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Role of the Avian Retroviral Protease in the Activation of Reverse Transcriptase during Virion Assembly

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The retroviruses of the avian sarcoma-leukosis virus group synthesize their viral protease (PR) in two precursor forms—as a carboxy-terminal domain of the Gag precursor and as an embedded domain within the Gag-Pol precursor. We have shown previously that the Gag-derived PR is fully capable of processing the Gag precursor in the absence of the embedded PR (R. P. Bennett, S. Rhee, R. C. Craven, E. Hunter, and J. W. Wills, J. Virol. 65:272–280, 1991). In this study, we examined the question of whether or not the PR domain of Gag-Pol has an essential role in the maturation of the Pol proteins. The Gag-Pol precursor was expressed in the absence of Gag by use of a simian virus 40-based vector in which the *gag* and *pol* reading frames were fused. The fusion protein accumulated to high levels in transfected cells without being released into the medium but could be rescued into particles by coexpression of the Gag protein from a second vector. The resulting particles contained mature Gag and Pol proteins and active reverse transcriptase (RT). Using this complementation system, the effects of PR defects in the Gag and/or Gag-Pol proteins on the activation of RT were examined. The results showed that the presence of a functional PR on the Gag precursor, but not on Gag-Pol, was required for full activation of RT. The embedded PR of Gag-Pol was unable to carry out any detectable processing of the Gag precursor and was able to activate RT to only a low level in the absence of a functional Gag PR domain. Finally, some point mutations in the Gag-Pol PR domain inhibited activation of RT in *trans* by a wild-type PR, suggesting that the correct conformation of the PR domain in Gag-Pol is prerequisite for activation of RT.

Rous sarcoma virus (RSV) and other members of the avian sarcoma-leukosis virus (ASLV) group of retroviruses synthesize two polyproteins, the Gag and Gag-Pol precursors, products of the *gag* gene and the *gag* and *pol* genes, respectively. The two precursors are cleaved after particle assembly to yield the mature internal structural and enzymatic proteins of the virion core (6, 14, 25, 26, 40, 46). Both precursors contain a protease (PR) domain. This situation, which differs from that found in other retroviruses, arises because of the unusual placement of the PR-coding sequence in the *gag* reading frame of the ASLV genome (34). Termination of translation at the end of *gag* yields a Gag protein that contains PR at its carboxy terminus. The Gag-Pol precursor, a fusion protein that is synthesized only when ribosomal frameshifting occurs at the end of *gag*, allowing translation to continue into the *pol* gene (15–17), carries an identical PR sequence as an embedded domain flanked by the rest of Gag on one side and the *pol* gene product on the other. In other retroviruses, the PR-coding sequence is located within *pol*, so that PR is synthesized only in the form of a Gag-Pol fusion protein, or in a separate *pro* reading frame, situated between *gag* and *pol*. In the latter case, two successive frameshift events produce three precursors, known as Gag, Gag-Pro, and Gag-Pro-Pol (10, 15). Thus, the ASLV are the only known replication-competent retroviruses that direct the synthesis of a PR domain as part of every Gag-containing precursor.

Once the Gag and Gag-Pol precursors are packaged into an immature virion, a process that is directed by the Gag protein itself, the viral PR is activated by a mechanism that is not understood (19, 24, 25, 46). Processing of the Gag protein yields the major structural proteins of the virion core, which in the case of the ASLV are known as MA (matrix protein), p10, CA (capsid), NC (nucleocapsid), and PR (listed in the order of the domains within the precursor) (23). Several short peptides are also released during processing. These include peptides of 9 and 11 amino acids that are located between the MA and p10 domains in the precursor and another of 9 amino acids that is released from the carboxy end of the CA protein (30, 31). What the structural role of these small peptides is remains unknown. Cleavage at two sites within the Pol domain of the Gag-Pol precursor releases the 95-kDa (β) polypeptide chain of reverse transcriptase (RT), separating it from a seven-amino-acid spacer peptide that is located between PR and RT in the precursor and from a 4-kDa carboxy-terminal peptide. Further processing of some β molecules at an internal site produces the 63-kDa (α) form of RT and the 32-kDa integration (IN) protein (1, 4, 33). The α and β proteins form the active RT that catalyzes the synthesis of a DNA copy of the viral RNA, while the IN protein catalyzes integration of the viral DNA into the host cell genome (3, 12, 35).

Proteolytic processing of the immature gene products is essential for virion infectivity; in all retroviruses in which it has been examined, mutations that block PR activity have been shown to block completely the proteolysis of both Gag and Gag-Pol and the morphological conversion of immature, noninfectious virions into mature, infectious ones (5, 11, 19, 20, 29, 37, 41). The failure to process the Gag-Pol protein also prevents full activity of the replication enzymes (5, 11, 22, 27–29, 37). Complete maturation of the ASLV precursors

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is a complex process, as outlined above, requiring at least seven cleavages on the Gag precursor and three within Pol to form the final products (36). Whereas the Gag-associated PR has been shown to be sufficient to carry out complete cleavage of the Gag precursor (2), it is not known whether the Gag-derived PR or the embedded PR domain of the Gag-Pol precursor is responsible for Pol maturation. It is conceivable that the Gag PR and embedded PR domains have separate and distinct functions in this process.

We have been using an in vitro mutagenesis approach to examine the functional role(s) of the two PR domains of Rous sarcoma virus (RSV) during virion assembly and maturation (2, 43, 44). Bennett et al. (2) demonstrated that mutations that prevent the synthesis of a ribosomal frameshift product do not interfere with Gag processing and, furthermore, that the fusion of amino acids derived from Pol onto the carboxy terminus of Gag cripples its proteolytic activity. These results suggested that the embedded PR domain is of little importance in the maturation of the Gag protein. The present study extends the earlier work by asking whether the PR domain of either the Gag or the Gag-Pol precursor has an essential role in the maturation of Pol proteins, specifically in the activation of RT. The results show that the proteolytic activity of the Gag-Pol PR domain does not have an essential role in RT maturation and that the Gag PR can activate RT via a *trans*-cleavage event. Furthermore, the Gag-Pol PR appears to have very little ability to activate RT in the absence of a functional Gag PR.

MATERIALS AND METHODS

DNAs and cells. The *gag* expression plasmid pSV.Myr1 is a simian virus 40 (SV40) late-region replacement vector containing the *gag* gene of the RSV Prague C strain on a fragment that extends from the *Sac*I site at nucleotide (nt) 255 to *Hin*dIII at nt 2740 (44). The product of expression of pSV.Myr1 is a hybrid, myristylated form of the RSV Gag protein (Myr1) in which the first 10 amino acids of the wild-type protein have been replaced by those of $p60^{v\text{-}src}$, the product of the *src* oncogene of RSV. Plasmid pSV.Myr1.3h encodes a truncated Myr1 protein that carries only the first seven amino acids of PR, followed by one foreign amino acid (Leu) (43). For construction of the *gag-pol* expression vector, the wild-type *gag* and *pol* genes were taken from pATV-8R, a plasmid containing an infectious copy of the RSV Prague C genome (kindly provided by Eric Hunter, University of Alabama, Birmingham). The D37I PR mutation, obtained from Volker Vogt (Cornell University, Ithaca, N.Y.) in a clone of the Schmidt-Ruppin A (SRA) *gag* gene, has been characterized in quail (37) and mammalian (44) cells. The D37S mutation was provided by Anna Marie Skalka (Fox Chase Cancer Center, Philadelphia, Pa.) in the form of pNC-PR(D37S), a plasmid carrying the NC- and PR-coding sequence of the RSV Prague C *gag* gene (21). DNA manipulations were done by using standard techniques (32). Recombinant plasmids were propagated in *Escherichia coli* DH-1 grown in LB medium containing ampicillin at 25 μg/ml. COS-1 cells, a line of SV40-transformed African green monkey kidney cells, were obtained from the American Type Culture Collection and grown in Dulbecco's modified Eagle medium (GIBCO BRL) supplemented with 3% fetal bovine serum and 7% bovine calf serum (Hyclone, Inc.).

Construction of the Gag-Pol expression vector. A Gag-Pol expression plasmid was developed for synthesis of the RSV Gag and Gag-Pol proteins in mammalian cells (see Fig. 1). The parent plasmid, pSV.Myr1, was cut with *Bss*HII (nt 2724), the sticky ends were made blunt by using the Klenow fragment of DNA polymerase I, and then an *Xho*I linker (CCCTCGAGGG) was attached by using T4 DNA ligase. Next, a *Cla*I linker (CCATCGATGG) was inserted at the *Eco*RV site in the bacterial plasmid sequence. The *Cla*I linker insertion destroyed the *Eco*RV site; insertion of the *Xho*I linker, however, created two *Bss*HII sites, one on either side of the linker. Finally, most of the *gag* sequence (nt 630 to 2724) was excised from this modified plasmid with *Xho*I and replaced with the *Xho*I fragment (nt 630 to 5258) from pATV-8R, which contains *gag*, *pol*, and the first 14 nt of *env*. This construction created a short duplication of 16 nt (nt 2724 to 2740) following the *Xho*I site at nt 5258. The resulting plasmid was named pSV.G1P (see Fig. 1).

PR mutations in pSV.G1P. Two different mutations (D37I and D37S) that modified the codon specifying the catalytic aspartic acid residue (D37) of the PR were introduced into pSV.G1P by restriction fragment exchanges. The D37I mutation (which causes an Asp-to-Ile substitution at position 37) was transferred into pSV.G1P on a *Bgl*II-*Eco*RI fragment (nt 1630 to 2319), replacing the wild-type fragment; the resulting plasmid was named pSV.G1P.D37I. The D37I mutation was originally constructed in an M13 clone containing the SRA *gag* gene (37) rather than that of the Prague C strain. Therefore, as a control to confirm that the SRA sequence in the chimeric *gag* gene of pSV.G1P.D37I had no adverse effect on RT, a similar construction was made by inserting the *Bgl*II-*Eco*RI fragment of the wild-type SRA gene into pSV.G1P. The resulting plasmid was named pSV.G1P.D37. Plasmid pSV.G1P.D37S, carrying the D37S mutation (changing Asp to Ser at position 37), was constructed by replacing the *Sma*I-*Eco*RI fragment (nt 1921 to 2319) of pSV.G1P.D37I with the corresponding fragment of pNC-PR(D37S). A third PR mutation (D37N, changing Asp to Asn) was introduced into pSV.G1P by Lance Stewart and Volker Vogt (Cornell University), who kindly shared it with us.

Construction of *gag-pol* fusion mutations. Two *gag-pol* fusion mutations were constructed by insertion of single base pairs at the junction of the two genes in pSV.G1P. Insertion mutations SR12 and SR15 were initially created by site-directed mutagenesis and characterized in pSV.Myr1 (2). For the present study, the frameshift mutations were moved into pSV.G1P by exchange of the *Eco*RI-*Bss*HII fragment (nt 2319 to 2724) that spans the *gag-pol* junction. The resulting plasmids were named pSV.GP12 and pSV.GP15, respectively.

Finally, a series of PR-defective derivatives of pSV.GP12 and pSV.GP15 were constructed. The D37I mutation was excised from pSV.Myr1.D37I on either an *Mlu*I-*Eco*RI fragment (nt 408 to 2319) or an *Sst*I-*Eco*RI fragment (nt 255 to 2319) and spliced into the *gag-pol* fusion plasmids to replace the corresponding wild-type fragments, thereby creating pSV.GP12.D37I and pSV.GP15.D37I, respectively. A D37S derivative of pSV.GP15 (pSV.GP15.D37S) was constructed by moving the SR15 frameshift mutation from pSV.Myr1.-SR15 into pSV.G1P.D37S by exchange of their *Eco*RI-*Eco*RV fragments (nt 2319 to 3655). A third PR-defective mutant of pSV.GP15 carrying a D37K (Asp-to-Lys) mutation (pSV.GP15.D37K) was constructed by replacing the *Bgl*II-*Eco*RI fragment (nt 1630 to 2319) of pSV.GP15 with the corresponding fragment from a mutant clone of the Prague C *gag* gene obtained from Volker Vogt.

To guard against the introduction of unwanted, spontaneous mutations during subcloning, all of the plasmids were

subjected to extensive restriction site analysis and multiple clones of each plasmid were analyzed in COS-1 cells. DNA sequencing was used to confirm the presence of the desired mutations.

Transfection of COS-1 cells. The *gag-pol* expression plasmids were prepared for transfection by cutting with *Cla*I to remove the bacterial plasmid and religating at a concentration of 20 μg/ml to place the SV40 polyadenylation site adjacent to *gag-pol*. The *gag* expression plasmids pSV.Myr1 and pSV.Myr1.3h were cut with *Xba*I and ligated similarly. COS-1 cells were transfected by the DEAE-dextran-chloroquine method (43, 44). For most experiments, 1 to 2 μg of ligated DNA was applied to a 60-mm-diameter plate of cells; however, for analysis of the Pol cleavage products in the medium of transfected cells, a total of 5 μg of DNA was applied to a 100-mm-diameter plate of cells. For cotransfection experiments, 100-mm plates of cells were treated with a mixture of Gag and Gag-Pol expression plasmids in a ratio of approximately 1 to 4 μg, respectively.

Immunoprecipitation. For analysis of Gag proteins, 60-mm plates of transfected cells were labeled at 48 h posttransfection with L-[^{35}S]methionine (50 μCi, >1,000 Ci/mmol, 2.5 h; ICN Biomedicals) as previously described (44). Cell lysates and medium samples were prepared and subjected to immunoprecipitation with anti-RSV or anti-CA serum in 1× lysis buffer B (25 mM Tris hydrochloride [pH 8.0], 0.15 M NaCl, 0.1% sodium dodecyl sulfate [SDS], 1% Triton X-100, 1% sodium deoxycholate) (43, 44). Immunoprecipitated proteins were separated by electrophoresis in an SDS-polyacrylamide gel (monomer-to-crosslinker ratio, 29.2:0.8) and visualized by fluorography. Except where indicated otherwise, the polyacrylamide concentration was 10%.

A variation of this method was used to characterize the Pol cleavage products released into the medium of transfected cells. Transfected cells in 100-mm plates were labeled with 150 μCi of L-[^{35}S]methionine for 6 h. After labeling, detached cells were removed from the labeling medium by low-speed centrifugation (3,500 × *g* for 10 min at 4°C); the labeled particles were then pelleted through a 3-ml layer of 5% sucrose (to remove serum proteins and labeled cellular proteins) by ultracentrifugation at 85,000 × *g* for 45 min in an SW41 rotor. The pelleted particles were dissolved in 500 μl of 1× lysis buffer B. Prior to addition of antibody, the samples were incubated with fixed *Staphylococcus aureus* cells (13) to remove labeled proteins that bind nonspecifically; the staphylococcal cells were then removed by centrifugation. Antibodies to RT and/or the IN protein were added to the lysed particles, and the antigen-antibody complexes were subsequently collected with fixed staphylococcal cells and analyzed as usual (43, 44). The antibodies used for analysis of RT and IN were from two sources. Rabbit sera against RT(α) and IN proteins, expressed by *E. coli*, were supplied by Lance Stewart and Volker Vogt. Goat anti-RT serum, raised against enzyme purified from virus, was donated by Duane Grandgenett (St. Louis University). When the goat serum was used, rabbit anti-goat immunoglobulin G (Cooper Biomedical, Malvern, Pa.) was added to the samples to facilitate binding of the immune complexes to the fixed *S. aureus* cells.

RT assays. Unlabeled particles for RT assays were harvested from the culture medium of transfected cells at 48 and 72 h posttransfection, and the two harvests were combined. The particles from a 100-mm plate of transfected cells were collected by centrifugation as described above, except that the pellet was suspended in 200 μl of 10 mM Tris-Cl, pH 8.0, with 1 mM EDTA and stored at −70°C. The RT activity associated with the particles was measured by incorporation of [^{32}P]TTP during synthesis of DNA on a poly(A) template as described by Goff et al. (9). Typically, 10-μl samples of the concentrated particles were added to 50 μl of reaction cocktail and the reactions were incubated at 37°C. At intervals over a 2- to 3-h period, 10-μl samples of the reaction mixture were transferred to DE81 filters (Whatman) and dried. The filters were washed twice, for 5 min each time, in 0.3 M sodium chloride–30 mM sodium citrate (2× SSC), twice in 95% ethanol, and once in distilled water and then counted in scintillation cocktail.

RESULTS

Previous studies have suggested that the uncleaved Gag-Pol precursor of RSV lacks significant RT activity and that active RT arises by the action of the viral PR on the precursor protein (25, 37). The experiments described below fully support that idea and address the role of the PR domains of the Gag and Gag-Pol precursors in this process. Specifically, we asked whether the PR domain embedded in the Gag-Pol protein has an essential role in the activation of RT or, alternatively, the PR on Gag is sufficient to carry out this function. To evaluate the two PR domains separately, it was necessary to express the Gag and Gag-Pol polyproteins from different plasmids so that mutations could be used to inactivate the PR of one or the other precursor separately. Previously we have described vectors for expression of the RSV *gag* gene in mammalian cells (7, 43, 44). For this study, we extended that earlier work by developing vectors for expression of the Gag-Pol precursor.

Construction and characterization of the *gag-pol* expression vector. The RSV Gag protein, when expressed in simian cells by an SV40 expression vector, has been shown to direct the assembly of noninfectious particles that resemble authentic RSV in the kinetics of assembly and processing, in the size and trypsin resistance of the mature Gag proteins, and in particle density and morphology (43–45). We predicted, therefore, that expression of the *pol* gene in concert with *gag* should allow packaging and processing of the Pol protein. To test this idea, the pSV.G1P expression vector was developed (Fig. 1). The parent plasmid for this construction was pSV.Myr1, a *gag* expression plasmid that encodes a myristylated Gag protein (44). This plasmid was chosen as the progenitor for the series of *gag-pol* expression vectors described below primarily because the *myr1* allele lacks the splice donor site in *gag* (nt 397), which might otherwise cause significant loss of Gag and Gag-Pol expression because of mRNA splicing between this donor and the splice acceptor at the 3′ end of *pol*. To construct pSV.G1P, a fragment of the RSV Prague C genome (including all of the *pol* gene through nt 5258 in *env*) was inserted into pSV.Myr1, as described in Materials and Methods, so that the frameshift signal between *gag* and *pol* was preserved.

To evaluate expression of the *gag* and *pol* genes, 60-mm plates of pSV.Myr1- and pSV.G1P-transfected cells (at 48 h posttransfection) were labeled for 2.5 h with [^{35}S]methionine. The cells were lysed in a detergent buffer, and proteins that reacted with anti-CA serum were collected from the lysates by immunoprecipitation and analyzed by SDS-polyacrylamide gel electrophoresis and fluorography, as described in Materials and Methods. The Gag precursor ($Pr76^{gag}$), the CA protein, and related cleavage products were detected in the lysates of both pSV.Myr1- and pSV.G1P-transfected cells (Fig. 2A). In addition, pSV.G1P-transfected cell lysates (lane 1) contained a protein of

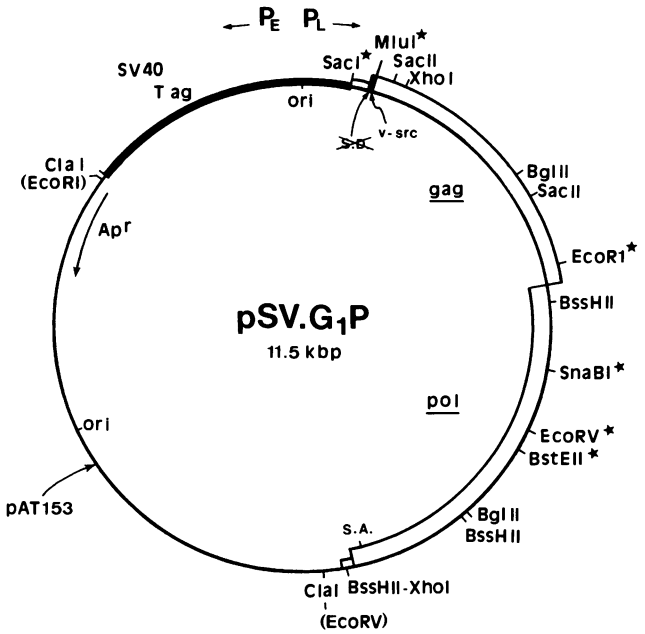

FIG. 1. Gag-Pol expression vector pSV.G1P. RSV sequences are shown as open bars. The *gag* and *pol* genes are out of frame, as in wild-type RSV, indicated by the open-bar offset. The first 10 codons of the *gag* gene have been replaced with those of v-*src* to create a myristic acid addition site; this mutation destroyed the splice donor (S. D.) at the 5′ end of *gag*, as indicated. The splice acceptor (S. A.) remains at the 3′ end of the *pol* gene. The bold line represents sequences from the SV40 genome. The early promoter (P_E) drives expression of the T antigen (T ag) gene; the late promoter (P_L) drives expression of the *gag* and *pol* genes. The SV40 origin of replication used in monkey cells is indicated (ori). The bacterial plasmid (pAT153) used to propagate the plasmid in *E. coli* is represented by the thin line, where Ap^r and ori signify the ampicillin resistance gene and the replicon, respectively. Unique restriction sites are indicated by stars. The *Eco*RI and *Eco*RV sites (in parentheses) were destroyed during the plasmid construction.

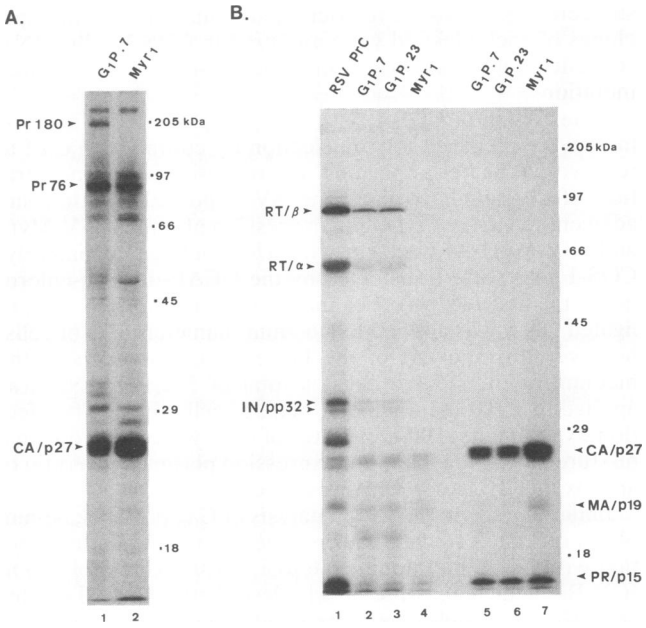

FIG. 2. Characterization of RSV Pol products produced in mammalian cells. COS-1 cells transfected with pSV.Myr1 (carrying the RSV *gag* gene) and with two clones of pSV.G1P (carrying the *gag* and *pol* genes) were labeled with [^{35}S]methionine. (A) Gag proteins from cell lysates were immunoprecipitated with anti-CA antibodies and analyzed by SDS-polyacrylamide gel electrophoresis. The positions of the Gag-Pol precursor (Pr180), the Gag precursor (Pr76), and the CA (p27) proteins are indicated by arrowheads. Fibronectin was detected above Pr180. (B) Pol cleavage products in [^{35}S]methionine-labeled particles collected from the medium by ultracentrifugation were analyzed by immunoprecipitation. [^{3}H]leucine-labeled RSV served as a control for anti-RT immunoprecipitation (lane 1). Lanes: 1 to 4, samples immunoprecipitated with goat anti-RT serum, followed by rabbit anti-goat immunoglobulin G; 5 to 7, samples immunoprecipitated with rabbit anti-RSV. The positions of the Pol cleavage products (α, β, and IN [pp32]) and the Gag cleavage products (CA [p27], MA [p19], and PR [p15]) are indicated by arrowheads. The positions of protein size standards are indicated to the right of each panel.

approximately 180 kDa that was not seen in cells expressing only Gag. This protein was also detected with anti-RSV and anti-RT sera (data not shown), confirming that it is the Gag-Pol precursor (Pr180$^{gag\text{-}pol}$). The labeled species located above the 205-kDa size marker in both lanes is fibronectin, a cellular protein that is secreted into the medium and binds specifically to staphylococcal cells in the absence of antibody; it was also seen in samples from untransfected cells (data not shown). Fibronectin was not evident in experiments in which the particles were collected by ultracentrifugation or when samples in lysis buffer were preabsorbed with fixed staphylococcal cells prior to addition of antibody.

To look for the mature Pol cleavage products in the medium, 100-mm plates of transfected cells were labeled for 6 h, after which the particles in the medium were collected by ultracentrifugation through a layer of 5% sucrose and then dissolved in lysis buffer. The mature Gag proteins were analyzed by immunoprecipitation with anti-RSV serum and the Pol cleavage products with goat anti-RT serum (Fig. 2B). In the material recovered from the medium of pSV.G1P-transfected cells (lanes 5 and 6), the mature Gag proteins (CA, MA, and PR) were detected, indicating that particles had been released from the transfected cells. Immunoprecipitation with anti-RT serum (lanes 2 and 3) revealed cleavage products that have electrophoretic mobilities identical to those of the authentic IN and RT (α and β) proteins of RSV (lane 1). The α and β forms of RT have been shown to differ by the presence or absence of the IN domain at the carboxy terminus of the protein (33). No uncleaved Gag or Gag-Pol precursors were evident in any of the medium samples (lanes 5 to 7), and no Pol-specific products were seen in the particles produced by pSV.Myr1-transfected cells (lane 4). These results indicate that the Gag-Pol protein expressed by pSV.G1P was packaged into particles that were dense enough to be pelleted though sucrose and that the polyprotein was cleaved normally. We also found in repeated experiments that the level of expression of *gag* from pSV.G1P was lower than that from pSV.Myr1 (compare the amount of CA protein seen in Fig. 2A, lanes 1 and 2, and 2B, lanes 5 to 7). This phenomenon may reflect a limit to the amplification of the pSV.G1P plasmid in COS-1 cells due to its larger size, although other explanations, such as aberrant or excessive mRNA splicing, have not been ruled out.

When G1P particles were assayed for RT activity by using an exogenous template and primer (Fig. 3), RT was readily

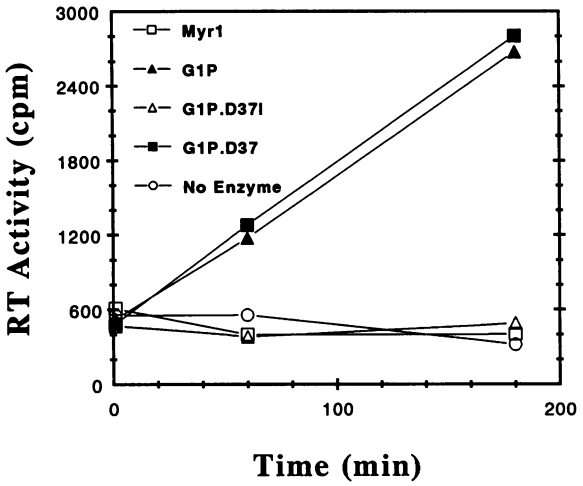

FIG. 3. RT activity in G1P particles. Particles were collected by centrifugation from the medium of transfected COS-1 cells, and RT assays were performed, using an exogenous template. Myr1 represents particles produced by the pSV.Myr1 vector that contains the *gag* gene but lacks *pol*. G1P particles were produced by the parent *gag-pol* expression vector (pSV.G1P). G1P.D37 particles were produced by a derivative of pSV.G1P in which the 3′ end of *gag* has been replaced with the wild-type sequence from the SRA strain of RSV as described in the text. The vector used to produce the G1P.D37I particles contains the same SRA sequence but also carries a mutation, D37I, that inactivates the viral PR. The reactions were set up as described in Materials and Methods, and samples were removed at 0, 1, and 3 h to determine the amounts of radioactivity incorporated.

detected. The enzyme exhibited linear kinetics similar to those of the RSV control (data not shown) over a 3-h period. As expected, no enzyme activity was found associated with Myr1 particles. The RT activity of the G1P particles produced in COS-1 cells was approximately equal to that of wild-type RSV when samples were normalized for CA protein content (data not shown), indicating that the two precursor proteins were packaged in the wild-type ratio (20 Gag molecules to each Gag-Pol molecule). We conclude from these experiments that expression of the RSV Gag-Pol protein together with Gag in COS-1 cells results in proper assembly into particles and maturation of Pol proteins.

Effects of PR mutations on RT activation. Although in authentic RSV little or no RT activity has been found in association with the uncleaved Gag-Pol precursor (37), the possibility existed that the protein would behave differently when produced in the mammalian cell expression system. To explore this, three derivatives of pSV.G1P were constructed, each containing a different mutation in the codon that specifies the aspartic acid at position 37 in the active site of the PR. These mutations inactivate the PR domains in both the Gag and Gag-Pol precursors. When the D37I mutant (containing an Asp-to-Ile substitution) was expressed in COS-1 cells, RT activity was virtually undetectable (<1% of the wild-type level) in the medium (Fig. 3). Since the *gag* gene of pSV.G1P.D37I is chimeric, containing fragments derived from both the PrC and SRA strains of RSV, we considered the possibility that the lack of RT activity was due to the chimeric structure of *gag* rather than the D37I substitution. However, expression from pSV.G1P.D37, another chimera which carries the wild-type (PR^+) version of the SRA fragment, yielded RT levels equivalent to those of pSV.G1P (Fig. 3). This result demonstrates that the D37I substitution itself prevented release of active RT. Moreover, as will be shown below, the absence of RT in the medium was not due to failure of the D37I Gag-Pol precursor to be incorporated into particles. Rather, these results confirm that the uncleaved D37I precursor produced in our mammalian cell system has no significant RT activity and are consistent with the report of Stewart and Vogt (37) that the D37I substitution caused a 900-fold reduction in the RT activity of the mutant virions relative to that of the wild type.

Although the D37I mutation has been shown to have no discernible effect on particle formation (37, 44), we were concerned that an altered conformation of the Gag-Pol precursor due to the D37I substitution (rather than the PR defect per se) could limit the RT activity of uncleaved Gag-Pol. Therefore, in addition to D37I, we also tested the D37S (Asp-to-Ser) and D37N (Asp-to-Asn) substitutions for their effects on RT activity by using the pSV.G1P vector. Both substitutions had the same effect as D37I; that is, no RT activity was found associated with particles containing the uncleaved, mutant Gag-Pol precursors (data not shown). Similar results have been reported by Stewart and Vogt in the accompanying report (38). Since multiple substitutions in the PR active site all have the same effect, it seems improbable that the lack of RT activity associated with the precursor is only an indirect effect due to alterations of the precursor conformation. Furthermore, as shown below and in the accompanying report (38), Gag-Pol precursors containing either the D37N or the D37S substitution could be processed in *trans* to yield active RT. Thus, we conclude that PR-mediated cleavage of the Gag-Pol molecule is indeed necessary for RT activation.

Fusion of the *gag* and *pol* genes. To create a vector that expresses the Gag-Pol fusion protein as the primary translation product (i.e., in the absence of Gag), plasmid pSV.G1P was modified by fusing the *gag* and *pol* reading frames. In the ASLV genome, these two genes are contiguous but out of frame by −1 bp; thus, insertion of a single base pair at the overlap site should fuse the two genes (Fig. 4). Two different mutants were constructed in this way. In pSV.GP12, a T was inserted in the last sense codon of the *gag* reading frame, a change that should result in substitution of phenylalanine in place of leucine at the last residue of the Gag domain. In the case of pSV.GP15, insertion of an A between the last *gag* codon and the termination codon TAG was made; this change should not alter the amino acid sequence of the Gag-Pol product.

Immunoprecipitation of labeled proteins from pSV.GP12-transfected cell lysates revealed the presence of the Gag-Pol precursor ($Pr180^{gag\text{-}pol}$) but no trace of the Gag ($Pr76^{gag}$) protein, confirming that the GP12 mutation had indeed fused the two reading frames (Fig. 5A, lane 3). If frameshifting had continued to occur just downstream of the insertion site, a product consisting of the Gag protein carrying 28 foreign amino acids at its carboxy terminus would be synthesized; however, no such protein was detected. Identical results were obtained with pSV.GP15-transfected cells (Fig. 5B, lane 2). Interestingly, both the GP12 and GP15 fusion proteins were trapped in the cell. Neither the uncleaved precursors nor mature cleavage products could be detected in the medium samples from cells expressing GP12 or GP15 (Fig. 5A, lane 3, and 5B, lane 2). This behavior is in striking contrast to that of the Myr1 protein (Fig. 5A and B, lanes 1) and that of the truncated Gag protein (3h), the latter of which lacks all but six amino acids of the PR (Fig. 5A, lanes 2). Consistent with the failure of Gag-Pol to be released, no RT

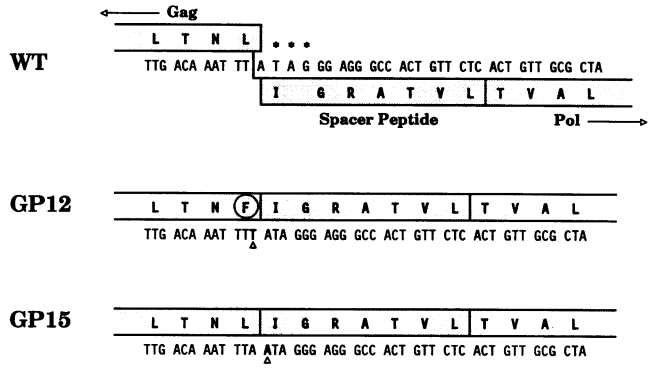

FIG. 4. Sequence at the *gag-pol* junction in the wild type (WT) and fusion mutants. The amino terminus of Gag extends to the left, and the carboxy terminus of Pol extends to the right. The stop codon in the wild-type construction is marked by the three asterisks. Normal frameshifting occurs 1 nt before the stop and is represented by the shift of the amino acid sequence from above to below the nucleotide sequence. Nucleotide insertions are indicated by the open triangles on the mutant sequences. These +1 insertions have the effect of fusing the *gag* and *pol* reading frames and synthesizing a single translation product. The circle indicates a single amino acid substitution.

activity was detectable in the medium from cells expressing GP12 (Fig. 6A) or GP15 (data not shown). Furthermore, cleavage products derived from the GP12 or GP15 Gag-Pol precursor could not be detected in the cell lysates, indicating that the PR embedded in Gag-Pol either has no activity or the Gag-Pol protein cannot undergo some (as yet uncharacterized) conformational changes that occur normally as part of virion morphogenesis and are prerequisite for PR activation. Finally, neither the D37I mutation (in GP12 [Fig. 5A, lane 4]) nor the D37S mutation (in GP15 [Fig. 5B, lane 3]) had any effect on the behavior of the trapped fusion proteins. We conclude, as do the authors of the accompanying report (38), that the RSV Gag-Pol protein itself is not capable of particle formation in the absence of the Gag precursor. A similar observation has been made for the Gag-Pol precursor of murine leukemia virus (8).

Rescue of GP12 and GP15 fusion proteins by wild-type Gag protein. Although the GP12 and GP15 fusion proteins were themselves incapable of particle formation, we expected that they would retain the ability to interact with the Gag precursor. Thus, we attempted to package the trapped fusion proteins into particles (i.e., rescue them) by complementation with the wild-type Gag protein. If this proved to be possible, then the wild-type and PR-defective versions of the Gag and Gag-Pol proteins could be tested in different combinations to evaluate the contribution of each PR domain to the proteolytic activation of RT.

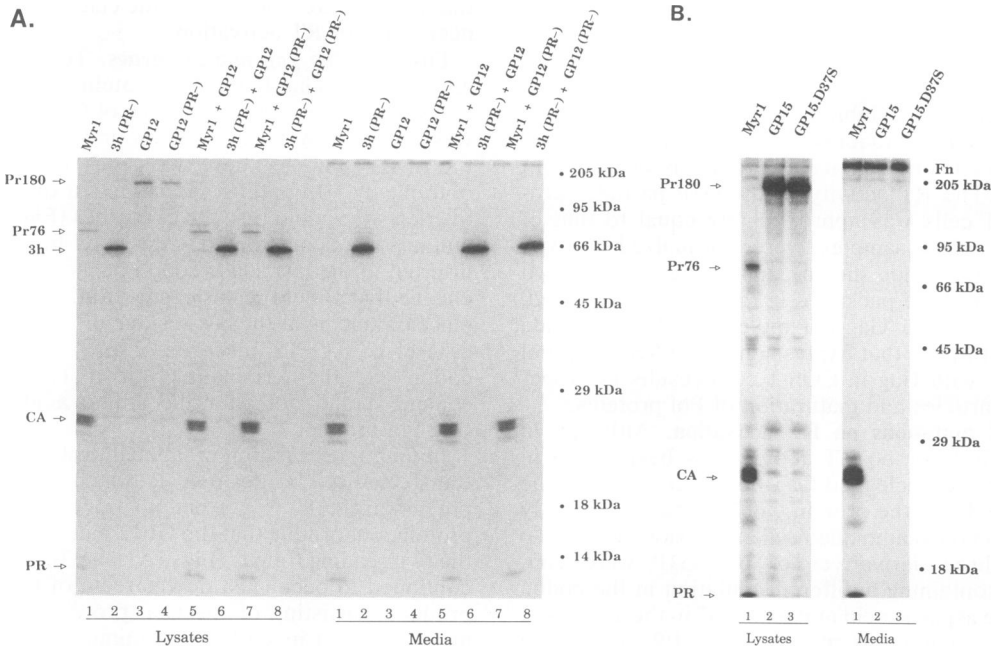

FIG. 5. Analysis of Gag and Gag-Pol proteins by immunoprecipitation. COS-1 cells were transfected with either a Gag or a Gag-Pol expression vector or cotransfected with a mixture of Gag and Gag-Pol expression plasmids, as indicated. After 48 h, the cells were labeled with [^{35}S]methionine and RSV-related proteins present in the cell lysates or particles in the growth medium were collected by immunoprecipitation with anti-RSV and analyzed by SDS-polyacrylamide gel electrophoresis and fluorography. For each sample, the transfected plasmid(s) is shown at the top of the lane. (A) Expression of Gag-Pol fusion protein GP12. Myr1 (lane 1) expresses a full-length Gag protein with wild-type PR; 3h (lane 2) produces a truncated Gag protein that lacks most of the PR domain and is therefore labeled (PR$^-$); GP12 (lane 3) expresses a Gag-Pol fusion protein that is wild type except for the presence of a single amino acid substitution at the carboxy terminus of PR (Fig. 4); GP12(PR$^-$) (lane 4) produces a Gag-Pol fusion protein that is PR$^-$ because of a D37I substitution. Lanes 5 to 8 show the results of cotransfection with a mixture of Gag and Gag-Pol expression plasmids, as indicated. Immunoprecipitated proteins were analyzed by electrophoresis in a 12% gel. (B) Behavior of the GP15 Gag-Pol fusion protein. Myr1 (lane 1) is defined in panel A. GP15 (lane 2) expresses a wild-type Gag-Pol protein. GP15.D37S (lane 3) produces a fusion protein that is (PR$^-$) because of a D37S substitution. The fibronectin band is indicated by the abbreviation Fn at the right. The positions of protein size standards are labeled at the right.

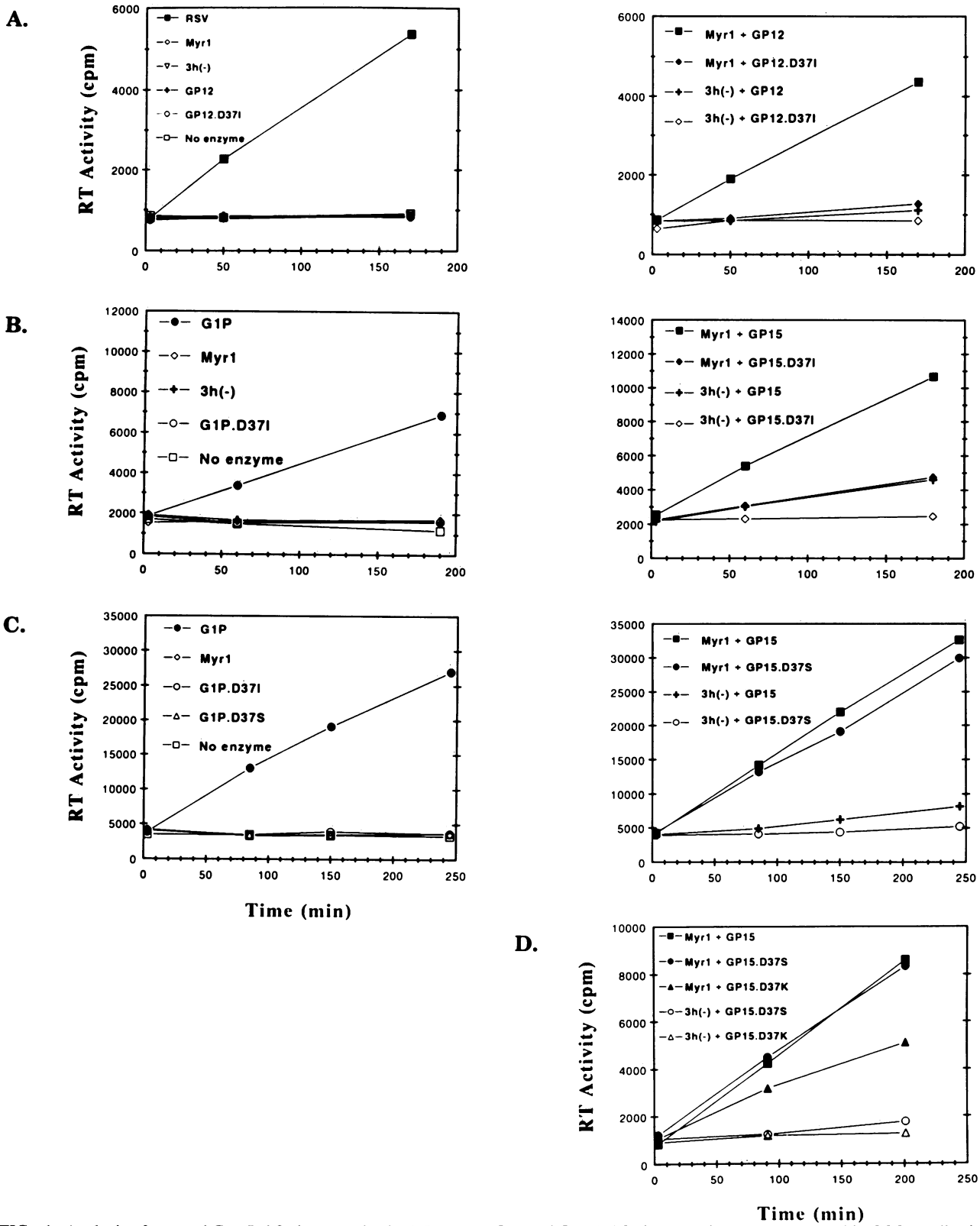

FIG. 6. Analysis of rescued Gag-Pol fusion proteins by RT assays. Gag and Gag-Pol fusion proteins were expressed in COS-1 cells either alone (control graphs on the left) or in combinations (graphs on the right) to evaluate the relative contributions of the Gag and Gag-Pol PR to RT activation. Particles released into the growth medium of transfected cells were collected by ultracentrifugation and assayed for RT activity by using an exogenous template, as described Materials and Methods. Samples were removed at various times during each RT assay to observe the reaction kinetics. Phosphate-buffered saline, rather than particles from transfected cells, was added to the no-enzyme control reaction. Myr1 and 3h express Gag-only proteins with and without a PR domain, respectively. The cotransfection experiments (right side) utilized *gag-pol* expression vectors that contained different combinations of the Gag-Pol fusion mutations and the PR mutations, as follows: A, GP12 and PR mutation D37I; B, GP15 and PR mutation D37I; C, GP15 and PR mutation D37S; D, D37I, D37S, and D37K, all contained within the GP15 fusion mutant. Details concerning the interpretations of these data can be found in the text.

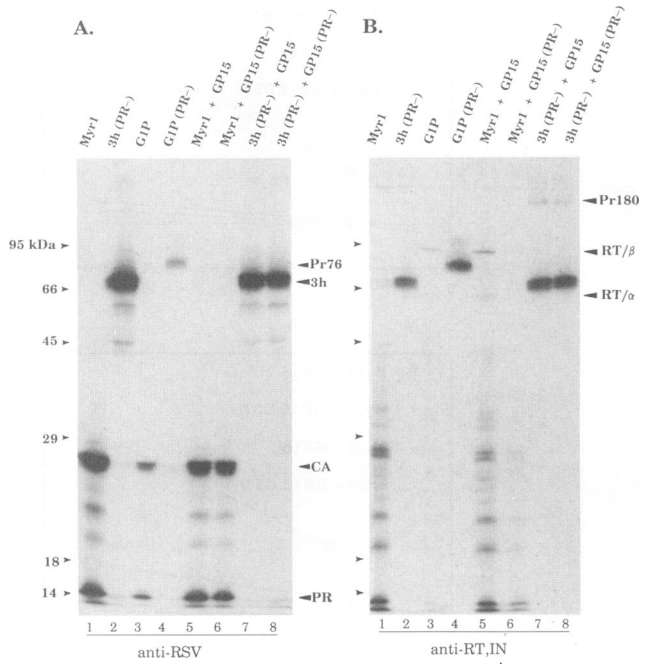

FIG. 7. Analysis of rescued Gag-Pol fusion proteins in particles. COS-1 cells were transfected with the indicated DNAs, either alone or in combination. After 48 h, the cells were labeled with [^{35}S]methionine and the particles were collected by ultracentrifugation. Vector G1P expresses the Gag precursor along with the Gag-Pol precursor by ribosomal frameshifting. GP15 expresses a Gag-Pol precursor with a fully wild-type PR domain. GP15(PR$^-$) produces the same fusion protein, but it is PR defective because of a D37I mutation. Myr1 and 3h(PR$^-$) are as defined in the legend to Fig. 5. (A) One-tenth of each sample was used in an immunoprecipitation with antibodies against RSV. The Gag precursor (Pr76), the 3h protein, and the CA and PR cleavage products are indicated. (B) The remainder of each sample was immunoprecipitated with a mixture of antibodies against RT(α) and IN. The Gag-Pol precursor (Pr180) and the mature RT proteins, α and β, are marked by arrowheads at the right. The positions of molecular mass standards are indicated by arrowheads to the left of each panel.

To evaluate whether the GP12 fusion protein could be packaged into particles by coexpression with a wild-type (PR$^+$) Gag protein, COS-1 cells were transfected with a mixture of plasmids: pSV.Myr1 (to direct Gag synthesis) and pSV.GP12 (for expression of Gag-Pol). At 3 days posttransfection, particles were recovered from the medium and assayed for the presence of RT activity. Neither pSV.Myr1 nor pSV.GP12 alone was capable of directing RT release (Fig. 6A, left panel); however, when the two plasmids were introduced together into cells, RT did accumulate in the culture medium (Fig. 6A, right panel). Similar results were obtained with pSV.GP15 (Fig. 6B and C, right panels). Moreover, the mature Pol cleavage products (RTα, RTβ, and IN) were detected in particles produced by cells cotransfected with pSV.Myr1 and pSV.GP15 (Fig. 7, lane 5) and no uncleaved Gag-Pol precursor was present. The same pattern of cleavage products was seen in particles formed by cells cotransfected with pSV.Myr1 and pSV.GP12 (data not shown). These results demonstrate that Gag-Pol fusion proteins GP12 and GP15, although themselves incapable of particle assembly, could be incorporated into particles when coexpressed with the Gag protein.

We found that cotransfection of pSV.GP12 with Gag expression plasmid pSV.Myr1 always resulted in a considerable decrease in the expression of Gag-Pol compared with that obtained when Gag-Pol was expressed alone (Fig. 5, lysates, lanes 3 and 5). In contrast, Gag expression by pSV.Myr1 was not noticeably affected by pSV.GP12 (compare the amounts of CA protein evident in lysates and medium samples in Fig. 5, lanes 1 and 5). The same phenomenon was observed in cells cotransfected with pSV.-GP15 and pSV.Myr1 (data not shown). These results suggest that the pSV.Myr1 expression plasmid competes with the pSV.GP12 and pSV.GP15 fusion vectors for replication or expression in COS-1 cells, but the exact molecular mechanism is not known. The number of particles produced in the cotransfections is, nevertheless, higher than that obtained from pSV.G1P alone (Fig. 7A, lanes 3 and 5), reflecting the higher level of Gag expression obtained with pSV.Myr1 than with pSV.G1P (as noted above). Consequently, the ratio of Gag-Pol to Gag molecules in the particle population is reduced and the specific activity of the RT is lower than for authentic RSV.

Activation of RT in *trans* by the Gag PR. Since the Gag-Pol fusion protein alone is incapable of particle formation, any Pol-containing particles that are released by coexpression of the fusion protein with a Gag protein must contain both types of precursors. Most importantly, those particles would be sure to contain both the Gag PR and the embedded PR of Gag-Pol. To examine the question of whether both PR domains must be functional to activate RT, COS-1 cells were transfected with mixtures of plasmids in which either the Gag or the Gag-Pol expression vector, or both, carried a mutation to inactivate the PR in the expressed polyprotein (Fig. 8). The particles produced by each combination of proteins were collected and analyzed for RT activity (Fig. 6) and for protein content (Fig. 7).

To test the hypothesis that the Gag-derived PR is capable of cleaving the Gag-Pol precursor to yield active RT, the Myr1 Gag protein (containing an active PR domain) was coexpressed with fusion proteins bearing each of several PR$^-$ mutations. Interestingly, the different mutations displayed different effects on RT activation (Fig. 6). From coexpression of Myr1 and GP15.D37S, fully wild-type levels of enzyme were obtained (Fig. 6C), indicating that the Asp-to-Ser substitution in Gag-Pol did not prevent RT maturation. These results demonstrate that a functional PR domain on the Gag-Pol precursor is not essential for RT maturation; rather, the requirement for a functional PR can be met fully by the action in *trans* of the Gag-derived PR. Although catalytic activity of the embedded PR is not essential, it is still possible that the PR domain plays a critical structural role in the Gag-Pol precursor (see Discussion).

In marked contrast to the results obtained with D37S, the presence of the D37I substitution in the Gag-Pol fusion protein sharply reduced the yield of active enzyme. In three replicate experiments, the yield of RT resulting from coexpression of Myr1 and GP15.D37I was only 15 to 25% of that obtained with the wild-type mixture (Myr1 and GP15) (Fig. 6B). The GP15 fusion protein containing the D37K substitution, when expressed with Myr1 (Fig. 6D), yielded a level of RT intermediate between that seen with the D37I and D37S mutants (50% of the wild-type level). When the GP12 mutation (which causes a Leu-to-Phe substitution at the carboxy terminus of the PR domain in GP12) was combined with the D37I substitution in GP12.D37I (Fig. 6A), the yield of active RT was reduced even further than with GP15.D37I (down to

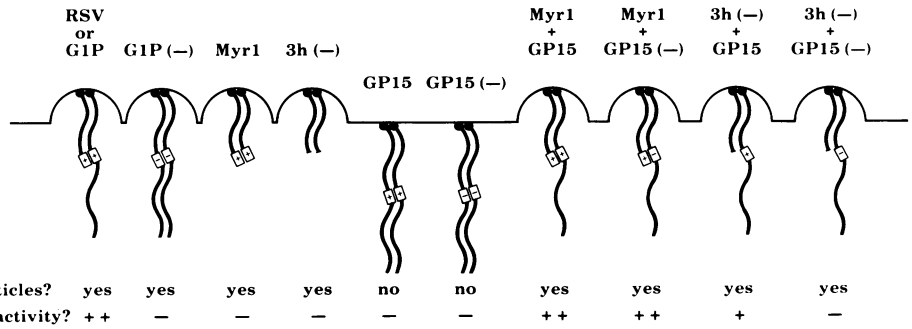

FIG. 8. Summary of phenotypes observed in coexpression experiments. The continuous line through the middle of the diagram represents the plasma membrane, and combinations of molecules that are capable of particle formation are indicated by bulges at the membrane. Gag precursors are indicated by short wavy lines, and Gag-Pol precursors are indicated by long wavy lines. The black balls represent the membrane-binding domains of Gag and Gag-Pol. Each PR domain is labeled as either positive or negative, except for mutant 3h, in which the PR domain was deleted. In GP15(−), the fusion protein is PR^- because of a D37S substitution. The arrangement of precursors at the membrane is not meant to imply anything about the true ratios of Gag and Gag-Pol in each situation. For each experiment, particle production and the presence of RT activity in the medium are indicated below the diagram.

7 to 15% of the wild-type level in the case of the GP12 mutant).

The presence of Gag and Pol proteins in the particles produced by coexpression of the Myr1 Gag protein with the GP15.D37I fusion protein was evaluated by immunoprecipitation. Analysis of the Gag proteins (Fig. 7A, lanes 5 and 6) showed that similar amounts of material were recovered from each of the plates of cells. The RT proteins (α and β), while clearly seen in the sample from the wild-type mixture (Myr1 and GP15 [Fig. 7B, lane 5]), are only faintly visible in the case of Myr1 and GP15.D37I (Fig. 7B, lane 6). That the mature RT subunits could be seen at all confirms that the Gag-derived PR is capable of cleaving the Gag-Pol precursor in *trans*. The low background of small Gag-specific bands in Fig. 7B, lane 6, suggests that some loss of material occurred during RT immunoprecipitation or electrophoretic analysis of this particular sample, but repeated experiments have confirmed that the amount of mature α and β RT is, indeed, reduced in the particles produced by Myr1 and GP15.D37I compared with fully wild-type particles.

The low yields of mature RT produced by *trans* processing of the D37I and D37K precursors led us to consider the possibilities that the mutant Gag-Pol precursors were not expressed to the same level as their wild-type parent or were not incorporated into particles with the same efficiency. The first possibility was tested by transfecting plates of COS-1 cells with either pSV.GP15 or its D37I, D37S, and D37K derivatives in the absence of a *gag* expression vector; the Gag-Pol precursor present in cell lysates was analyzed by immunoprecipitation (Fig. 9A, lanes 2 to 5). Densitometric scans of the film shown in Fig. 9A showed that the fusion proteins accumulated to similar levels (i.e., within 20% of one another) in cells transfected with each of the four plasmids. This demonstrates that the mutants do not differ significantly from their wild-type parent in the ability to express *gag-pol*. In addition, Gag-Pol expression levels did not change when the amount of DNA applied was varied over a sixfold range (Fig. 9A, lanes 5 to 7), indicating that the plasmid was used in these experiments at a saturating level. Thus, slight variations in expression levels from one plate of transfected cells to another are not due to differences in the amounts of DNA applied to the cells.

To evaluate the ability of the D37I, D37S, and D37K forms of GP15 to be rescued into particles with a Gag precursor, the mutant proteins and their wild-type parent (GP15) were coexpressed with the 3h protein (the truncated version of the Myr1 protein that lacks all but six residues of the PR domain) (Fig. 9B). The 3h protein, rather than the full-length Myr1 Gag protein, was used for this experiment to prevent proteolytic processing and thus make it easier to visualize the *pol* gene product (Gag-Pol). To quantitate the amount of Gag-Pol incorporated into particles, densitometric scans were performed on the film shown in Fig. 9B to obtain a relative measure of the amount of Gag-Pol precursor in each sample; the 3h precursor was measured by scanning a shorter exposure of the same gel, and the ratio of Gag to

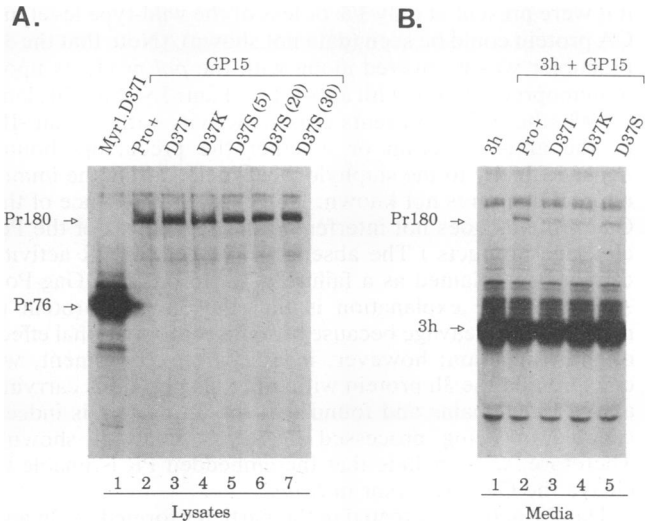

FIG. 9. Effects of PR mutations in pSV.GP15 on expression and packaging of Gag-Pol fusion proteins. (A) COS-1 cells were transfected with pSV.Myr1.D37I, wild-type pSV.GP15, and the D37I, D37K, and D37S variants of pSV.GP15. Lanes: 1 to 4, 35-mm-diameter plates of cells treated with 20 μl of ligated plasmid DNA as described in Materials and Methods; 5 to 7, DNA volumes of 5, 20, and 30 μl, as indicated. (B) COS-1 cells were transfected with 3h DNA alone (lane 1) or 3h DNA mixed with the GP15 plasmid carrying either the wild-type PR allele or the D37I, D37K, and D37S alleles (lanes 2 to 5).

Gag-Pol precursors present in each sample was calculated. The results indicated that the D37I and D37S precursors were present in similar amounts (within 14% of one another). In this particular experiment, the Gag to Gag-Pol ratio measured for the wild-type particles (lane 2) was twice that found for the D37S and D37I particles (lanes 3 and 5); the experiment whose results are shown in Fig. 7B, lanes 7 and 8, revealed a much smaller effect, however. From these results and others (data not shown), we conclude that the Gag-Pol mutants and their wild-type parent generally differ by 20% or less in incorporation. In no case did we find a difference that could account for the four- to sixfold lower RT activity consistently observed in particles containing the D37I Gag-Pol precursor compared with particles composed of the wild-type or D37S proteins. These results suggest to us that the low level of RT activity in particles produced by coexpression of Myr1 and GP15.D37I is not due to an effect of the PR mutation on Gag-Pol packaging but, rather, that the *trans* processing of the mutant Gag-Pol protein is incomplete or occurs inappropriately.

Limited activity of the embedded PR domain. To evaluate whether the embedded PR of the Gag-Pol precursor could function alone (in the absence of a functional Gag-derived PR) to release active RT, the wild-type GP12 and GP15 molecules were coexpressed with truncated Gag protein 3h (Fig. 6A to D). In all cases, very little RT activity was detected in the medium (usually about 10% of the wild-type level, but as high as 25% in one experiment). As demonstrated above, this low level was not due to failure of the Gag-Pol molecules to be packaged.

The Gag- and Pol-related proteins present in the particles produced by coexpression of 3h and GP15 were examined by immunoprecipitation (Fig. 7A and B, lanes 7). The Gag and Gag-Pol precursors were detected, but no mature cleavage products were evident. On very long exposures, where we would expect to be able to see the mature CA protein even if it were present at only 1% or less of the wild-type level, no CA protein could be seen (data not shown). (Note that the 3h precursor was recovered along with the *pol* products upon immunoprecipitation with anti-RT and anti-IN [Fig. 7B, lane 7]. Whether this represents cross-reactivity with the anti-IN or the anti-RT serum or whether the precursors bound nonspecifically to the staphylococcal cells during the immunoprecipitation is not known. However, the presence of the Gag proteins does not interfere with the analysis of the Pol cleavage products.) The absence of detectable PR activity cannot be explained as a failure of 3h to package Gag-Pol. An alternative explanation is that the 3h Gag protein is refractory to cleavage because of some conformational effect of the truncation; however, in a separate experiment, we coexpressed the 3h protein with other Gag proteins carrying active PR domains and found that the 3h protein is indeed capable of being processed in *trans* (data not shown). Therefore, we conclude that the embedded PR is unable to cleave the Gag precursor in *trans*.

The RT activity detected in the particles formed by 3h and GP15 suggests that some proteolysis of the Gag-Pol fusion protein occurred under these conditions. It seems unlikely that this activation of RT is due to a cellular or serum PR, since RT activity was undetectable in the case in which both precursors were PR deficient. Rather, it seems likely that the PR domain of Gag-Pol has the ability to cleave the Gag-Pol precursor to a limited extent, yielding an active, although perhaps incompletely processed, enzyme. We were not able to identify the cleavage products, however.

DISCUSSION

In ASLVs, as in all other retroviruses that have been examined, the viral PR appears to be responsible for all of the proteolytic cleavages that occur during maturation of the Gag and Gag-Pol precursors. Unlike all other retroviruses, however, those of the ASLV group produce a PR domain at the carboxy terminus of every Gag precursor, as well as within the Gag-Pol protein. Studies presented previously have shown that the Gag-derived PR is capable of carrying out complete maturation of the Gag protein (2). However, the question of whether the embedded PR of the RSV Gag-Pol precursor has an obligate role in the maturation of the Pol proteins has not been addressed until now. A direct approach to this question has been limited by the overlapping nature of the *gag* and *pol* genes—mutations that block the activity of the PR domain of Gag also affect the PR of Gag-Pol. Our strategy, therefore, was to develop an expression vector that could direct the synthesis of Gag-Pol separately from the Gag precursor and thereby allow the contribution of each precursor to the proteolytic maturation of Pol proteins to be assessed.

The *gag-pol* expression vector. By using an SV40-based vector that carries the *gag* and *pol* genes of RSV in their normal out-of-frame relationship, we have shown that expression of the Gag and Gag-Pol precursors in COS-1 cells results in packaging of the proteins into particles and processing to yield mature Gag and Pol products, all of which have electrophoretic mobilities identical to those of their counterparts in authentic RSV. Since the Gag-Pol fusion protein is synthesized by the same ribosomal frameshifting mechanism that operates in virally infected cells, the two precursors are produced in the normal 20:1 ratio and the RT activity associated with the particles is equal to that of RSV on a per-particle basis.

To express the Gag-Pol precursor separately from the Gag protein, a derivative of the *gag-pol* expression plasmid was constructed by fusing the *gag* and *pol* reading frames. We found that the resulting Gag-Pol fusion protein, in the absence of Gag, accumulated to a high level in the transfected cells without releasing particles. This observation is consistent with that of Felsenstein and Goff (8), who characterized a similar mutant of Moloney murine leukemia virus, and with our earlier results, which showed that fusion of the first 85 amino acids of RSV Pol onto the carboxy terminus of Gag severely reduced the ability of Gag to form particles (2). The reasons for the inability of Gag-Pol fusion proteins to form particles are unclear. It may be that the conformation of the Gag domain is affected by fusion with the Pol domain such that it is unable to function in particle assembly. Alternatively, it may be that the Pol domains, when present on every Gag molecule, cannot be accommodated in a particle of fixed diameter and thus may interfere with the budding process by a steric mechanism. Whatever the reasons, the block to particle formation could be relieved by coexpressing the Gag protein in the same cell; this was accomplished by simple cotransfection of COS-1 cells with a mixture of Gag and Gag-Pol expression plasmids. Under these conditions, mature Gag and Pol products and RT activity accumulated in particles in the medium. Since the fusion protein can be packaged only in the presence of the Gag protein, it follows that the Gag-Pol proteins that are released into the medium must be contained within particles that also contain Gag.

That the Gag-Pol precursor of the avian virus would be able to function in mammalian cells was not obvious, since

past studies have found that RSV-transformed mammalian cells produce little or no virus (39, 42). However, the work presented here and elsewhere (43, 44) has shown that this phenomenon is not due to inability of Gag and Gag-Pol precursors to function in mammalian cells. An explanation for the earlier observations may be that the RSV *gag* and *pol* genes, in the context of the RSV genome, are expressed at too low a level to drive assembly or that they express abnormal Gag proteins as a result of unusual splicing events. Use of SV40 vectors apparently avoids these blocks to RSV Gag-mediated assembly.

Activation of RSV RT. The presence of a functional PR in the viral particle appears to be an absolute requirement for activation of RSV RT. Stewart and Vogt (37) have shown that substitution of an isoleucine for the aspartic acid in the catalytic site of RSV PR blocked proteolytic processing and RT activation. Our study and the accompanying report (38) demonstrate that substitution of serine, asparagine, or lysine for Asp-37 of PR all have the same effect as the isoleucine and, thus, provides additional support for the notion that the Gag-Pol precursor lacks significant RT activity. Because of this dependence on PR function, we were able to use RT as a sensitive indicator of the processing of the Pol protein during assembly.

In all of the retroviruses that have been examined, a defect in the viral PR has been found to result in a decrease in RT activity of the resulting particles relative to the wild-type strain (where RT activity is defined as the ability to catalyze DNA synthesis by utilizing an exogenously supplied RNA template and DNA primer). However, the extent of the effect seen by different investigators using different retroviruses has been variable. Our work (reported here) and that of Stewart and Vogt (37, 38) have shown that inactivation of the PR by an amino acid substitution in the active site reduces the RT activity of the particles to less than 1% of the wild-type level. In the case of murine leukemia virus, studies utilizing mutant particles that were temperature sensitive for PR activity suggested that the unprocessed Gag-Pol precursor was enzymatically inactive until shifted to the nonrestrictive temperature (46). In contrast, a mutant, *dl*2905, in which much of the 3′ half of the PR coding sequence was deleted (leaving the codons for the PR active site intact) produced particles that contained one-third to one-half of the wild-type RT activity even though no processing of Gag-Pol was detected (5). Biochemical characterization of this same mutant by other investigators, however, found that the levels of RT activity were only 1 to 2% of the wild-type level (27). These conflicting results have not been resolved in the literature. The results obtained with human immunodeficiency virus are equally confusing. Two different studies of PR mutations in human immunodeficiency virus have shown that the RT activity of PR-defective particles is reduced by as little as twofold (28, 29) or as much as sixfold (11). In contrast, uncleaved Pol precursor proteins produced in *E. coli* were found to be enzymatically inactive in the absence of PR-mediated cleavage (22). Thus, no generalizations regarding the presence or absence of polymerase activity in unprocessed Gag-Pol proteins from different viruses can be drawn.

None of the studies cited above has addressed a possible weakness in the interpretation of the effects of PR mutations on RT activity. Even after treatment with detergent to solubilize the viral membrane, the immature PR-defective cores may be less permeable to the exogenously supplied template and primer than are mature wild-type cores. This may be a real concern, since the immature cores of RSV, but not mature ones, have been shown to be resistant to disruption by nonionic detergent at a concentration 10-fold higher than those used in the RT assay cocktail (9, 37). Furthermore, most of these studies evaluated only the ability of the immature *pol* products to initiate reverse transcription on an exogenous template and not the ability to complete synthesis of viral DNA in vivo. The exception is the study of Crawford and Goff (5), which showed that mutant particles with a significant level of RT activity were able to initiate but could not complete the synthesis of viral DNA in vivo. Thus, at least in the case of murine leukemia virus, it appears that one or more functions of the mature Pol proteins, aside from the RT activity (template strand switching, for instance), cannot be performed by the unprocessed enzyme.

Processing of Pol in ASLV. How is processing of the Pol domain actually carried out? One could imagine at least two possibilities. The first is that complete processing of Pol can be performed by mature, Gag-derived PR dimers acting in *trans* on the Gag-Pol precursor; in other words, the embedded PR domain of Gag-Pol could be entirely dispensable. In the second model, catalytic activity of the embedded PR domain of Gag-Pol is required to accomplish one or more of the cleavages of the Gag-Pol fusion protein. In this case, an active PR may be formed by pairing one Gag-Pol precursor with another Gag or Gag-Pol protein, and the PR dimer formed in this way would act either in *cis* or in *trans* on another precursor to catalyze RT release.

The results presented in this report appear to support the first model. When an active PR was present on the Gag precursor but Gag-Pol was PR defective because of the D37S substitution, RT was activated to the same level obtained when both precursors were PR^+, demonstrating that an active PR domain in Gag-Pol is not essential for RT activation. In the accompanying report, a D37N substitution was used and the results were the same (38). Since the uncleaved mutant Gag-Pol precursor has no significant RT activity of its own and the only active PR in this experiment was that supplied by the Gag precursor, it is clear that activation can occur in vivo by *trans* cleavage. Cleavage of the RSV Gag-Pol precursor and activation of RT in *trans* has previously been demonstrated by supplying exogenous PR to immature virions in vitro (25, 37).

The full activation of the GP15.D37S RT in this *trans*-processing experiment argues against the second model, that a catalytically active Gag-Pol PR domain is necessary to carry out cleavages in Pol, although the possibility that the Gag-Pol PR carries out a cleavage that is not prerequisite for RT activation cannot be ruled out. Further evidence against the second model comes from experiments in which a PR-defective Gag protein (mutant 3h) was coexpressed with a Gag-Pol fusion protein carrying a wild-type PR domain. Under these conditions, the wild-type Gag-Pol precursor was readily packaged into particles but cleavage of the Gag-Pol and Gag precursors was virtually undetectable, although a limited amount of RT activity was obtained. These results suggest that the Gag-Pol PR domain is able to carry out, at best, only very limited cleavage of Gag or Gag-Pol, which we were unable to detect by immunoprecipitation. Stewart and Vogt (38), in a study described in the accompanying report, found no detectable PR or RT activity associated with particles produced by a PR mutant Gag protein and a wild-type Gag-Pol protein.

The reasons for the limited activity of the RSV Gag-Pol PR domain are not understood, especially since the PR of human immunodeficiency virus and murine leukemia virus function well as embedded domains. A possible explanation

is that RSV PR, when embedded in the large fusion protein, has a much lower catalytic activity than its counterpart enzyme in murine leukemia virus and human immunodeficiency virus. We have presented this free carboxy terminus hypothesis in a recent report (2), which shows that fusion of 85 amino acids from the Pol reading frame onto the end of the RSV Gag protein completely blocked PR activity on the Gag substrate. The possibility existed, however, that the lack of PR activity in that case was due to an inappropriate conformation of the PR domain caused by fusion of only part of the Pol protein to Gag. The present work demonstrates that the PR domain of the full-length Gag-Pol protein is also limited in function and supports the idea that the activity of RSV PR is encoded in *gag* so that a free carboxy terminus on the PR can be formed without proteolysis. It will be interesting to learn whether the same is true for those viruses, such as the mouse mammary tumor virus, which direct the synthesis of both a carboxy-terminal PR (on the Gag-Pro precursor) and an embedded PR (on Gag-Pro-Pol).

Although catalytic activity of the Gag-Pol PR domain is not essential for RT activation, it is possible that the embedded PR domain plays a critical role in the structure of the precursor, allowing it to achieve a conformation that is capable of being activated. The behavior of the D37I Gag-Pol mutant suggests such a possibility. It has been established (above) that the Gag-derived PR is able to activate RT by *trans* cleavage of a Gag-Pol precursor that contains a serine in the PR active site. However, the same PR was not able to *trans*-activate the RT fully when isoleucine was present instead of serine. A possible interpretation is that the D37I substitution affects the structure of the precursor in a way that prevents complete and appropriate processing by the PR supplied in *trans*. An effect of the D37I substitution on the conformation of the precursor is conceivable, since the isoleucine side chain would not be able to participate in the network of hydrogen bonding that has been shown to stabilize the structure of the mature PR around the active site (18). The serine residue, because of its smaller size and ability to form hydrogen bonds, may not have a drastic effect on the structure of the enzyme, although it does prevent its catalytic activity.

If the D37I substitution exerts its inhibitory effect by making cleavage sites in Gag-Pol inaccessible or by exposing cryptic cleavage sites, then one would expect to find incomplete or inappropriate processing of the molecule by the Gag-derived PR. In our experimental system, however, identification of the cleavage products has proven difficult because of the limited quantity of material typically obtained and the fact that the Pol proteins represent only a very small fraction of the total viral protein. The cleavage products have been particularly difficult to detect in cases in which only one of the PR domains is active (Fig. 5 and 7). Efforts are under way to refine the detection of the Pol products so that cleavage of the mutant Gag-Pol proteins can be analyzed. However, to obtain enough material for definitive analysis, it may be desirable to construct cell lines that express the wild-type Gag protein along with the D37I or D37S Gag-Pol protein, an approach that has been used in the study described in the accompanying report to analyze the effects of the D37N mutation (38).

Another intriguing possibility is that the D37I substitution does not directly affect the structure of the Pol domain but rather may influence the conformation indirectly by preventing proper pairing of the PR domains of two Gag-Pol molecules. This dimerization of PR domains may, in turn, be needed to allow the Pol domains to reach the proper conformation for processing to begin. This hypothesis could be tested by making deletions of the entire PR domain. If the PR domain of Gag-Pol could be deleted without preventing Pol processing, it would follow that the PR does not have an essential structural role.

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