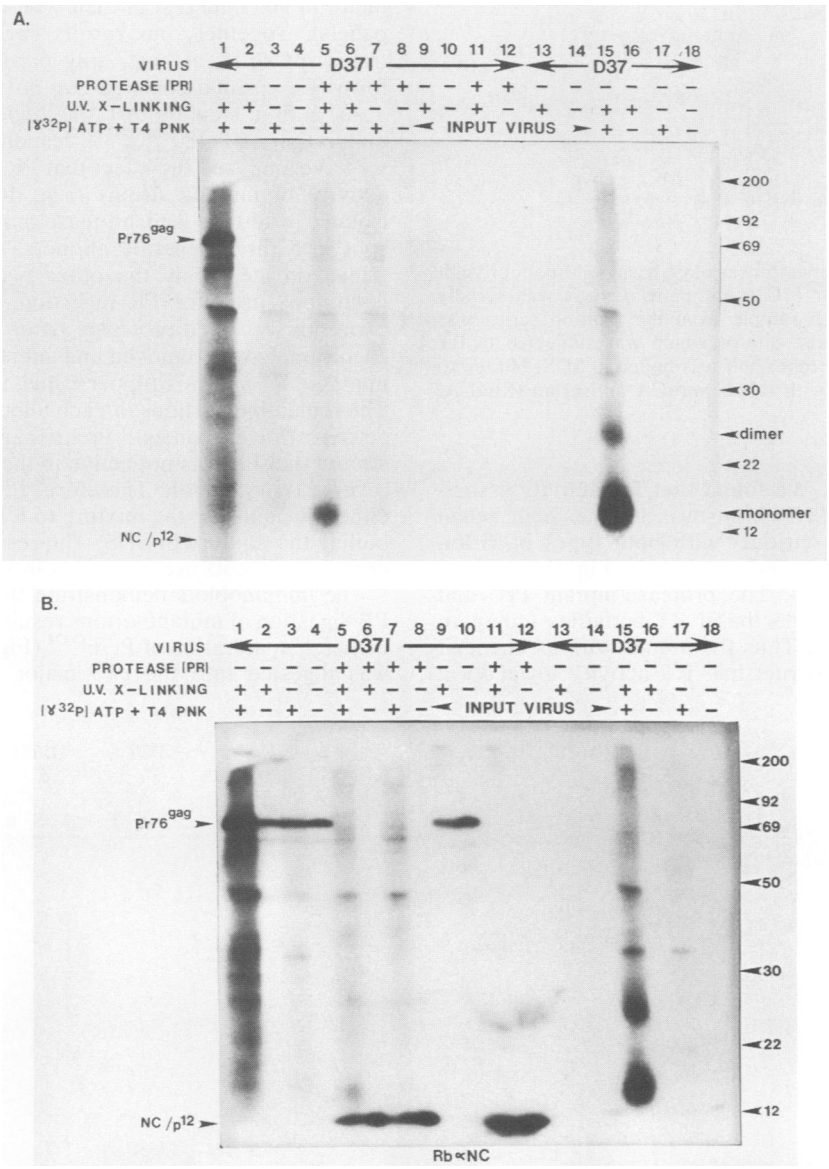

FIG. 8. UV cross-linking analysis. (A) Wild-type (D37) and protease mutant (D37I) viruses were subjected to UV cross-linking and then disrupted with DOC. Proteins were immunoprecipitated with rabbit anti-NC antiserum and protein A-Sepharose beads. Alternatively, the previously cross-linked or non-cross-linked, disrupted D37I virus was digested with PR prior to immunoprecipitation. Immunoprecipitates were incubated with RNase A and then split into two halves. One half was incubated at 37°C with T4 polynucleotide kinase (PNK) and [γ-^{32}P]ATP, while the other was maintained at 37°C for 30 min in kinase buffer. Finally, the immunoprecipitates were washed, disrupted by boiling in SDS, fractionated by SDS-PAGE, and electrotransferred to a membrane. The blot was wrapped while wet and submitted to autoradioagraphy for 15 h. Lanes 1 to 4, Immunoprecipitated D37I virus (250 ng); lanes 5 to 8, immunoprecipitated, PR-digested D37I virus (250 ng); lanes 9 and 10, input D37I virus without immunoprecipitation (250 ng); lanes 11 and 12, input PR-digested D37I virus without immunoprecipitation (250 ng); lanes 13 and 14, input D37 virus without immunoprecipitation (5 ng); lanes 15 to 18, immunoprecipitated D37 virus (5 ng). Above each lane, positive and negative signs indicate that the sample received (+) or did not receive (−) UV cross-linking, digestion with protease, or labeling with kinase. (B) The blot shown in panel A was subsequently probed with rabbit anti-NC serum (RbαNC) followed by ^{125}I-labeled protein A and then exposed again to X-ray film for 12 h. The autoradiograph thus shows a superposition of ^{32}P and ^{125}I labels.

is thought to be activated through proteolytic maturation of the initially inactive *gag-pol* precursor (43, 47). If this notion is correct, protease mutant virions would be expected to display reduced RT activity. To assess RT activity, we subjected freshly isolated mutant and wild-type virions to exogenous RT assays and parallel anti-CA Western slot blot analysis to normalize the amount of viral protein. In over 10 experiments, we consistently found that the mutant SR-A virions exhibited RT activity reduced by a factor of 30 relative to wild-type virions (Fig. 9, left panels). Since SR-A virus encoded by the RCAS*neo* vector has very low infectivity in quail cells (Hughes, personal communication), we decided to test the effect of the D37I mutation on RT activity in another ALV strain, PrC. The major portions of the *gag* and *pol* genes of PrC were substituted for the equivalent SR-A sequences in the RCAS*neo* vector, as described in

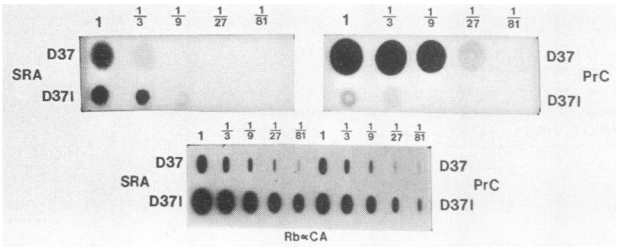

FIG. 9. Exogenous reverse transcriptase assays. Mutant or wild-type SR-A (left panels) or PrC (right panels) virus was serially diluted (1:3) in STE. Each sample from the dilution series was divided into two equal parts, one of which was subjected to RT assays (upper panels). The other half was boiled in SDS, blotted to a membrane, and probed with rabbit anti-CA antiserum (RbαCA, lower panel).

Materials and Methods. We found that RT activity associated with the wild-type PrC virions was 30 times higher than that in wild-type SR-A virions, with both types of virion harvested from batch-selected quail cells (Fig. 9; compare D37 SR-A with D37 PrC). The protease-mutant PrC and SR-A virions showed similar basal RT activities (compare D37I SR-A to D37I PrC). Thus the mutant virions encoded by the PrC*neo*D37I construct had RT activity lower by a factor of 900 than that of their wild-type parent (Fig. 9, right panels). Together, our results suggest that ALV reverse transcriptase is, at best, only partially active when in the form of a virion-associated *gag-pol* polyprotein.

As a first step to test the hypothesis that proteolytic maturation of $Pr180^{gag\text{-}pol}$ is responsible for activating RT, we have analyzed the effect that PR digestion has on the RT activity of mutant virions (Fig. 10). To do this, freshly isolated wild-type and protease-mutant SR-A virions were split into three separate aliquots. One aliquot was maintained on ice, while the other two were adjusted to the conditions used for PR digestion, either with or without exogenously added protease. After incubation, a portion of each sample was removed and subjected to SDS-PAGE and immunoblot analysis (upper panels; anti-CA and anti-MA). The remaining portions of each aliquot were assayed for RT activity (lower panels). Preliminary experiments demonstrated that PR was not active in the buffer and salt used for reverse transcription. Therefore, PR digestion was stopped either by adjusting the mixture to RT assay conditions or by boiling the mixture in SDS. The results from three independent experiments are presented in the three sets of panels.

The immunoblots demonstrate that in these experiments PR digestion of mutant virions resulted in complete or nearly complete maturation of $Pr76^{D37I}$ (Fig. 10, lanes 6). $Pr180^{D37I}$ was digested into the two major *pol*-related species de-

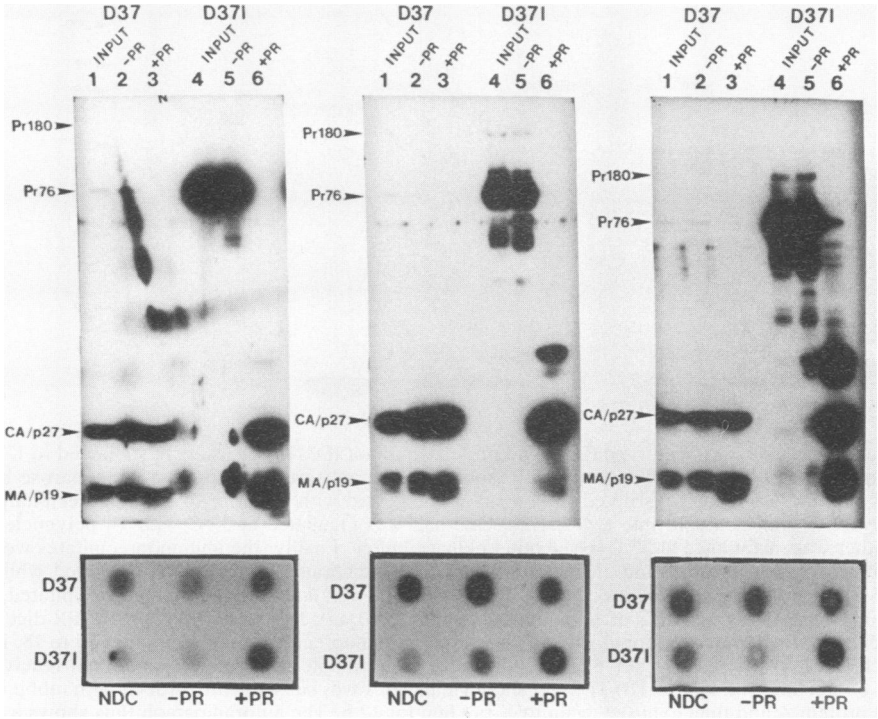

FIG. 10. Stimulation of RT activity in mutant virions by PR digestion. Mutant (D37I) and wild-type (D37) SR-A viruses were split into three equal 30-μl samples (1 plate-day per 10 μl). One sample was maintained on ice (no digestion conditions [NDC or input]). The other two samples were subjected to PR digestion conditions (45-μl final volume) with (+PR) or without (−PR) added protease. Following incubation, a portion of each sample was removed and immediately boiled in SDS. These small portions were subsequently analyzed by SDS-PAGE and immunoblotting (upper panels). The remaining virus in each sample was then subjected to exogenous RT assays. The reaction volumes were sufficiently large to ensure that the high salt concentration and low-pH conditions of PR digestion were effectively diluted to the final RT assay conditions as described in Materials and Methods. To ensure that all samples were assayed under the same conditions, the NDC samples were adjusted to PR digestion conditions on ice immediately before being adjusted to exogenous RT assay conditions. (Upper panels) Immunoblot analysis with a mixture of rabbit anti-CA (RbαCA) and rabbit anti-MA (RbαMA) antisera. Lanes 1 to 3, Wild-type virus (D37); lanes 4 to 6, protease-mutant virus (D37I). (Lower panels) Exogenous RT assays. Upper rows, Wild-type virus (D37); lower rows, protease-mutant virus (D37I).

scribed above, 120-kDa$^{gag\text{-}pol}$ and 40-kDa^{pol} (data not shown). Without added PR, the mutant polyproteins remained intact (Fig. 10, lanes 5). RT activity was stimulated by a factor of approximately 5 by PR digestion in these and similar experiments. For example, compare the D37I spot intensity for the no-digestion-condition (NDC) control or −PR samples with that of the +PR samples in the lower panels (Fig. 10). The RT activity of wild-type virions was relatively unaffected by PR digestion conditions, with or without added PR (lower panels; compare D37 spot intensity for NDC to −PR or +PR samples). These results indicate that digestion of mutant Pr180^{D37I} by exogenous protease does not lead to complete activation of RT. Our working model to account for these results states that the incomplete activation of RT by PR is due to the observed incomplete cleavage of Pr180^{D37I}. According to this model, the *gag* sequences that remain attached to the N terminus of RT act in *cis* to inhibit the enzyme. Experiments to test this idea are in progress.

DISCUSSION

To better define the function of the viral protease in the assembly of ALV, we mutated the PR-coding sequence at the active site and studied the properties of the resulting mutant virus particles. Some of our findings corroborate observations in other systems, while several are new. For example, in all cases that have been examined (8, 14, 23, 48, 64), inactivating PR does not interfere with assembly or release of particles but prevents morphological maturation (i.e., condensation) of the core after release and renders the virus noninfectious. In ALV, the morphology of protease mutant particles was noted earlier (64) but was not examined in detail. Previous reports of immature MuLV cores (37, 68) and cores of the protease-deficient Gazdar murine sarcoma virus (49) indicated their stability in the presence of detergents, but this aspect of protease-defective particles has not been studied in a systematic manner. Using both centrifugation and electron microscopy, we have shown that protease-mutant ALV cores are stable in 0.5% Triton X-100 at 37°C, a condition that completely disrupts wild-type virus. Since immature MuLV behaved identically in these assays, it seems likely that all retroviruses share this property. This stability of immature retroviruses suggests that part of the function of proteolytic cleavages may be to facilitate disassembly of the virus particle after infection of a cell. Stability in the presence of detergents may be of practical importance, since complete disruption of cores presumably is necessary to liberate soluble forms of *gag* and *gag-pol* proteins that are needed for some in vitro analyses. For example, the tacit assumption of Panet and Baltimore (46) that nonionic detergent leads to solubilization of RT activity may help to explain the discrepancy between their data and those of Crawford and Goff (8) on the RT activity of a protease mutant of MuLV. It is noteworthy that by electron microscopy, immature cores that have been treated with detergent still show two major rings of electron dense material. The outer ring coincides approximately with the outer ring in intact mature or immature particles, which often has been considered to represent the "membrane" of the virus. But since at least 95% of the phospholipid is stripped from the immature cores by detergent treatment (Southard and Vogt, unpublished observations), the outer ring is clearly protein. We suggest that it represents the MA domain of the *gag* precursor.

Incubation of mutant virus with PR and detergent resulted in proper cleavage of Pr76^{D37I} into the mature forms of MA, CA, NC, and PR. With few exceptions (43), complete maturation of MA has rarely been observed when Pr76^{gag} is digested with PR in vitro. The intermediate MA-related cleavage products Pr32^{gag} and p23 were produced instead (43, 62–64). In nearly all of these reports, the polyprotein substrates were soluble, having been obtained by immunoprecipitation of infected cell lysates or by in vitro translation. By contrast, the substrate in our experiments was virion associated. Thus, we considered the possibility that efficient processing of MA may require the self-association of Pr76^{gag} in a particle. This possibility appears to be ruled out by the observation that predisruption of mutant virions in 1.0% DOC did not alter proteolytic maturation of Pr76^{D37I} (Fig. 8B) or release of mature MA (data not presented). However, we cannot exclude the possibility that small aggregates of Pr76^{D37I} could remain after this treatment. Perhaps the *gag* precursor in virions, but not in cell lysates or reticulocyte extracts, bears secondary modifications that facilitate complete cleavage. In contrast to Pr76^{D37I}, Pr180$^{D37I gag\text{-}pol}$ was not completely cleaved in our experiments. A similar observation was made with wild type *gag-pol* precursor several years ago (10). The major *pol* species generated still carries *gag* sequences at its N terminus. Further studies will be required to determine the exact structure and possible functional significance of the *pol*-related PR digestion products of mutant virions.

Although several reports in the literature have described protease-defective viruses (8, 14, 23, 26, 48, 64), only one included a partial characterization of viral RNA. Crawford and Goff utilized endogenous reverse transcription experiments to show that the primer tRNA^{Pro} is properly annealed to genomic RNA within *dl*2905 protease-defective MuLV virions (8). Our Northern slot blot and tRNA end-labeling experiments lead to a similar conclusion for the protease-mutant ALV particles. Therefore, in both ALV and MuLV, polyprotein processing is not necessary for genomic RNA encapsidation and primer tRNA annealing. However, processing does appear to be necessary for complete or proper dimerization of the viral RNA. Previous studies have not examined the possible role of proteolysis in this function. We found that a fraction, typically about one-half, of the genomic RNA from mutant virions remains as a monomeric species, while the rest is in the form of higher-order structures that migrate at a rate similar to or slower than that of dimeric RNA of wild-type virus. We hypothesize that encapsidation precedes dimerization during assembly and that core maturation is necessary for complete and proper dimerization. This hypothesis was proposed many years ago on the basis of analyses of the genomic RNA obtained from rapidly harvested (3 to 5 min) ASLV virions, which contain largely monomeric RNA (5, 7, 27). Those experiments revealed that dimerization of genomic RNA was coincident with polyprotein cleavage and core maturation (7, 27). It is also of interest to note the similarities between the D37I RNA we have studied and the RNA extracted from virions of the temperature-sensitive RSV mutant *LA*334. When obtained from virus harvested at the nonpermissive temperature, this RNA is characterized by nearly equal proportions of a 35S species and a heterogeneous collection of RNAs in the range of 80S to 125S (16). At this temperature, *LA*344-infected cells demonstrate suppression of particle release and a concomitant accumulation of partially budded virions at the plasma membrane (12). Those virions that are released have abnormal morphology, are noninfectious, and contain aberrantly processed Pr76^{gag} (19, 55). In contrast, *LA*334

particles released at the permissive temperature have wild-type properties and contain properly dimerized 70S RNA. These characteristics of the *LA*334 mutant also suggest that accurate polyprotein processing and core maturation are necessary for proper dimerization.

The nucleocapsid proteins of retroviruses are highly basic nucleic acid-binding proteins that contain one or two conserved cysteine-histidine amino acid sequence motifs (Cys-His boxes) and that bind along the length of the RNA genome to form the nucleocapsid structure (reviewed in reference 65). Data from UV cross-linking (38, 53), mutational analyses (39–41), and in vitro biochemical studies (4, 53, 54) indicate that NC functions in encapsidation of RNA, dimerization of RNA, and annealing of primer tRNA. Since virions are initially formed from precursor polyproteins, it seems likely that the NC domain functions in some of these tasks while it is still embedded in the *gag* precursor. Our UV cross-linking results indicate that the *gag* polyprotein of protease mutant virions interacts with RNA primarily through its NC domain. However, the apparent NC-RNA cross-linking efficiency of mutant virions is dramatically reduced, by a factor of approximately 1,000, when compared with that of wild-type virions. Since protein-RNA adducts are detected by their ability to be immunoprecipitated and then ^{32}P labeled with polynucleotide kinase after RNase A digestion, either of these steps in principle could be responsible for the apparent reduction of cross-linking. However, it is unlikely that these factors are significant, since the ^{32}P signals from mutant virions were not altered by prior PR digestion or by the use of different anti-*gag* antisera for immunoprecipitation (data not presented). We consider it most probable that the ^{32}P signals produced by the cross-linking protocol actually are a good reflection of the relative quantities of protein-RNA adducts generated. Given that protease-mutant virions encapsidate normal quantities of viral RNA, the cross-linking results imply that the interactions between viral RNA and the NC domain of $Pr76^{D37I}$ differ from the NC-RNA interactions within wild-type virions. Either there are fewer protein-RNA contacts within mutant virions or the contacts differ in geometry or proximity and thus do not lead to efficient cross-linking by UV irradiation. We envision the possibility that the *gag* polyprotein of immature virions interacts only with certain sequence elements of genomic RNA, such as encapsidation signals (24).

Protease-mutant virions provide a source of virion-associated *gag-pol* polyprotein and thus can be used to test the hypothesis that ALV reverse transcriptase is activated by proteolytic maturation. We find that mutant virions demonstrate low but measurable levels of RT activity. Depending on the ALV strain used, they have a reduction by a factor of either 30 (SR-A) or 900 (PrC) in RT relative to wild-type virions. While we cannot completely rule out the possibility that the template and primer used in the RT assays for steric reasons fail to enter the mutant core, we favor the explanation that $Pr180^{D37I}$ inherently has feeble RT activity. The strain differences result from the fact that wild-type PrC virions exhibit 30 times more RT activity than similar quantities of wild type SR-A virions. Experiments designed to define the factor(s) responsible for such differences are now in progress. The apparent deficiency in the amount of the RTα subunit of wild-type SR-A virions might well explain their reduced RT activity. In any case, the results from two different strains suggest that ALV *gag-pol* polyprotein is relatively inactive as a reverse transcriptase. Similar results have been obtained by J. Wills and R. Craven (personal communication) by expression of ALV *gag-pol* (PrC) constructs in monkey COS cells.

Moelling et al. originally reported that rapidly harvested RSV virions, which are enriched in unprocessed polyproteins, have low RT activity and that this activity is augmented by incubation under conditions under which processing occurs (43). Furthermore, digestion with added PR in the presence of detergent accelerated the appearance both of the mature RT subunits and of RT activity. This evidence prompted us to test the effect of PR digestion on the RT activity associated with mutant virions. We found that digestion reproducibly increases RT activity fivefold for mutant SR-A virions. The PR-mediated stimulation was incomplete, however, since it did not produce the levels of RT activity associated with equal quantities of wild-type SR-A virions under the same conditions. PR digestion also failed to completely digest $Pr180^{D37I}$, yielding instead two major *pol*-related products of 120 and 40 kDa. Since both processing and enzymatic activation were incomplete, we hypothesize that amino acid sequences that remain attached to the N terminus of RT act in *cis* to inhibit activity. We are now attempting to develop both in vitro translation and bacterial-expression systems to delineate which N-terminal *gag* amino acids are inhibitory to RT activity.

The reason for incomplete processing of $Pr180^{D37I}$ remains unclear. It is formally possible that the particular D37I mutation used in this study leads to a conformational change in the *gag-pol* precursor such that it can not be properly cleaved. To address this possibility, we are currently analyzing virions with other PR mutations. A more likely reason for the incomplete maturation of $Pr180^{D37I}$ is that the conditions for cleavage in vitro are not optimal, perhaps because auxiliary factors are missing. One such factor could be phosphorylation. Evidence suggests that phosphorylation of the IN domain may be a necessary prerequisite for complete proteolytic maturation of $Pr180^{gag\text{-}pol}$ in ALV (10). For MuLV, it has been suggested that phosphorylation of the *gag* precursor, $Pr65^{gag}$, is required for correct cleavage (69). Phosphorylation sites on the *gag* and *gag-pol* precursor proteins have yet to be mapped for any retroviral system.

The low RT activity associated with protease-mutant ALV virions is in direct contrast to what has been observed for protease-mutant MuLV, which demonstrates normal RT activity (8, 23). Perhaps proteolytic maturation of the *gag-pol* precursor is not necessary for activation of RT in this system. Alternatively, since the PR mutations in MuLV were deletions, the appearance of RT activity could result from coincidental deletion of a domain that would otherwise have an inhibitory effect on RT. In HIV, extensive analyses of bacterially expressed RT clearly demonstrate that proteolytic removal of both the PR domain (at the N terminus of RT) and the IN domain (at the C terminus of RT) is necessary for full activation of RT activity (32–34). Furthermore, PR active-site mutations in HIV result in virions with RT activity reduced by a factor of 6 relative to wild-type virions (14). The interplay between proteolysis and activation of virion-associated RT is an area of retrovirology that remains largely unexplored. To gain a better understanding of the role of proteolysis in the activation of ALV RT, we have begun experiments to test the ability of virion-associated $Pr180^{D37I}$ to be matured and activated in *trans* when copackaged with wild-type *gag* polyprotein.

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ADDENDUM IN PROOF

We have constructed an alternative PR mutant, D37N, and found that it behaves in every manner like the D37I mutant described herein. We also found that coexpression of the wild-type *gag* protein with D37I or D37N *gag* and *gag-pol* proteins leads to efficient *trans* cleavage of the mutant Pr180 and consequent activation of RT.

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