Reverse Transcriptase and Protease Activities of Avian Leukosis Virus Gag-Pol Fusion Proteins Expressed in Insect Cells

LANCE STEWART† AND VOLKER M. VOGT*

Section of Biochemistry, Molecular and Cell Biology, Biotechnology Building, Cornell University,
Ithaca, New York 14853

Received 29 June 1993/Accepted 7 September 1993

Protease (PR)-defective avian leukosis virus particles display 300-fold-reduced levels of reverse transcriptase (RT) activity relative to wild-type particles. This observation suggests that during virion assembly RT is activated by proteolytic maturation of the Gag-Pol polyprotein precursor. To study the relationship between proteolytic cleavage and RT activation, we subjected PR-defective virion cores to digestion with purified viral PR and analyzed the structure of the major polypeptides produced as well as RT activity. Under conditions in which Gag precursors were fully matured, the RT domain was only incompletely released from the Gag-Pol precursor, remaining tethered to the upstream Gag domains PR or NC-PR. In the same reaction, RT activity was stimulated only three-fold, or 100-fold less than expected for a fully active RT. The poor activation suggested that the NC or PR domains could repress RT activity. To test this idea, we constructed recombinant baculoviruses expressing 19 different fusion proteins with upstream Gag or downstream Pol sequences attached to RT. Each protein was partially purified and assayed for its inherent RT activity. The results are consistent with the idea that Gag sequences can inhibit RT activity but indicate that the size of the Pol domain as well as the status of the PR domain (wild-type or mutant) also can profoundly influence activity. Several of the constructed Gag-Pol fusion proteins contained a wild-type PR domain. Some of these underwent intracellular PR-mediated processing, while others did not. All proteins in which the PR domain was preceded by upstream Gag sequences showed specific proteolysis. By contrast, all proteins initiated with a methionine placed one residue upstream of the natural N terminus of PR failed to show specific proteolysis. Amino-terminal sequencing of one such protein yielded the correct amino acid sequence and showed that the initiating methionine was not removed. One interpretation of these findings is that activation of PR requires the generation of the precise N terminus of the mature PR.

Together, the *gag* and *pol* genes of retroviruses encode the major structural and enzymatic components of viral cores. These two genes are translated as Gag and Gag-Pol polyprotein precursors, which are cleaved during virion morphogenesis to give rise to the mature core proteins (7). In the avian leukosis virus (ALV) system, the *gag* gene product Pr76^{gag} is composed of the following five major protein domains, from the N to the C terminus: matrix (MA), p10, capsid (CA), nucleocapsid (NC), and protease (PR) (see reference 37 for nomenclature). PR, an aspartate protease whose crystal structure is known (26; for reviews, see references 6, 10, 32, and 46), is the enzyme that processes both Gag and Gag-Pol polyproteins. The *gag-pol* gene product Pr180^{gag-pol}, which is translated via a ribosomal frameshift (24), is composed of Gag followed by the reverse transcriptase (RT) and integrase (IN) domains. In addition, at the C terminus of Pol in ALV is a short stretch of polypeptide called 4kDa^{pol} which is proteolytically removed by PR (1) and which is dispensable for formation of infectious particles (28). Most of the RT molecules isolated from ALV particles are α/β heterodimers, in which α is the RT domain proper (here referred to as RT α) and β is composed of RT plus IN (here referred to as RT β). Thus, RT β and RT α have common N termini but differ by the absence or presence of the 32-kDa IN domain (1, 3, 13). RT not only synthesizes the double-stranded viral DNA but also contains the RNase H activity that degrades the RNA template. Both subunits of the

ALV RT have DNA polymerase as well as RNase H activities when individually purified (17) or expressed in *Escherichia coli* (50). However, as expected from its structure, only the RT β subunit (in addition to IN itself) displays the endonuclease activity associated with integrase (8, 12).

Virus particles contain roughly equal numbers of RT α , RT β , and IN polypeptides, and this stoichiometry may be critical for viral infectivity (16). Three forms of RT can be isolated from ALV particles: monomeric RT α , homodimeric RT β -RT β , and the predominant heterodimeric RT α -RT β . The β subunit purified from virions or expressed in *E. coli* is homodimeric. In contrast, the α subunit was reported to be monomeric when isolated from virions (17) but dimeric when isolated from *E. coli* (50). At 20°C and in the presence of 4% glycerol, the native active form of ALV RT appears to be a globular tetramer composed of two α/β heterodimers (39). Prolonged incubation under such conditions results in the dissociation of the tetramer into individual α/β heterodimers that are inactive.

Evidence suggests that in the ALV system, RT is activated by proteolytic maturation of the Gag-Pol precursor. In 1975, it was reported that a Pol-related protein (presumably Pr180^{gag-pol}) isolated from Rous sarcoma virus (RSV) (a close relative of ALV)-infected cells has low RT activity (45). It was demonstrated in 1980 that rapidly harvested RSV virions, which are enriched in unprocessed polyproteins, have low RT activity and that this activity increases during incubation under conditions in which processing occurs (40). In addition, exogenous PR digestion of the rapidly harvested RSV particles, in the presence of detergent, was reported to accelerate the appearance of mature RT and of RT activity (9, 40). More recently it was shown that the RT activity in PR-defective

* Corresponding author.

† Present address: Department of Microbiology, University of Washington, Seattle, WA 98195.

particles is reduced at least 100-fold relative to that of the wild type (41, 42, 51). We and others also have performed a series of in vivo complementation experiments wherein wild-type or PR-mutant Gag precursors were separately coexpressed with wild-type or PR-mutant Gag-Pol precursors (4, 41, 52). These experiments revealed that when wild-type Gag precursors are coassembled with PR-mutant Gag-Pol precursors, the resulting virions undergo complete and proper maturation, have wild-type levels of RT activity, and even are fully infectious (52). These results imply that PR supplied by Gag precursors is sufficient for proper *trans* maturation of Gag-Pol proteins and activation of RT.

We reported previously that incubation of PR-defective virus particles with purified PR in the presence of detergent leads to the complete and correct maturation of Pr76^{gag} (51). However, under these conditions Gag-Pol proteins are incompletely matured and RT activity is stimulated only slightly. In particular, the amino acid sequence between Gag and Pol remains refractory to cleavage. These observations led us to hypothesize that protein domains upstream of RT could act in *cis* to inhibit activity. To test this hypothesis, we constructed recombinant baculoviruses expressing a variety of Gag-Pol fusion proteins and then analyzed the inherent RT activity of each partially purified protein. The results demonstrate that RT activity depends on the size of the Gag domain, the size of the Pol domain, and whether or not the PR domain is mutated.

MATERIALS AND METHODS

DNA constructs and cell culture conditions. All plasmids were constructed by common subcloning techniques and propagated in the DH5 α strain of *E. coli*. Nucleotides of the RSV genome are numbered according to Schwartz et al. (49). Plasmids PrCneo.D37, PrCneo.D37I, and PrCneo.D37N have been described previously (52).

Mutagenic oligonucleotides and polymerase chain reaction techniques were used to engineer initiating methionine codons immediately 5' to the first codons of the NC, PR, and RT α coding regions of the Prague C (PrC) strain of ALV. The same techniques were also used to engineer stop codons immediately 3' to the last codons of the RT α and IN coding regions. The subcloned sequences that had been subjected to polymerase chain reaction were sequenced to confirm proper construction of translation initiation and termination signals and to ensure that no other mutations were present.

The baculovirus transfer vector pJV(*NheI*) (58) has a unique *NheI* cloning site, located immediately downstream of the baculovirus polyhedrin gene, into which we inserted ALV sequences encoding 23 different open reading frames (see Fig. 3). Nineteen of the pJV(*NheI*)-based derivatives consisted of PrC sequences encoding truncated Gag-Pol proteins. The open reading frame of each construct is implied by its name. Thus, plasmid pJV.RT α contains the sequences from nucleotides 2503 to 4219 (encoding the open reading frame for RT α) flanked by translation start and stop signals. Similarly, pJV.RT β and pJV.Pol have sequences that begin at nucleotide 2503 and end at 5076 and 5188, respectively. The PrC sequences of pJV.PR-RT α , pJV.PR-RT β , and pJV.PR-Pol each begin at nucleotide 2111. In the same manner, pJV.NC-RT α , pJV.NC-RT β , and pJV.NC-Pol have sequences that begin at nucleotide 1844. Plasmids pJV.CA-RT β and pJV.CA-RT α .D37N each contain an open reading frame that initiates at a naturally occurring methionine codon immediately 5' to the first codon of CA, which starts at nucleotide 1097. The latter construct was also engineered to contain the D37N

and D41Y PR mutations, which are indicated by the "D37N" suffix for simplicity. Plasmids pJV.PR-RT α .D37N, pJV.PR-RT β .D37N, pJV.PR-Pol.D37N, pJV.NC-RT α .D37N, pJV.NC-RT β .D37N, pJV.NC-Pol.D37N, and pJV.CA-Pol.D37N also encode mutant PR domains.

Plasmid pJV.PR-RT β .COLL is similar to pJV.PR-RT β except that it contains a consensus cleavage site for collagenase Pro-Leu-Gly-Pro in place of the Leu-Thr residues normally located at the PR-RT α cleavage site. Plasmid pJV.E-RT β (the "E" signifies that the PrC sequences begin at the *EcoRI*²³¹⁹ site) contains an open reading frame that initiates at a naturally occurring methionine codon for residue 73 within PR (nucleotide 2327) and terminates at nucleotide 5076.

Each of the above-mentioned constructs that encode both Gag and Pol domains was engineered to contain a single nucleotide (A) insertion immediately preceding the gag termination codon. This insertion aligns the Gag and Pol reading frames (frame correction [FC]) without altering the Gag-Pol coding sequence and hence eliminates the frameshift requirement for Pol translation.

The entire Gag-Pol open reading frame was also cloned into pJV(*NheI*). Three such constructs—pJV.GP.D37N.FC, pJV.GP.D37.FC, and pJV.GP.D37—are composed entirely of PrC sequences, while a fourth—pJV.Gag-Pol.D37N.FC—is composed of PrC sequences up to nucleotide 4995 followed by RAV-1 sequences through the rest of *pol*. Both pJV.Gag-Pol.D37N.FC and pJV.GP.D37N.FC contain the D37N plus D41Y mutations in PR as well as the FC that fuses Gag and Pol. pJV.GP.D37.FC encodes a wild-type PR domain and contains the FC mutation. pJV.GP.D37 encodes a wild-type PR domain and lacks the FC mutation and thus contains the *gag* and *pol* genes as they are present in infectious ALV.

QT35 quail cells were propagated as previously described (52). *Spodoptera frugiperda* insect cells (SF21) were propagated at 28°C in Grace's medium (GIBCO) supplemented with 1 $\times$ Yeastolate (GIBCO), 1 $\times$ lactalbumin hydrolysate (GIBCO), 10 μ g of gentamicin per μ l, and 5% fetal calf serum.

Isolation of recombinant baculoviruses. Recombinant baculoviruses were generated by cotransfecting SF21 cells with wild-type *Autographica californica* multiple nucleocapsid nuclear polyhedrosis virus DNA together with transfer vector DNAs. Homologous recombination between the transfer vector and the polyhedrin gene of virus results in the formation of recombinant baculoviruses that encode both β -galactosidase and the gene that was introduced into the *NheI* cloning site of the transfer vector (58). Transfection was performed by mixing *A. californica* multiple nucleocapsid nuclear polyhedrosis virus (5 μ g) and transfer vector DNA (5 μ g) (100 μ l total in Tris-EDTA) with 30 μ l of Lipofectin reagent (Bethesda Research Laboratories). The transfection cocktail was incubated at 28°C for 10 min before being added dropwise to a 60-mm-diameter plate containing 5 $\times$ 10⁵ SF21 cells bathed in 3 ml of serum-free insect cell medium. The cells were incubated at 28°C for 24 h, at which time 3 ml of complete Grace's medium was added to the plate, which was then adjusted to a final concentration of 10% fetal calf serum. Culture supernatants were harvested 4 days after transfection. The recombinant viruses were then purified by reiterative plaque purification of viruses that encode β -galactosidase, as determined by the conversion of the chromogenic substrate X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) into blue product. This was accomplished by plating $\sim$ 100- μ l drops of serially diluted culture supernatants into 96-well culture plates containing SF21 cells. Four days later, 1 drop of X-Gal (1 mg/ml) was added to each well. Color was allowed to develop for 24 h at 28°C. The medium from wells containing blue reaction product

and the fewest polyhedrin occlusion bodies was then harvested. This procedure was reiterated until pure recombinant virus was isolated. The names of the recombinant viruses are prefixed by "Ac," which is followed by the name of the ALV open reading frame carried by the virus. For example, Ac-PR-RT α encodes PR-RT α . Titers in virus stocks were determined by plating ~100- μ l drops of serially diluted virus into 96-well culture plates containing SF21 cells. Four days later, X-Gal was added and color was allowed to develop for 24 h. The highest dilution that still gave blue wells at this time was considered to contain at least 1 infectious unit per 100 μ l. Generally, stocks contained between 10^{10} and 10^{11} infectious units per ml.

Partial purification of recombinant proteins. Subconfluent (50%) 100-mm (diameter) plates of SF21 cells were infected with purified recombinant baculoviruses at a multiplicity of infection greater than 100. After incubation at 28°C for 72 h, the cells were washed twice with phosphate-buffered saline (PBS) and then resuspended on ice in 1.0 ml of sonication buffer (20 mM Tris-HCl [pH 7.9], 25% glycerol, 700 mM NaCl, 0.01% Nonidet P-40, 2 mM dithiothreitol). After sonication (three times, 6-s pulses with a model 350 Sonifier cell disruptor at output level 6), samples were centrifuged for 30 min at $14,000 \times g$ and 4°C in a microcentrifuge. Supernatant fractions were adjusted to 50% glycerol and then stored at -20°C for no more than 2 days before being fractionated by DEAE-ion-exchange chromatography. Whole-cell sonicates were diluted fivefold by the addition of 4 volumes of buffer A (20 mM Tris-HCl [pH 7.9], 25% glycerol, 0.01% Nonidet P-40, 2 mM dithiothreitol) and then passed through a 0.22- μ m-pore-size syringe filter. The filtrate was then pumped over a DEAE MemSep (Millipore) column that had been preequilibrated with buffer A (1.0-ml/min flow rate, using the Pharmacia fast-performance liquid chromatography apparatus). One-milliliter fractions were then eluted with a linear 10-ml salt gradient from no salt to 600 mM NaCl (1.0-ml/min flow rate). The fractions were immediately adjusted to 50% glycerol and stored at -20°C for no more than 10 days before being subjected to RT assays.

Retrovirus isolation and immunoblotting. The procedures for retrovirus isolation from culture medium and immunoblotting have been described previously (52). The amount of virus shed in 24 h from a 100-mm-diameter plate is defined as 1 plate day. Equilibrium density sedimentation of virions was achieved by centrifugation of 20 to 60% (wt/vol) sucrose-STE (100 mM NaCl, 10 mM Tris-HCl [pH 7.5], 1 mM EDTA) step gradients at 40,000 rpm for 12 h or 50,000 rpm for 4 h at 4°C in a Beckman SW-60 rotor. A 1-ml syringe was used to recover the virions from the 20 to 60% sucrose interface.

Anti-MA and anti-CA antisera were raised in rabbits that had been injected with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)-purified avian myeloblastosis virus (AMV) viral antigens MA (p19) and 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. Anti-RT α and anti-IN-4kDa^{pol} antisera were raised in rabbits that had been injected with SDS-PAGE-purified proteins that were overproduced in *E. coli* according to the methods of Soltis and Skalka (50) and Alexander et al. (1). Immunoaffinity purification of the anti-RT α and anti-IN-4kDa^{pol} antisera was achieved by incubating a 1:5 dilution of crude antisera—in RB (150 mM NaCl, 10 mM Tris-HCl [pH 7.5], 1 mM EDTA, 0.1% Tween-20 [Sigma]) plus 1% lowfat milk—with an Immobilon-P (Millipore Corp.) membrane disk (2 cm²) to which 200 μ g of SDS-PAGE-purified antigen had previously been immobilized. After immunoabsorption overnight at 4°C, the membrane disk was washed once in RB at room temperature and then

incubated in 1.0 ml of 10 mM glycine (pH 2.5) for 10 min at room temperature to elute the immunoglobulin G (15). The pH of the eluate was then adjusted to 8.0 with the addition of 100 μ l of 1 M Tris-HCl (pH 8.0). Affinity purification of anti-NC antiserum was performed in a similar manner except that the immobilized antigen was bacterially expressed CA-NC protein.

PR digestion and RT assays. AMV PR was isolated from disrupted AMV by chloroform-methanol extraction (27). The final PR digestion conditions that were common among all samples were 83 mM Tris acetate (pH 6.8), 6 mM MgCl₂, 0.6 mM EDTA, ~20% (wt/vol) sucrose, 0.1% Triton X-100, 6% glycerol, 1 mM dithiothreitol, presence or absence of 100 μ g of PR per ml, and incubation at 37°C for 60 min. The NaCl concentrations present in the digestions are described in the appropriate figure legends. Digestions were stopped either by boiling in SDS or by further adjusting the sample to RT assay conditions.

RT activity was measured as the incorporation of [α -³²P]dTTP into high-molecular-weight DNA through utilization of an exogenously provided homopolymeric RNA template, poly(rA), and 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 ~35% sucrose-STE, PR-digested virus, or partially purified recombinant proteins. Two different final assay conditions were utilized. (i) The final RT assay condition used in the PR digestion experiments was 17 mM Tris acetate (pH 6.8), 50 mM Tris-HCl (pH 8.3), ~4% (wt/vol) sucrose, 1.2% glycerol, 180 mM NaCl, 7.2 mM MgCl₂, 20 mM dithiothreitol, 0.1 mM ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 0.05% Nonidet P-40, 0.02% Triton X-100, 5 μ g of oligo(dT)₁₂₋₁₈ per ml, 10 μ g of poly(rA) per ml, 100 μ Ci of [α -³²P]dTTP (3,000 Ci/mmol; Amersham) per ml, and 10 μ M dTTP (final specific activity, 10 Ci/mmol of [α -³²P]dTTP). (ii) The final RT reaction condition for assaying activity of recombinant proteins was 50 mM Tris-HCl (pH 8.3), 92 mM NaCl, 6 mM MgCl₂, 20 mM dithiothreitol, 0.1 mM EGTA, 0.05% Nonidet P-40, 5 μ g of oligo(dT)₁₂₋₁₈ per ml, 10 μ g of poly(rA) per ml, 100 μ Ci of [α -³²P]dTTP (3,000 Ci/mmol; Amersham) per ml, and 10 μ M dTTP (final specific activity, 10 Ci/mmol of [α -³²P]dTTP). Reaction mixtures of 50 μ l each were incubated for 60 min at 37°C, and reactions were stopped by the addition of an equal volume of Tris-EDTA-equilibrated phenol, followed by vortexing. A 5- μ l aliquot of the aqueous phase was spotted onto DE-81 anion-exchange paper (Whatman) which was washed and subjected to autoradiography (11). After autoradiography, the radioactive spots were quantified with the aid of a Betascope (Betagen).

Amino-terminal sequence analysis of PR-RT α . Ten plates (100-mm diameter) of SF21 cells were infected with Ac-PR-RT α isolate 9-3. After 3 days of incubation at 28°C, the infected cells were harvested and washed twice in ice-cold PBS. The cells were then resuspended in 10 ml of buffer A (20 mM Tris-HCl [pH 7.9], 25% glycerol, 0.01% Nonidet P-40, 2 mM dithiothreitol) and incubated on ice for 10 min. This total cell lysate was centrifuged at $120 \times g$ for 10 min. The pellet, consisting mostly of nuclei, was resuspended in 10 ml of buffer A containing 1 M NaCl and then sonicated. The sonicate was centrifuged at $12,000 \times g$ for 30 min to pellet the insoluble PR-RT α , which was fractionated by SDS-8% PAGE, transferred to an Immobilon-P membrane (Millipore), and then subjected to amino-terminal sequencing (23).

RESULTS

PR digestion of PR-mutant ALV virions. ALV virions with a defective PR are immature in morphology and contain the unprocessed polyprotein precursors Pr76^{gag} and Pr180^{gag-pol} (51, 60). We reported previously that the RT activity associated with PR-defective virions released from stably transfected quail cells is reduced 300-fold compared with that in wild-type virions of the standard PrC strain (51). Incubation of the immature cores with purified PR leads to proper and complete maturation of the Gag protein but incomplete maturation of the Gag-Pol protein as well as only a feeble stimulation of RT activity. To gain insight into the poor activation of RT, we have now studied the Gag-Pol cleavages more systematically. In the first set of experiments, virus particles were isolated from QT35 cells expressing PR mutant (PrCneo.D37N) constructs or, as a control, wild-type (PrCneo.D37) viral DNA constructs (52). The PR mutant construct has two point mutations in the PR coding region, D37N and D41Y. The former is an active site mutation, while the latter also changes a conserved amino acid. Particles purified by sucrose density gradient sedimentation were subjected to one of the following treatments: (i) maintenance on ice, (ii) incubation with PR at different concentrations of salt, and (iii) incubation without PR. A portion of each sample then was assayed for RT (Fig. 1B), with the final salt concentration in the assay being held constant. The remaining portion was analyzed by SDS-PAGE and immunoblotting either with a mixture of anti-MA and anti-CA sera (Fig. 1A) or with affinity-purified anti-RT α serum (Fig. 1B).

Digestion at salt concentrations above 660 mM yielded complete or nearly complete Gag processing (Fig. 1A, lanes 10 and 11), as previously reported (51), while digestion at lower salt concentrations yielded a series of Gag cleavage intermediates (Fig. 1A, lanes 12 to 14). In the same reactions, the Pol-related cleavage products shifted from high to low molecular weight as the salt was increased (Fig. 1B, compare lanes 10 through 14). However, even at high salt concentrations the Gag-Pol precursors were only incompletely processed. Only very small quantities of fully mature RT α and RT β were generated by exogenous digestion of mutant virions. RT assays showed that digestion stimulated RT activity (Fig. 1C, compare spots 10 through 16). However, the maximal stimulation was only threefold in this and in two independent experiments, i.e., the activity reached only about 1% of the activity of wild-type RT. Under the same conditions, digestion had little effect on the RT activity of wild-type virions (Fig. 1C, compare spots 1 through 7). Similar stimulation and effects of salt on cleavage were seen with immature virus particles carrying a different PR mutation, D37I (Fig. 2). Thus the major Pol-related polypeptides formed both at high and at low salt concentrations appear to be poorly active as RTs.

What are the structures of the Pol-related products generated by PR? At the different salt concentrations, at least four prominent polypeptides could be visualized by immunoblotting with anti-RT α serum. We have identified these polypeptides as CA-Pol and PR-Pol at the lower salt concentrations (Fig. 1B, lanes 12 to 14) and NC-RT α and PR-RT α at the higher salt concentrations (Fig. 1B, lanes 10 and 11; see Fig. 3 for diagrammatic structures of Pol-related proteins). The identification is based on SDS-PAGE comparison with a set of similar polypeptides produced in the baculovirus expression system and electrophoresed in parallel and on reactivity with specific antisera. For example, the logic for the identification of the NC-RT α cleavage product is as follows. This protein did not react with anti-IN-4kDa^{pol} serum, indicating that it is missing

the IN domain or at least the portions of IN that are recognized by the antiserum (Fig. 2A). By size it should thus contain about 200 amino acid residues extending upstream into Gag. Reprobing of the blot with affinity-purified anti-NC serum resulted in the appearance of a faint new band at the position of NC-RT α (Fig. 2B, lanes 7 to 11). (Under the conditions used for reprobing, the original visualized bands remain on the blot). The anti-NC serum also reacted with the large amounts of intact Gag precursor Pr76^{D37I} and various Gag-related processing intermediates (marked with dots), but these proteins did not interfere with the analysis. Probing of the blot a third time, with anti-RT α serum, greatly enhanced the faint band seen with anti-NC serum (Fig. 2C, lanes 7 to 11) and showed that this polypeptide had exactly the same mobility as the PR-mutant NC-RT α made in the baculovirus system and used as a marker (Fig. 2C, lane 13). Thus, we conclude that the primary structure of NC-RT α is exactly as its name implies. The identity of the other three major products was inferred similarly (53). In careful comparisons of gel mobilities, we noticed that the mutations in PR (D37N plus D41Y, as well as D37I) led to an increase in mobility of all polypeptides carrying the PR domain. For example, this can be seen from the positions of the PR-Pol marker protein (which has a wild-type PR domain) and that of the PR-Pol protein generated by digestion (Fig. 1B, compare lane 9 to lanes 10 through 14). This mobility shift, which presumably results from the loss of negative charges, thus is a useful diagnostic marker for the presence and status of the PR domain.

While it is conceivable that under different digestion conditions a different pattern of Pol cleavage intermediates might be formed and a more substantial activation of enzymatic activity might be achieved, we have seen no evidence for qualitative differences in cleavage specificity at either different salt concentrations, pH values, or concentrations of PR. In summary, since neither high-salt nor low-salt digestion led to RT activation to more than about 1% of the wild-type level, we conclude that the major cleavage products generated under these conditions—CA-Pol, NC-Pol, NC-RT α , and PR-RT α —have at most minimal enzymatic activity.

Recombinant baculoviruses expressing Gag-Pol fusion proteins. To try to delineate which cleavages of the Gag-Pol protein lead to activation of RT, and thus which domains inhibit enzymatic activity, we constructed 19 recombinant baculoviruses expressing a variety of Gag-Pol and Pol polypeptides that might occur as cleavage intermediates. Each polypeptide (except E-RT β) was engineered to contain an artificial initiating methionine positioned immediately before the N-terminal amino acid normally generated by PR-mediated cleavage. Similarly, a translation stop signal was positioned to recreate the C-terminal sequence normally generated by PR. For the viruses that encode Gag-Pol fusion proteins, the reading frames for Gag and Pol were genetically aligned by a single base (A) insertion immediately 5' to the Gag stop codon (4, 52), thereby eliminating the frameshifting requirement for translation of Gag-Pol. As controls, four additional baculoviruses that express intact Gag or Gag-Pol proteins were derived. The names and predicted structures of all 23 of these polypeptides are shown schematically in Fig. 3. Note that some proteins have the wild-type PR domain while others carry the inactivating D37N plus D41Y mutations (marked D37N for simplicity). One protein (E-RT β) begins with a natural methionine residue in the middle of PR, just downstream of the *EcoRI* site used in cloning. Another (PR-RT β ^{COLL}) carries a mutation that creates a consensus collagenase cleavage site at the beginning of Pol. To analyze the properties of these expressed proteins, immunoblotting with Gag or Pol antisera

FIG. 1. Exogenous PR digestion of PrC D37N virions. Virions were isolated from culture medium that was used to support the growth of QT35 cells that had been stably transformed with PrCneo.D37 (lanes 1 to 7) or PrCneo.D37N (lanes 10 to 16) plasmid constructs. The particles were purified by sucrose equilibrium density sedimentation before being subjected to the following conditions: no treatment (INPUT, lanes 1 and 16); PR digestion conditions with a final NaCl concentration of 890 mM and no added PR (–PR, lanes 2 and 15); and PR digestion conditions with PR (+PR) and final NaCl concentrations of 160 mM (lanes 3 and 14), 260 mM (lanes 4 and 13), 460 mM (lanes 5 and 12), 660 mM (lanes 6 and 11), and 890 mM (lanes 7 and 10). After incubation, a portion of each sample was subjected to an exogenous RT assay. The remaining portion was immediately boiled in SDS. A fraction of each sample was then analyzed by SDS-PAGE and immunoblotting. (A) Samples (~0.2 plate day) were fractionated by SDS–15% PAGE and then analyzed by immunoblotting with anti-CA (αCA) and anti-MA (αMA) sera (30 s of chemiluminescent exposure). Lane 8 contained a 20-ng CA standard. Lane 17 contained the equivalent amount of PR that was loaded as part of each digested sample. Dots indicate the positions of Gag-related cleavage intermediates. (B) Samples (~2.0 plate day) were fractionated by SDS–8% PAGE and then analyzed by immunoblotting with affinity-purified anti-RTα (αRTα) serum. Lanes 8 and 9 each contained a mixture of recombinant protein markers produced in the baculovirus-insect cell system (described below). Lane 8, NC-RTβ^{D37N} and NC-RTα^{D37N} (~50 ng each). Lane 9, PR-Pol, PR-RTβ, Pol, RTβ, PR-RTα, and RTα (~50 ng each). (C) Exogenous RT assays of each sample (~0.6 plate day). Radioactivity was quantified with a Betascope, and values for spots were as follows: 1, 6,120 cpm; 2, 10,730 cpm; 3, 10,160 cpm; 4, 10,650 cpm; 5, 8,500 cpm; 6, 8,320 cpm; 7, 11,210 cpm; 10, 180 cpm; 11, 140 cpm; 12, 120 cpm; 13, 110 cpm; 14, 110 cpm; 15, 70 cpm; and 16, 70 cpm. The background from spotting the RT assay cocktail alone was 20 cpm (data not shown).

FIG. 2. Identification of the NC-RT α cleavage product. Virions were isolated from culture medium that was used to support the growth of QT35 cells that had been stably transformed with *PrCneo.D37* (lanes 1 to 3) or *PrCneo.D37I* (lanes 5 to 11) plasmid constructs. The particles were purified by sucrose equilibrium density sedimentation before being subjected to the following conditions: no treatment (INPUT, lanes 1 and 5); PR digestion conditions with a final NaCl concentration of 890 mM and no added PR (-PR, lanes 2 and 6); and PR digestion conditions with PR (+PR) and final NaCl concentrations of 160 mM (lane 7), 260 mM (lane 8), 460 mM (lane 9), 660 mM (lane 10), and 890 mM (lanes 3 and 11). After incubation, a portion was subjected to an exogenous RT assay. The remaining portion of each sample was immediately boiled in SDS. A fraction of each portion was then analyzed by SDS-PAGE followed by sequential immunoblotting. (A) Samples (~2.0 plate day) were fractionated by SDS-8% PAGE and then analyzed by immunoblotting with affinity-purified anti-IN-4kDa^{pol} (α IN-4kDa^{pol}) serum (1 min of chemiluminescent exposure). Lane 4, the equivalent amount of PR that was loaded as part of each digested sample. Lanes 12 and 13, mixtures of recombinant protein markers produced in the baculovirus-insect cell system. Lane 12, PR-Pol, PR-RT β , Pol, RT β , PR-RT α , and RT α (~50 ng each). Lane 13, NC-RT β ^{D37N} and NC-RT α ^{D37N} (~10 ng each). (B) The immunoblot in panel A was probed a second time with affinity-purified anti-NC (α -NC) antiserum (10 min of chemiluminescent exposure). Dots indicate the positions of Gag-related cleavage intermediates. (C) The immunoblot in panel A was probed a third time with affinity-purified anti-RT α (α RT α) antiserum (1 min of chemiluminescent exposure). (D) Exogenous RT assays. Samples are numbered as described above. Radioactivity was quantified with a Betascope, and values for spots were as follows: 1, 8,000 cpm; 2, 8,100 cpm; 3, 7,500 cpm; 5, 450 cpm; 6, 550 cpm; 7, 950 cpm; 8, 1,000 cpm; 9, 1,130 cpm; 10, 1,250 cpm; 11, 1,120 cpm.

was carried out with insect cell lysates to determine whether PR-mediated proteolysis had occurred. Relevant proteins were partially purified and tested for RT activity. A summary of all of the resulting data is presented in Table 1.

The Gag-Pol proteins were observed to fall into two categories with regard to proteolysis, those for which the PR domain was inactive and those for which PR was active and led to partial and apparently correct processing of the protein. An immunoblot analysis of the PR-inactive proteins is shown in Fig. 4. Each crude lysate prepared from sonicated insect cells 3 days after infection contained a single major polypeptide of the predicted size which represented approximately 5% of total cell protein. The only case in which more than one major polypeptide was apparent was PR-RT α , for which a second band about 7 kDa larger than authentic PR-RT α appeared consistently. The upper band could be due to a secondary modification such as ubiquitination or glycosylation, but we have not pursued this question. The absence of lower-molecular-mass bands indicates that cellular PRs do not cleave these proteins in vivo. For the proteins containing a mutant PR

domain, the lack of processing due to PR was expected. However, we were surprised to find that all three proteins with a wild-type N-terminal PR domain (PR-Pol, PR-RT α , and PR-RT β ; lanes 4, 5, and 7, respectively) also showed no evidence of processing. The recombinant DNAs for these proteins were constructed independently from a clone in which the PR domain had been sequenced, ruling out a trivial error in their construction. Furthermore, the mobility difference created by the mutations in PR allowed the wild-type PR domain to be recognized in all three of these proteins (shown for PR-RT β in lanes 5 and 6). As discussed further below, it is possible that the extra initiating methionine residue is not removed and inhibits activity or that the PR domain folds differently in the context of these fusion proteins.

Six of the baculovirus constructs showed evidence of active PR. In each case, at least 10 times less recombinant protein was observed in immunoblots of lysates from cells infected with viruses that do not encode active PR (data not shown). This difference probably is the result of PR toxicity, which has previously been noted for insect cells expressing Pol precursors

A

Transfer Vector	Open Reading Frame	Size	Protein	Isolates
pJV.CA-Pol.D37N		153	CA-Pol ^{D37N}	1
pJV.CA-RTB		149	CA-RTB	3
pJV.CA-RTα.D37N		117	CA-RTα ^{D37N}	1
pJV.NC-Pol		126	NC-Pol	2
pJV.NC-Pol.D37N		126	NC-Pol ^{D37N}	1
pJV.NC-RTB		122	NC-RTB	2
pJV.NC-RTB.D37N		122	NC-RTB ^{D37N}	1
pJV.NC-RTα		90	NC-RTα	3
pJV.NC-RTα.D37N		90	NC-RTα ^{D37N}	1
pJV.PR-Pol		114	PR-Pol	2
pJV.PR-Pol.D37N		114	PR-Pol ^{D37N}	2
pJV.PR-RTB.COLL		110	PR-RTB ^{COLL}	1
pJV.PR-RTB		110	PR-RTB	2
pJV.PR-RTB.D37N		110	PR-RTB ^{D37N}	2
pJV.E-RTB		101	E-RTB	1
pJV.PR-RTα		78	PR-RTα	4
pJV.Pol		99	Pol	1
pJV.RTB		95	RTB	1
pJV.RTα		63	RTα	2

B

FIG. 3. Proteins expressed by recombinant baculoviruses. Recombinant baculovirus transfer vectors were used to generate recombinant baculoviruses as described in Materials and Methods. The primary structure (open reading frame), predicted size (in kilodaltons), and name of each protein are listed. Dotted vertical lines indicate sites of proteolytic cleavage mediated by PR during in vivo maturation of the Gag and Gag-Pol precursors. The number of independent baculovirus isolates obtained is also shown. (A) Vectors that encode truncated Gag-Pol-related proteins. Nineteen transfer vectors that encode truncated Gag-Pol proteins were constructed. The open reading frame for each is encoded entirely by sequences from strain PrC. Initiating methionine codons were engineered immediately 5' to the first codons of the NC, PR, and RT α coding regions located, respectively, at nucleotides 1844, 2111, and 2503 of the RSV genome. In addition, stop codons were engineered immediately 3' to the last codons of the RT α and IN coding regions located, respectively, at nucleotides 4219 and 5076. A naturally occurring methionine codon immediately 5' to the first codon of CA, at nucleotide 1094, was used to initiate translation of the proteins that contain CA at their amino termini. In a similar manner, the methionine codon at position 73 within PR was used to initiate translation of E-RT α . For each construct that contains both *gag* and *pol* coding sequences, a single nucleotide insertion (A) immediately 5' to the *gag* termination codon was used to align the two reading frames without altering amino acid coding (FC) (52). Some of the constructs were engineered to contain the D37N plus D41Y PR mutation and are indicated with the "D37N" suffix. The pJV.PR-RT β .COLL expression vector contains a COLL mutation which replaces the Leu-Thr residues of the PR-RT α cleavage site with the consensus cleavage site for collagenase, Pro-Leu-Gly-Pro. Symbols: $\otimes$, D37N plus D41Y; $\odot$, D37. (B) Vectors that encode full-length Gag-Pol. Four transfer vectors were constructed to carry the entire *gag-pol* coding sequences. The Gag-Pol proteins encoded by the first three—pJV.GP.D37, pJV.GP.D37.FC, and pJV.GP.D37N.FC—are derived entirely from sequences of strain PrC. In contrast, the Gag-Pol open reading frame of pJV.Gag-Pol.D37N.FC is composed of PrC sequences up to nucleotide 4995, followed by RAV-1 sequences through to the *pol* termination codon. pJV.GP.D37 encodes fully wild-type Gag-Pol. Lacking the FC mutation, this construct primarily encodes Gag protein, with occasional frameshifting leading to translation of Gag-Pol. pJV.GP.D37.FC is identical to pJV.GP.D37, except that it has the FC mutation. pJV.GP.D37N.FC and pJV.Gag-Pol.D37N.FC each encode frame-corrected Gag-Pol precursors that have the D37N plus D41Y mutation in PR.

of human immunodeficiency virus (HIV) (22). The recombinant proteins undergoing specific proteolysis all have Gag sequences upstream of the PR domain. Five of these proteins carry the FC that fuses Gag to Pol and begin either with the upstream domain preceding PR (NC-RT α , NC-RT β , and NC-Pol), with the next upstream domain preceding NC (CA-RT β), or with the entire Gag protein (Gag-Pol). In addition and as expected, baculovirus-infected cells expressing wild-type Gag plus Gag-Pol, i.e., without the FC mutation, also made active PR. In this case, the PR domain is part of the Gag protein as well as part of the Gag-Pol protein. The Gag-related polypeptides observed for wild-type Gag plus Gag-Pol are shown in Fig. 5A (lane 5). Prominent cleavage products of the Gag precursor Pr76 included bands of 66 and 60 kDa plus some mature CA protein. The 66- and 60-kDa proteins comigrated with two of the cleavage intermediates generated by PR digestion of D37I virions in vitro under low-salt conditions (lane 1). Similar products, though quantitatively diminished, were observed when frame-corrected Gag-Pol alone was expressed (lane 6). We have not identified the strong band migrating at about 80 kDa, but its size could be consistent with a Gag protein extended 7 amino acids at its C terminus, i.e., generated by cleavage of Gag-Pol at the N terminus of RT α . The intracellular processing of Gag-Pol alone was unexpected, since we had previously demonstrated that the same

protein expressed in QT35 cells remains intact (52). This difference could be due to the different levels of expression in insect and quail cells.

In four cases—NC-RT β , NC-Pol, CA-RT β , and NC-RT α —intracellular processing was observed for portions of the wild-type Gag-Pol protein expressed in insect cells. In each case, the Pol-related fragments corresponded to polypeptides expected to be generated by the action of PR at known cleavage sites (shown in Fig. 5B). In these experiments, the marker proteins used were pools of the partially purified baculovirus-expressed proteins indicated. When a cell extract containing NC-RT β was fractionated by DEAE-anion-exchange chromatography (lanes 2 to 10), the eluted fractions were seen to contain a small amount of the intact protein plus three major products of PR-mediated processing, PR-RT β , RT β , and PR-RT α . The peak fractions displayed a cleavage pattern identical to that of the cell sonicate (data not shown). Very little if any mature RT α was generated. Similar fractionation of an extract containing NC-Pol showed intact NC-Pol plus the cleavage products PR-Pol, PR-RT β , Pol, RT β , and PR-RT α (lanes 12 and 13). Again, very little if any mature RT α was produced. Crude extracts of cells expressing CA-RT β contained the intact species, migrating as a doublet, plus several cleavage products, including NC-RT β , PR-RT β , RT β , NC-RT α , PR-RT α (lane 19). A small amount of mature RT α

TABLE 1. Properties of recombinant proteins^a

Protein (no. and name)	RT activity	PR activity	No. of RT assays ^b
1. RT α	+++ ^c	*	>5
2. RT β	+++	*	>5
3. Pol	+++	*	>5
4. PR-Pol	+++	○, N	>5
5. PR-Pol ^{D37N}	++ ^d	⊗	4
6. NC-Pol ^{D37N}	—	⊗	3
7. CA-Pol ^{D37N}	—	⊗	3
8. Gag-Pol ^{D37N,PrC}	—	⊗	1
9. Gag-Pol ^{D37N}	—	⊗	2
10. PR-RT β	+++	○, N	>5
11. PR-RT β ^{D37N}	— ^d	⊗	5
12. E-RT β	—	⊗	2
13. NC-RT β ^{D37N}	—	⊗	3
14. PR-RT β ^{COIL}	+++	○, N	3
15. PR-RT α	— ^d	○, N	>5
16. NC-RT α ^{D37N}	—	⊗	3
17. CA-RT α ^{D37N}	—	⊗	3
18. NC-RT α	ND	○, Y	0
19. NC-RT β	— ^c	○, Y	1
20. CA-RT β	ND	○, Y	0
21. NC-Pol	— ^c	○, Y	1
22. Gag-Pol	ND	○, Y	0
23. Gag + Gag-Pol	ND	○, Y	0

^a Symbols and abbreviations: +++, specific RT activity is similar to that from PrC virus particles; ++, specific RT activity is three times less than the activity of RT β or Pol; Y, wild-type PR domain is active, with processed proteins observed; N, wild-type PR domain is inactive, with no processed proteins observed; ○, PR domain is wild type, D37; *, PR domain is absent; ⊗, PR domain is mutant, D37N plus D41Y; —, RT activity at least 100 times less than the RT activity of RT β or Pol; ND, not determined.

^b Number of RT assays refers to the number of times RT assays have been performed with proteins purified on separate occasions.

^c RT activity is unstable.

^d The same result was obtained for the proteins expressed by at least two independently isolated baculoviruses.

^e DEAE fractions contain a mixture of cleavage products, including RT β , and display significant RT activity.

also was apparent. Notice the expected mobility difference between the NC-RT α ^{D37N} marker (lane 18) and NC-RT α . Finally, extracts containing NC-RT α showed the partially processed form PR-RT α as well as RT α (lane 20).

RT activity of the recombinant proteins. To determine the specific activity of the several Pol and Gag-Pol proteins, we first subjected infected cell sonicates to fractionation by DEAE-anion-exchange chromatography. This step removed inhibitors (probably nucleases) found in both the cytoplasmic and nuclear fractions of infected cells. Fractionation was carried out in 25% glycerol and was completed within several hours of sonication of the cells. All of the recombinant proteins bound to DEAE at pH 7.9 and were eluted at NaCl concentrations of 400 to 600 mM, with the exception of RT α , which was eluted at about 200 mM NaCl. After DEAE chromatography, the fractions were adjusted to 50% glycerol in a buffer reported to stabilize activity (39) and stored at -20°C for no longer than 10 days. To determine specific activity, portions of the stored fractions were serially diluted and then subjected to exogenous RT assays and immunoblot analyses. As a control, DEAE fractions from an extract of wild-type baculovirus-infected cells were prepared. The latter were assayed both for RT activity and for inhibition of RT from wild-type virus particles as a source of the enzyme. Neither enzymatic activity nor inhibitory activity was found (data not shown).

Initially we compared the activities of RT α and RT β with that of purified wild-type virus particles (Fig. 6). The absolute

quantities of enzyme were estimated by comparison of the immunoblot signals with that of a known quantity of highly purified PR-RT α . One nanogram of recombinant RT β incorporated 0.04 nmol of dTMP into high-molecular-weight DNA in 10 min at 37°C , a value that is similar to that of purified commercially available AMV RT. The activity in purified virions, normalized for the amount of RT, also was similar. The specific activity of recombinant RT α was found to be three times less than that of RT β . This result differs from that reported for RT α and RT β proteins individually purified from AMV particles (18) or expressed in *E. coli* (50), in which the two subunits were found to have the same specific activities. Possibly this difference is due to the instability of RT α when stored at less than 1 mg/ml (14). In our study, after storage at -20°C for 2 weeks the peak DEAE fractions, which contained $\sim 10\ \mu\text{g}$ of RT α per ml, had lost 80% of their activity (data not shown). The analysis shown in Fig. 6 was repeated several times with independently purified proteins, and in each case similar specific activity was observed.

Having established that the recombinant RT α and RT β proteins are active, we then analyzed the other relevant proteins in a similar manner (Table 1). These data are summarized as follows. The 4kDa^{pol} C-terminal domain that distinguishes RT β from Pol did not affect activity (see results for protein no. 3), as might be expected from the fact that this domain is dispensable for virus replication (28). The addition of the N-terminal PR domain onto Pol also did not affect activity (protein no. 4). Thus, in the context of this protein, PR is not a *cis*-acting inhibitor of RT. However, when the same construct carried the PR mutations D37N and D41Y, a threefold diminution of activity was observed (protein no. 5), a result that was reproduced at least three times for proteins produced by two independently isolated viruses expressing PR-Pol^{D37N}. The further addition of the NC domain onto PR-Pol^{D37N} to make NC-Pol^{D37N} (protein no. 6) virtually inactivated RT, with a further 50- to 100-fold drop in the activity of the Pol domain. Proteins including still longer Gag sequences (CA-Pol^{D37N} [protein no. 7], Gag-Pol^{D37N} [protein no. 9], and Gag-Pol^{D37N,PrC} [protein no. 8]) were all also inactive. These results imply that the NC domain can inhibit the RT activity of Pol.

When the PR domain was fused to RT β , the resulting PR-RT β protein (protein no. 10) was fully active, supporting the conclusion that the upstream PR is not necessarily a *cis*-acting inhibitor. A similar protein (PR-RT β ^{COIL}) with several amino acid substitutions near the N terminus of RT β also was active (protein no. 14). Surprisingly however, when the PR domain carried the D37N plus D41Y double mutation, the resulting PR-RT β ^{D37N} protein (protein no. 11) was nearly inactive, with 300-fold less RT activity than PR-RT β . This result was reproduced at least three times for protein expressed by two independently isolated baculoviruses. It is difficult to understand how the two single amino acid mutations could have such a drastic effect in the context of the RT β constructs yet such a modest effect in the context of the Pol constructs. The importance of the PR structure for the RT activity of PR-RT β is further demonstrated by the fact that the E-RT β protein (protein no. 12), which lacks the amino-terminal two-thirds of PR, also was largely inactive. As expected given the inhibitory effect of the NC domain, the NC-RT β ^{D37N} protein (protein no. 13) lacked substantial activity as well.

While fusion of the wild-type PR domain to Pol or to RT β did not affect activity, fusion to RT α to give PR-RT α resulted in complete loss of activity (protein no. 15). The differences in the effects of PR in these different constructs suggests that the

FIG. 4. Immunoblot analysis of recombinant proteins that do not undergo processing. One plate (100-mm diameter) of SF21 cells was infected with each of the following recombinant baculoviruses: Ac-RT α (lanes 1 and 14), Ac-RT β (lanes 2 and 15), Ac-Pol (lanes 3 and 16), Ac-PR-RT α (lanes 4 and 17), Ac-PR-RT β (lanes 5 and 19), Ac-PR-RT β^{D37N} isolate 4-4 (lanes 6 and 20), Ac-PR-RT β^{D37N} isolate 12-3 (lane 21), Ac-E-RT β (lane 18), Ac-PR-RT β^{COLL} (lane 22), Ac-PR-Pol (lane 7), Ac-PR-Pol D37N (lane 23), Ac-NC-RT α^{D37N} (lanes 8 and 24), Ac-NC-RT β^{D37N} (lanes 9 and 25), Ac-NC-Pol D37N (lane 26), Ac-CA-RT α^{D37N} (lane 27), Ac-CA-Pol D37N (lane 28), and Ac-gp D37N,FC (lane 10). Whole-cell sonicates (~ 1 μ g of protein prepared 72 h after infection) were analyzed by SDS-8% PAGE and immunoblotting with a mixture of affinity-purified anti-RT α (α RT α) and anti-IN-4kDa pol (α IN-4kDa pol) sera (5 s of chemiluminescent detection). Lanes 12 and 29 each contained 3 plate days of PR-mutant virus released from QT35 cells expressing PrC $_{neo}$.D37I (51). Lanes 11 and 13 each contained 3 plate days of wild-type virus released from QT35 cells expressing PrC $_{neo}$.D37 (52). The two panels are from separate experiments.

IN domain may play a role in RT activity, perhaps by contacting the PR domain and thus buffering against its influence. The lack of activity of NC-RT α^{D37N} (protein no. 16) and CA-RT α^{D37N} (protein no. 17) might be expected from the analogous constructs based on RT β . We have not succeeded in isolating a baculovirus that expresses PR-RT α^{D37N} .

As described above, the NC-RT α (protein no. 18), NC-RT β (protein no. 19), CA-RT β (protein no. 20), NC-Pol (protein no. 21), and Gag-Pol (protein no. 22) proteins undergo PR-mediated processing and are poorly expressed. We performed only qualitative assays with two of these proteins. DEAE elution fractions of NC-RT β (protein no. 19) and NC-Pol (protein no. 21) showed significant levels of RT activity, as expected since they contained a mixture of cleavage products that included RT β .

In summary, the activities of the baculovirus-expressed Gag-Pol fusion proteins imply that upstream Gag sequences can act as potent inhibitors of RT. The NC domain in particular acts in this way. The PR domain inhibits activity in some but not in other constructs, depending on whether PR is mutant or not and depending on whether the Pol sequences downstream of the RT domain are present or not.

DISCUSSION

The experiments described in this report were undertaken to try to explain the mechanism by which PR-mediated processing of the Gag-Pol precursor polypeptide leads to activation of RT. Our previous results had shown that PR-defective virus particles composed of the unprocessed Gag plus Gag-Pol proteins manifest only very low levels of RT (51). Digestion of these particles with PR in the presence of detergent led to proper and complete cleavage of the Gag protein at high salt

concentrations but incomplete cleavage at low salt concentrations, which is consistent with the known preference of PR for high ionic strength (30). At neither salt concentration was the Gag-Pol protein completely cleaved, and at neither salt concentration was RT activity activated by more than threefold. We now have identified the major Pol-related cleavage products generated at high salt concentrations as NC-RT α and PR-RT α and at low salt concentrations as NC-Pol and PR-Pol (in which the PR domain carried either the D37N plus D41Y mutations or the D37I mutation). Thus, in both cases most of the Gag protein was cleaved off efficiently, leaving either the NC-PR domains together or just the PR domain tethered to the intact or partially processed Pol protein. Under none of the many conditions we have tried was the amino acid sequence between PR and RT α cleaved (53). Since RT activity was activated at most to about 1% of its wild-type level at either high or low salt concentrations, we infer that the four predominant products of digestion are enzymatically inactive or poorly active.

To study how amino acid sequences upstream or downstream of the RT domain affect enzymatic activity, we constructed baculovirus vectors that express fusion proteins like those generated by *in vitro* cleavage of Gag-Pol with PR. Each protein was partially purified from insect cell extracts. The Gag-Pol-related and Pol-related proteins fall cleanly into two classes. The first class is composed of proteins exhibiting a RT-specific activity of at least 30% of that found for the RT β standard, while the second is composed of proteins with less than 1% of this specific activity. Inspection of the structures of proteins in each class leads to the general conclusion that both upstream Gag sequences and downstream Pol sequences can inhibit RT activity *in cis*. However, interpretations of the results are complicated by the fact that apparently similar

proteins responded remarkably differently to the fusion of the same extra domain. For example, fusion of PR to the RT domain itself (PR-RT α) virtually abolished activity, while fusion of PR to RT plus IN (PR-RT β) had no effect on activity. Thus, it appears that IN can act to mask the negative effect of PR. This masking action of IN seems to require a wild-type PR sequence, since both a partial deletion (E-RT β) and mutations

(PR-RT β^{D37N}) in the PR portion of PR-RT β blocked RT activity. We postulate from these results that there is a specific interaction between the IN and PR domains when both are present on the same polypeptide. Surprisingly, fusion of the 4kDa.^{pol} domain onto inactive PR-RT β^{D37N} , recreating the entire Pol sequence, restored activity (PR-Pol^{D37N}). Finally, the NC domain appears to have a profound and consistent effect on enzymatic activity; fusion of NC, or larger portions of Gag containing NC, to PR-Pol^{D37N} reduced RT activity by more than 50-fold (NC-Pol^{D37N}, CA-Pol^{D37N}, and Gag-Pol^{D37N}).

The complexity of these results might be taken as an indication that some of the data on RT activity are artifactual. We made considerable effort to minimize such possibilities. All of the critical assays were reproduced with independently prepared proteins, and some of the experiments also were repeated with independently isolated clones of recombinant baculoviruses. Although it cannot be ruled out rigorously that mutations might have occurred during cloning, the several transfer vector clones were constructed on independent occasions and all polymerase chain reaction-generated DNAs were sequenced. Furthermore, trivial mixups between recombinant viruses carrying wild-type and mutant PR sequences were excluded by the characteristic mobility alteration associated with active-site mutations in PR.

Given that RT activity in one of the baculovirus-expressed proteins, PR-RT β , is sensitive to mutations in the PR domain, it might be argued that the lack of activity of PR-defective virions is irrelevant for the regulation of RT in the wild-type virus. We have addressed this argument previously by examin-

FIG. 5. Immunoblot analyses of recombinant proteins that undergo processing. (A) Three days after infection with either Ac-gp^{D37} (lane 5) or Ac-gp^{D37,FC} (lane 6), SF21 cells were lysed in SDS. Total cell proteins (~1 μ g) were analyzed by SDS-15% PAGE and immunoblotting with anti-CA (α CA) and anti-MA (α MA) sera (30 s of chemiluminescent exposure). For comparison, PR-mutant D37I virus was subjected to either no treatment (lane 1), PR digestion conditions in the absence of PR (lane 2), or PR digestion in the presence of 160 mM NaCl (lane 3). Lane 5 contained 20 ng of CA standard. Dots indicate the position of Gag-related cleavage intermediates. The question mark indicates the position of a major Gag-related cleavage product that is found in cells infected with Ac-gp^{D37,FC}. (B) Samples were analyzed by SDS-8% PAGE and immunoblotting with anti-RT α serum (α RT α) (1 min of chemiluminescent detection). Lanes 1 and 14, AMV standard containing 1 μ g of CA. Lanes 2 to 10, fractions from DEAE-anion-exchange chromatography of an Ac-NC-RT β -infected cell sonicate. Lane 11, Ac-NC-Pol-infected cell sonicate. Lanes 12 and 13, peak fractions from DEAE-anion-exchange chromatography of an Ac-NC-Pol-infected cell sonicate. Lane 15, partially purified PR-RT β . Lanes 16 and 17, mixture of partially purified PR-Pol, PR-RT β , Pol, RT β , PR-RT α , and RT α . Lane 18, mixture of partially purified NC-RT β^{D37N} and NC-RT α^{D37N} . Lane 19, an Ac-CA-RT β -infected cell sonicate. Lane 20, an Ac-NC-RT α -infected cell sonicate.

FIG. 6. Specific activity of recombinant proteins. Recombinant proteins were partially purified from whole-cell sonicates by DEAE-anion-exchange chromatography. The proteins, or, in parallel, sucrose-gradient purified virions, were serially diluted 1/3 in dilution buffer (20 mM Tris-HCl [pH 7.9], 50% glycerol, 0.006% Nonidet P-40, 1 mM dithiothreitol, 0.46 M NaCl). One-third of each dilution was subjected to an RT assay. The remaining two-thirds was fractionated by SDS-8% PAGE and analyzed by immunoblotting with affinity-purified anti-RT α (α RT α) serum. All of the proteins used in this analysis were prepared in parallel at the same time. The arrowheads point in the direction of increasing dilution. Also shown is a specific activity analysis of wild-type (D37) virus (released from QT35 cells expressing PrC $_{neo}$.D37) as a source of RT, relative to partially purified RT α , RT β , and a mixture of RT α plus RT β . Protein standards were 30, 10, and 3 ng of PR-RT α protein. Radioactivity was quantified with a Betascope, and values were as follows: D37, 12,040, 10,570, and 5,560 cpm; RT α , 4,880, 1,870, and 680 cpm; RT β , 9,250, 3,160, and 1,020 cpm; RT α plus RT β , 10,000, 4,120, and 1,570 cpm.

ing virions composed of PR-defective Gag but wild-type Gag-Pol proteins (52). In our experiments these phenotypically mixed virions undergo no proteolytic processing, apparently because the wild-type PR domain embedded in the Gag-Pol protein does not function as a protease and show only the same low RT activity as PR-defective virions. When the wild-type Gag-Pol protein in these mixed virions is digested with PR in vitro, RT activity is poorly activated, exactly as for PR-mutant Gag-Pol (data not shown). Hence, the status of the PR domain is not responsible for the feeble activation of RT by in vitro digestion of immature virions.

The properties of the baculovirus-expressed proteins explain some but not all of the observations on RT activity of PR-defective virus particles. Previous studies with immature virus particles could not entirely exclude the possibility that lack of activity was due to structural constraints, for example, exclusion of the exogenous rA/dT template/primer pair from the virus core. This possibility appeared plausible in view of the finding that unlike mature cores, immature cores are stable in nonionic detergents (48, 51). The observation that soluble baculovirus-expressed Gag-Pol protein was a very poor RT obviates the need to postulate such structural constraints. Digestion of the immature particles with PR at high salt concentrations led to the two predominant products, NC-RT α ^{D37N} and PR-RT α ^{D37N}, which appeared to be inactive. Consistent with this inference, the corresponding products purified from insect cells also were inactive. The two major products from immature virions digested at low salt concentrations also appeared to be inactive. One of these proteins expressed in insect cells, NC-Pol^{D37N}, also lacked substantial RT activity. However, the other, PR-Pol^{D37N}, had an activity that was close to that of the wild type. We do not have an explanation for this apparent discrepancy, which suggests that the structure of the in vitro digestion product differs in some

way from that produced in insect cells. In principle, the discrepancy could be due to an error in identification of the product, to a secondary modification, or to a difference in folding of the polypeptides. Although the antigenicity and size of the product are in agreement with the identification, without amino acid sequence analysis an error cannot be rigorously concluded. The only modification reported for Pol is a phosphorylation near the C terminus IN (19), and this modification is not required for infectivity of the virus (20). The possibility that the PR-Pol^{D37N} polypeptide folds differently when it is generated by cleavage than when it is generated directly by translation is difficult to address experimentally but draws support from our observations that the PR-RT α cleavage site of proteins produced in the baculovirus-insect cell system (or by in vitro translation) is refractory to cleavage by exogenous PR yet is efficiently cleaved during particle maturation (53).

A large body of evidence suggests that the RT of ALV is activated during virion assembly by proteolytic maturation of Gag-Pol. This conclusion appears to extend to many but not all other retroviruses and retroviral elements. For example, a PR-defective murine leukemia virus mutant exhibited nearly normal levels of RT (5), although there has been some controversy surrounding this result (44). It was reported that in the HIV type 1 system, PR-defective virus particles have 10-fold-reduced levels of RT activity (31), or nearly normal levels of activity compared with wild-type virus (47). Inconsistent with the latter result is the finding that bacterially expressed PR-RT and RT-IN fusion proteins of HIV type 1 demonstrate little if any inherent RT activity yet can be activated by *trans* cleavage with coexpressed PR (34, 35, 38). However, this *trans* activation has not been reproduced in vitro with purified components. Interpretation of these results is further complicated by the observations that different methods of purification (metal chelate or ion-exchange chromatography) or expression (bac-

teria or insect cells) can drastically affect the level of RT activity of HIV PR-RT (22, 33). In the pararetrovirus cauliflower mosaic virus, RT was reported to be inactive as a PR-RT fusion (55) but active when the amino-terminal PR domain (56) was proteolytically removed (54) or genetically deleted (55).

The recent X-ray crystallographic analysis of HIV type 1 RT provides insight into the relationship between proteolytic cleavage and activity (29). This enzyme is composed of the two subunits, p51 and p66, which have common amino termini but differ by the respective absence or presence of the C-terminal 15-kDa RNase H domain (2, 57). The p51-p66 heterodimer results from the asymmetric processing of a p66-p66 homodimer by PR and is resistant to further cleavage (25). This implied asymmetry is explained by the three-dimensional model, which shows that the conformations of the p66 and p51 subunits are quite different (29). Specifically, the residues that are predicted to form the active site for polymerization are located in a cleft of the p66 subunit but are buried in the p51 subunit. This result explains the previous observations that mutations predicted to wreck the active site in fact have no effect on RT activity if they occur only in the p51 subunit of the heterodimer (21, 36). Thus alternative folding can generate an inactive RT polypeptide. In addition, in the three-dimensional model the cleavage site at the RT-RNase H boundary in the p66 subunit is buried, which explains the inability of PR to cleave it. Thus, alternative folding can lead to internalization of a cleavage site. We propose that both of these themes also are applicable to the ALV system.

Our observations on the processing of Gag-Pol proteins in insect cells are relevant for models to explain the activation of PR late in assembly. The functionality of the embedded PR domain of the ALV Gag-Pol polyprotein is controversial. We reported previously that phenotypically mixed virions composed of PR-defective Gag and wild-type Gag-Pol proteins contained only uncleaved proteins, indicating that when PR is surrounded by upstream Gag sequences and downstream Pol sequences it does not function as a PR in virions (52). In these experiments, virus particles were obtained from stably transfected quail cells. A similar result was reported for virus-like particles obtained from COS cells transiently transfected with simian virus 40-based vectors (4). The mixed particles released from COS cells showed no evidence of PR activity but did display RT activity at a level reduced approximately 10-fold relative to that of the wild type. Those authors had difficulty detecting the Gag-Pol precursor in the particles, so it is unclear whether the residual RT activity originated from a polyprotein or was due to a small amount of mature RT that escaped detection. By contrast with these results, similar phenotypically mixed particles from transiently transfected chicken embryo cells were reported to contain high levels of RT activity and some mature CA, implying either that the embedded PR domain itself was active or that it was somehow proteolytically released (41). A possible explanation of the discrepancy between our results and those of Oertle et al. is that their mutation used to align the Gag and Pol reading frames introduced an extra amino acid between Gag and Pol (41). The extra amino acid occurs in, and thus could disrupt, a sequence potentially capable of forming a coiled coil in the intergenic region between Gag and Pol (53).

The results presented here show that in insect cells a PR domain embedded in a Gag-Pol protein can be a source of PR activity. Processing was observed in all Gag-Pol-related fusions carrying a wild-type PR domain and the upstream NC domain (i.e., Gag-Pol, CA-RT β , NC-Pol, NC-RT β , and NC-RT α). We do not know whether the activity is manifested while PR is still

embedded, after it has been proteolytically released, or both. Processing was not observed in any of the three Gag-Pol fusions that carry an N-terminal PR domain (i.e., PR-RT α , PR-RT β , and PR-Pol). In one model to explain the apparent difference in proteolytic activity of these several proteins, the extra N-terminal methionine residue in the proteins beginning with PR, which we showed is retained at least in the case of PR-RT α (data not shown), is inhibitory to PR activity. In the crystal structure of AMV PR (26), the N-terminal amino group of each monomer forms a network of interactions with the side chains of E92 and N86 within the same polypeptide chain and N123 in the adjacent monomer (59). The interaction with N123 occurs within the anti-parallel β -sheet structure that forms the dimer interface of PR. If this interaction is a major contributor to the stability of the dimer, then a single amino acid extension at the amino terminus of PR might prevent dimerization of the PR domain, thereby rendering it inactive. In a second model, PR, when tethered to the RT α domain, adopts an alternative conformation that is incompatible with dimerization. The leucine zipper-like region that spans the junction between the PR and RT α domains (53) might be able to assume an α -helix or coiled-coil conformation (43), which would preclude formation of the β -sheet important for dimerization of PR. In this model, the presence of upstream NC sequences would somehow stabilize the dimeric PR structure or destabilize the coiled structure postulated by the model. Work with purified fusion proteins containing the PR domain will be needed to evaluate these models.

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