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Role of the Foamy Virus Pol Cleavage Site in Viral Replication[▽]

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Foamy virus Pol precursor protein processing by the viral protease occurs at only one site, releasing a protease-reverse transcriptase and an integrase protein. To examine whether the cleavage of the Pol precursor protein is necessary for enzymatic activities and efficient viral replication, several mutations were generated around the cleavage site. All cleavage site mutants synthesize wild-type levels of Pol precursor protein. Mutants containing more than two amino acid substitutions around the cleavage site exhibit no detectable Pol processing. The Pol cleavage site is not required for the production of infectious particles in a single round of infection, but is important for subsequent rounds of viral infection. Mutations around the cleavage site affected the enzymatic activities of the protease and reverse transcriptase and prevented replication after two rounds of infection. Interestingly, Pol encapsidation is significantly reduced in some of the mutants.

The synthesis of polyprotein precursors used to initiate the viral assembly process is a common feature of retroviruses (5, 40). For example, the efficient maturation of human immunodeficiency virus (HIV) particles depends strongly on the coordinated synthesis of the large structural polyprotein Gag and the Gag-Pol containing the enzymes, as well as their proteolysis into active components. Orthoretroviral enzyme genes are translated as a Gag-Pro-Pol precursor, and the expression of Pro and Pol occurs by frameshifting or translational suppression (39, 40). Both Gag and Gag-Pol are synthesized from full-length viral mRNA, which is identical to the viral genome. The use of Gag and Pol protein precursors minimizes the number of individual components that need to be targeted to the site of assembly. Once these polyproteins reach their destination, proteolysis generates mature viral proteins. This proteolysis provides a means for regulating functions needed at different stages of the replication cycle. Additionally, the highly conserved order of the MA (matrix), CA (capsid), NC (nucleocapsid), PR (protease), RT (reverse transcriptase), and IN (integrase) sequences within the Gag and Gag-Pol proteins corresponds to their relative locations in the virion (39). The Gag and the Gag-Pol polyproteins play a central role in assembly, and Gag initiates the direct budding of virus particles from the cell, even when expressed in the absence of other virus-encoded proteins.

The replication strategy of foamy viruses (FVs), in the spumaretroviral subfamily, is characterized by several unusual features that distinguish them from orthoretroviruses (21, 26, 34). For example, reverse transcription is a late event in the viral morphogenesis and the viral particles contain an infectious DNA genome (27, 36, 44). One of the most intriguing FV characteristics is the Gag-independent expression of the Pol protein from a subgenomic mRNA (23, 42). This lack of a Gag-Pol fusion protein raises questions regarding the mechanism of Pol encapsidation into particles. It was previously shown that the incorporation of genomic RNA is essential for Pol encapsidation (14). Moreover, two Pol encapsidation sequences (PES) seem to be required for Pol incorporation, one located in the 5′ untranslated region of the viral genomic RNA (PES I) and one in the 3′ region of the *pol* gene (PES II) (14, 16). The C terminus of Gag was also found to be involved in Pol processing and incorporation (38).

In contrast to orthoretroviruses such as HIV, in which multiple PR cleavage sites (CSs) are found within Gag and Pol, FVs undergo only limited proteolytic processing of structural and enzymatic proteins by the aspartic viral PR (8, 22, 33). Compared to HIV, where the Gag precursor protein is cleaved into MA, CA, and NC, only a subset of FV Gag molecules is cleaved once by the FV PR, near the C terminus (11, 12, 19, 28). The partial processing at the C terminus of Gag, resulting in a p71/p68 kDa protein doublet, has been shown to be critical for infectivity (8, 45). Another cleavage occurring in Gag early after infection appears to be essential for viral replication, possibly by allowing the disassembly of incoming CAs (20). Pol is synthesized as a 127-kDa precursor protein (PR-RT-IN) and is processed into only two cleavage products, an 85-kDa protein containing PR and RT and a 40-kDa IN protein (29, 33). Although only a PR-RT protein is found in the virions, expression in vitro of either PR or RT without the other domain leads to enzymatically active protein (18, 32).

Given the fact that PR-RT is a unique protein with both proteolytic and polymerase activities, we wondered whether the PR-RT-IN precursor has enzymatic activity. IN is essential for efficient viral replication (9, 25), but it is not known whether IN is active in the Pol precursor.

There are two approaches to studying the effects of polyprotein cleavage on the activities of the viral enzymes and viral infectivity: disruption of the PR activity or deletion of the CS. A mutant with a mutation in the PR domain (D/A) lacking enzymatic activity has been described previously (19). However, this mutant fails to cleave Gag, which is essential during the early phase of infection and for the efficient disassembly of incoming CAs (20). For this reason we chose to examine Pol CS mutants. In the present study, we analyzed the enzymatic activities of PR and RT in full-length Pol and found reduced

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TABLE 1. Sequence at the CS in wild-type FV and CS mutants[a]

Virus or construct	Amino acid sequence
wt PFV	YV**V**N ↓ CNTK
RT-IN2	YVGH ↓ WNTK
ΔCS	Amino acids deleted
CS2	YVVA ↓ ANTK
CS4	YVAA ↓ AATK
CS6	YAAA ↓ AAAK
CS8	AAAA ↓ AAAA

[a] CS consensus sequence is taken from reference 33. ↓ indicates the CS. Mutated amino acids are underlined; the bold Val indicates the P2 residue in wt PFV.

levels of PR and RT activity. Although decreased levels of Pol were detected in cell-free virions, packaging of the Pol precursor protein is sufficient for the production of infectious viral particles. Interestingly, these particles cannot undergo subsequent rounds of infection.

MATERIALS AND METHODS

Mutagenesis and cloning. All Pol CS mutants were generated in the full-length viral clone pcHFV13 (24). CS mutations (Table 1) were created through PCR mutagenesis using the outer primer pair 5′-TAACTACAATGCACAAAGCC-3′ and 5′-TAGGACCCTCTCCTTCACCAC-3′. The Pol CS was mutated from TATGTGGTTAATTGTAATACCAAA to TATGCGGCTAATTGTAATAC CAAA in mutant pRT-IN2, to TATGTGGTTGCTGCTAATACCAAA in mutant pCS2, to TATGTGGCTGCTGCTGCTACCAAA in mutant pCS4, to TA TGCGGCTGCTGCTGCTGCCAAA in mutant pCS6, to GCTGCGGCTGCT GCTGCTGCCGCA in mutant pCS8, and in mutant pΔCS (Δ24 nucleotides [nt]), the whole CS was deleted. In pRTstop1, the two stop codons TGA and TAG were introduced into pcHFV13 at position 5221 (forward primer TAAT ACCAAAAAATGATAGCTGGATGCAGAGTTG and reverse primer AACT CTGCATCCAGCTATCATTTTTTGGTATTAC), and in pRTstop2, the two stop codons TGA and TGA were introduced at position 5182 (forward primer TTGCCACCCAATGATGATATGTGGTTAATTG and reverse primer CAAT TAACCACATATCATCATTGGGTGGCAA). pcHFV13-PR$^-$ is similar to pcHFV13 except that the PR-encoding region contains the active-site mutation D24A, which renders the human FV (HFV) PR inactive (19, 35). Additional mutants used in these studies are mutant ΔGag, lacking Gag protein expression (3), and mutant IN$^-$, lacking IN activity (25). In pcHFVΔRT, the 4 amino acids (aa) of the active center of the RT (YVDD) were changed to alanines (forward primer AATGTACAAGTGGCTGCTGCTGCTATATATTTAAG and reverse primer CTTAAATATATAGCAGCAGCAGCCACTTGTACAT).

Sequence analysis. The sequences of the mutated CSs were determined on both strands using an ABI Prism BigDye Terminator v3.1 and automated DNA analysis devices (Applied Biosystems). In each pcHFV13-derived clone, nucleotide changes from the published sequence (24) were found at positions 6664 (G to A) and 6667 (A to G).

Cell culture. 293T cells (6), BHK cells (ATCC CCL-10), FAB cells (43), and diploid human embryonic lung (HEL) cells (ATCC CCL-137) were cultivated in Dulbecco's modified Eagle's minimal essential medium supplemented with 10% fetal bovine serum and antibiotics. FV stocks were obtained by transfection of 293T cells with either PolyFect reagent (QIAGEN) or 1 mg/ml polyethylenimine (Polysciences, Inc.) as described previously (7). For viral transfer experiments, HEL cells were infected with cell-free supernatants (0.45-μm-pore-size filter) for 8 h, washed once with fresh medium, and cultivated in fresh medium. Forty-eight hours after infection, all medium was removed and cells were passaged into new dishes. Viral supernatants were harvested and filtered 48 h after passaging, and titration was performed on FAB cells. The foamy virus-activated β-galactosidase (β-gal) (FAB) assay of viral infectivity was done as described previously (43).

Western blotting. Cell lysates and viral supernatants were harvested between 42 and 45 h posttransfection and prepared for immunoblotting as described previously (38). Viral supernatants were collected, passed through a 0.45-μm-pore-size filter, and then pelleted for 2 h through a 20% sucrose cushion by ultracentrifugation (24,000 rpm, 4°C; Beckman). Cell lysates and viral pellets were resuspended in 1× sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample loading buffer. After protein separation on 10% sodium dodecyl sulfate-containing polyacrylamide gels, proteins were transferred to Immobilon-P membranes (Millipore) by using semidry electrophoresis. Western blot analyses were performed using anti-Gag polyclonal rabbit antiserum (4) at a 1:5,000 dilution, anti-Pol monoclonal mouse antibody (38) at a 1:800 dilution, anti-PR polyclonal rabbit antiserum at a 1:5,000 dilution, and anti-IN polyclonal rabbit antiserum at a 1:5,000 dilution. Rabbit polyclonal antiserum against prototype FV (PFV) PR (aa 1 to 128 of Pol) was generated using a glutathione-*S*-transferase (GST)-PR fusion protein. The antibody was shown to react to both the 127-kDa precursor Pol protein and the 85-kDa PR-RT cleavage product (data not shown). The anti-IN polyclonal rabbit serum was generated using a GST-IN fusion protein (aa 763 to 1123 of Pol) and specifically recognizes the 127-kDa Pol precursor protein and the IN domain of Pol. Blots were visualized using ECL Plus reagent (Amersham Biosciences) or the Odyssey infrared imaging system (LI-COR Biosciences). The levels of Pol were determined by using the Odyssey application software, version 1.2 (LI-COR Biosciences). This assay is roughly linear over an eightfold range of protein (data not shown).

Assay of virion-associated RT activity. Virion-associated RT assays were performed as described previously (35). This assay uses poly(A) poly(dT)$_{10}$ as the primer-template, and incorporation of [α-^{32}P]dTTP is measured. Viral supernatants from transfected BHK cells were collected, filtered, and concentrated with Centriprep YM-50 spin columns (Amicon). For the RT reaction, 100 μl RT cocktail was added to a normalized amount of viral supernatant (total volume of 260 μl) and incubated at 37°C. After 90 min, 25 μl of the reaction mixture was taken for the analysis. Normalization of Pol molecules was done using an anti-Pol monoclonal antibody at a 1:800 dilution, and the protein was roughly quantitated using the Odyssey application software, version 1.2 (LI-COR Biosciences).

RESULTS

Gag and Pol expression in Pol CS mutants. Previous work has shown that the FV Pol precursor is cleaved only once, into a PR-RT/RNase H (RH) and an IN subunit (Fig. 1A). The RT-IN CS sequence YVVN^{554} ↓ C^{555}NTK is recognized by an aspartic PR that is part of the FV Pol precursor protein and is absolutely required for infectivity and processing (19). To analyze the role of the RT-IN CS for FV replication, Pol CS mutants were generated in the context of the full-length virus pcHFV as shown in Fig. 1 and Table 1. Wild-type (wt) and mutant proviruses were transiently transfected into 293T cells, and equal amounts of cell lysates were tested for viral protein expression. Following Western blot analyses using a polyclonal anti-Gag serum (Fig. 2A and B, lanes 2 to 8), we quantitated the total amount of Gag expressed in the cell for all CS mutants and compared these numbers to

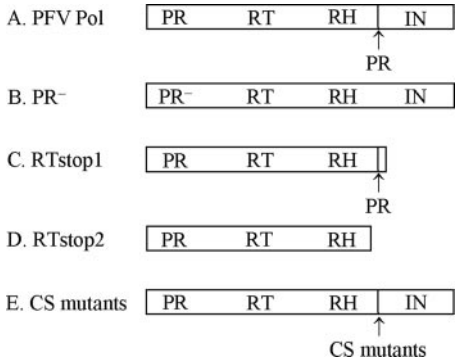

FIG. 1. Schematic representation corresponding to the PFV *pol* gene and its domains. (A) The locations of PR, RT, RH, and IN are shown. The arrow in wt PFV Pol indicates the position of the proteolytic CS between the RT and IN domains. (B) The D/A mutation in the PR$^-$ mutant results in the expression of the Pol precursor molecule only. (C) Mutant RTstop1 contains two stop codons downstream of the Pol CS, and the IN domain is absent. (D) Mutant RTstop2 contains two stop codons at the 3′ end of the RT/RH domain upstream of the Pol CS. (E) CS mutations are described in Table 1.

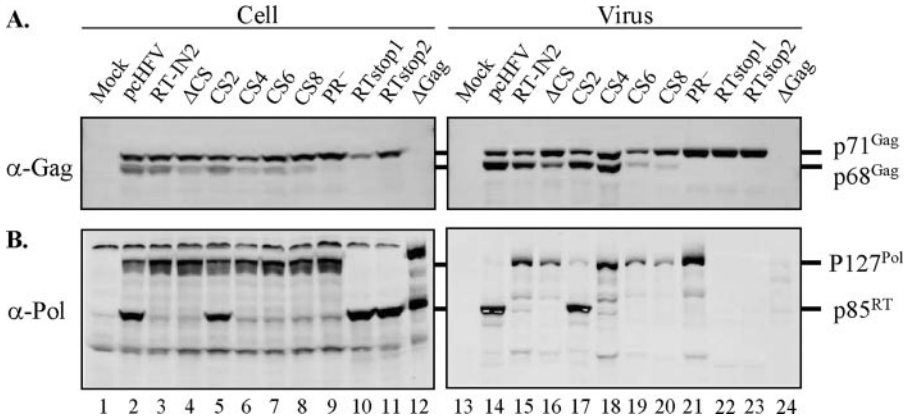

FIG. 2. Analysis of Gag and Pol expression and cleavage. 293T cells were transfected with control or viral DNA, and cell lysates as well as viral supernatants were harvested at 2 days posttransfection. (A) Western blot analysis of cell lysates and cell-free virus using polyclonal anti-Gag antiserum. (B) Western blot analysis of cell lysates and cell-free virus using a monoclonal anti-Pol antibody.

those for the wt (Table 2, first column). Mutant PR$^-$ is mutated at the active center of the viral PR and is used as a control, as there is no viral PR cleavage activity (Fig. 1B). Gag expression levels are similar for all CS mutants, albeit with slightly reduced amounts for mutants CS4 and PR$^-$ (24% and 27% less Gag expression, respectively).

To analyze the requirement of the IN protein for virus production, particle release, and viral replication, full-length infectious viral constructs expressing only PR-RT were generated (Fig. 1C and D, RTstop1 and RTstop2). Although both mutants contain an intact PR enzyme, they expressed only the 71-kDa Gag precursor protein and we did not detect Gag protein cleavage (Fig. 2A, lanes 10 and 11). Thus, it appears that the full-length Pol precursor protein is necessary for interaction with Gag and the proteolytic processing of the viral CA proteins. As expected, no Gag expression was detectable in the negative control ΔGag, which contains a large deletion of the gene (nt 1120 to 2368) (Fig. 2A, lane 12) (3). The Western blot results for released virus showed slightly reduced particle release for the ΔCS, CS6, and CS8 mutants (Fig. 2A, lanes 16, 19, and 20). The blots were quantitated using ImageJ software.

TABLE 2. Gag expression, particle release, and Gag p68/p71 ratio in wt FV and mutants[a]

Virus or construct	Gag expression and particle release (%) in[b]		p68/p71 ratio in	
	293T cells	Viral particles	293T cells	Viral particles
pcDNA[c]	0	0	0	0
pcHFV	100	100	0.96 ± 0.19	2.15 ± 0.39
RT-IN2	114 ± 25	85 ± 17	0.78 ± 0.17	1.81 ± 0.49
ΔCS	103 ± 21	68 ± 6	0.44 ± 0.11	0.54 ± 0.39
CS2	116 ± 38	109 ± 60	0.97 ± 0.19	1.95 ± 0.60
CS4	76 ± 22	108 ± 11	0.49 ± 0.18	1.18 ± 0.39
CS6	95 ± 19	50 ± 23	0.56 ± 0.21	0.89 ± 0.70
CS8	94 ± 21	58 ± 21	0.41 ± 0.24	0.5 ± 0.43
PR$^-$	73 ± 17	73 ± 11	0	0

[a] Results are mean values ± standard deviations from three independent experiments.
[b] Gag expression and cleavage are normalized to those of wt pcHFV.
[c] pcDNA, a plasmid containing a polylinker site (Invitrogen, Carlsbad, CA).

We have determined that this assay is linear over an eightfold range (data not shown). To determine the relative number of particles released for each mutant, the amount of Gag released from the cell was divided by the total amount of Gag expressed in the transfected 293T cells. The results show that all mutants except CS2 and CS4 release fewer particles into the supernatant (Table 2, second column). However, in no case was the effect greater than twofold. The PR$^-$, RTstop1, and RTstop2 mutants are all able to release the uncleaved Gag CAs into the supernatant (Fig. 2A, lanes 21, 22, and 23).

To compare cleavage of the mutants to cleavage of wt Gag, the amounts of p68 and p71 protein in transfected cells and released particles were quantitated. Equal amounts of cell lysates and viral supernatants were analyzed by Western blot analysis. The extent of cleavage in mutant PR$^-$ was set to 0 and the ratio of p68 to p71 in pcHFV and the mutants was normalized to the ratio in mutant PR$^-$. Gag cleavage in cells is reduced in most of the mutants, with about a twofold reduction (Table 2, third column). A similar pattern was observed for the p68 to p71 ratio in cell-free virus (Table 2, fourth column). Whereas minimal mutation of the CS (mutants CS2 and CS4) resulted in 1.2-fold- to 2.4-fold-reduced cleavage, more severe mutations, e.g., mutant ΔCS or CS8, resulted in about a fourfold-decreased p68/p71 ratio compared to that in the wt virus.

The PR-RT-IN precursor protein (P127^{Pol}) is cleaved into an 85-kDa PR-RT protein (p85$^{PR\text{-}RT}$) and a 40-kDa IN protein (p40^{IN}). We used a mouse monoclonal antibody directed against the RH domain of PR-RT (38) to detect both the Pol precursor protein and the cleaved PR-RT protein (Fig. 2B). Compared to the amount in the wt virus (Fig. 2B, lane 2), the amount of total Pol expressed in transfected cells was roughly similar in all mutants (Fig. 2B, lanes 3 to 8). Quantitation showed that for most mutants, Pol expression is reduced from 1.3-fold to 1.8-fold, except for mutant CS2, in which there was a 1.4-fold increase in Pol expression (Fig. 3A, black bars). However, proteolytic cleavage of the Pol precursor into mature PR-RT was observed only for the CS2 construct that contains a 2-aa substitution in the residues flanking the RT-IN CS (Table 1 and Fig. 2B, lane 5). The mutants RT-IN2, ΔCS, CS4, CS6, and CS8, which contain a greater number of mutated

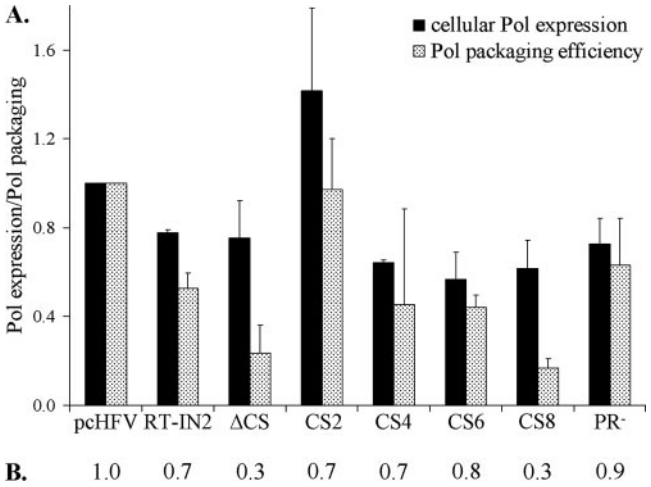

FIG. 3. Pol expression and Pol packaging efficiency. Equal amounts of cellular lysates or supernatants were loaded in each lane. (A) Pol expression and Pol incorporation into viral particles were normalized to wt virus Pol protein levels by using Western blot analysis and the Odyssey infrared imaging system. The mean values and standard deviations from three separate 293T transfections are normalized to the pcHFV control set as 1. Using dilutions of control wt FV Pol protein, the Western blot results were linear over an eightfold range (data not shown). (B) Ratios of viral Pol packaging efficiency to Pol expression in transfected 293T cells.

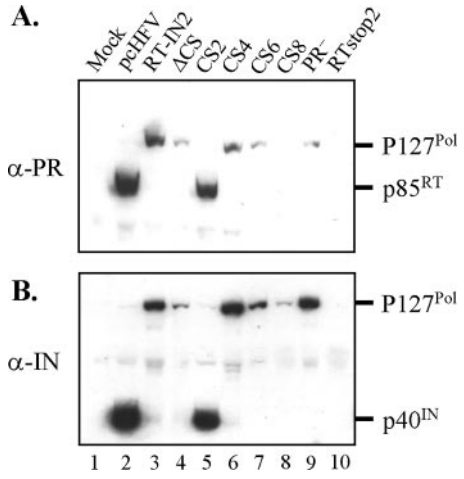

FIG. 4. Measurements of Pol incorporation into virus-like particles using anti-PR and anti-IN antibodies. (A) Pelleted viral supernatants from transfected 293T cells were probed in Western blot analyses using an anti-PR (A) and an anti-IN (B) antibody. Enhanced chemiluminescence reagents were used for signal detection with X-ray films. The levels of Gag expression in transfected cells and the levels of released CAs were the same in all mutants (data not shown).

amino acids, showed little or no Pol cleavage (Table 1 and Fig. 2B, lanes 3, 4, 6, 7, and 8). As PR$^-$ lacks PR activity, this mutant expresses only the 127-kDa precursor protein (Fig. 2B, lane 9). Both RTstop mutants showed efficient expression of the p85$^{PR\text{-}RT}$ protein with no full-length Pol protein detectable (Fig. 2B, lanes 10 and 11). Transfection of ΔGag resulted in both expression of the Pol precursor protein and cleavage into PR-RT (Fig. 2B, lane 12), showing that particle formation is not required for Pol cleavage. This implies that Pol can dimerize intracellularly, resulting in active PR.

Although the CS mutants produce significant levels of viral particles, Pol incorporation into viral particles was dramatically inhibited in some of the mutants. To determine the Pol packaging efficiency, the amount of Pol protein in viral particles was divided by the amount of Pol expressed in cells to determine the relative amount of Pol present in the particles for each mutant. Next, the amount of Pol in particles was divided by the relative number of particles released (using Gag), to calculate the relative packaging efficiency of each mutant (Fig. 3A, gray bars). We found that incorporation of the 127-kDa protein into PR$^-$ virions is slightly reduced (Fig. 2B, lane 21, and 3A), in contrast to previous work that showed no difference (3). As reported previously (31) the 85-kDa PR-RT proteins made by the RTstop mutants could not be detected in cell-free virus-like particles (Fig. 2B, lanes 22 and 23). No extracellular Pol was detected in viral supernatants obtained from ΔGag-transfected cells, confirming that the detection of extracellular Pol requires particle formation (3). The major Pol product found in wt viral particles is the 85-kDa PR-RT protein (Fig. 2B, lane 14), and a similar profile was observed for mutant CS2 (Fig. 2B, lane 17). For the other CS mutants, the Pol precursor protein was still packaged into virions, but to a much lower extent (Fig. 2B, lanes 15, 16, 18, 19, and 20, and 3A). As shown in Fig. 3B, the mutations in the ΔCS and CS8 constructs showed the most severe effects on the amounts of cellular Pol that were packaged, with threefold-reduced Pol encapsidation into viral particles (Fig. 2B and 3A, gray bars, and 3B).

To confirm the Pol cleavage results, we probed the blots with two polyclonal antisera that were raised against purified GST-IN (anti-IN) or GST-PR (anti-PR) fusion protein. Anti-PR recognizes p127^{Pol} and p85$^{PR\text{-}RT}$, and anti-IN recognizes P127^{Pol} and p40^{IN}, although for both sera, the cleaved products are recognized better than the precursor (Fig. 4A and B and data not shown). In the wt virus (Fig. 4, lane 2), the packaged Pol was efficiently cleaved to PR-RT and IN, and little or no precursor protein was detected. This was also the case for mutant CS2 (Fig. 4, lanes 5). In contrast, little or no cleaved Pol was seen in any of the other CS mutants or in mutant PR$^-$ (Fig. 4, lanes 3, 4, 6, 7, 8, and 9). As reported previously (31), no detectable Pol was seen in RTstop2 particles (Fig. 4, lanes 10). As these antisera react very strongly with Pol subunits, these results confirm the lack of processed proteins in all of the CS mutants except for CS2.

Virion-associated RT assay. We were interested in determining whether the uncleaved precursor has enzymatic activities. As shown in Fig. 2, Gag cleavage is decreased in most of the CS mutants, indicating that PR activity is affected. We used a previously described assay (35) to measure RT activity in the CS-mutant virions. Virus was concentrated and the level of Gag in the concentrated virus was assayed by Western blotting (data not shown). Equal aliquots from each concentrated viral preparation were also analyzed by Western blotting using a monoclonal anti-Pol antibody. For the RT assays, the aliquots to be tested were normalized to the amount of Pol in the virions, so that equal amounts of Pol protein were assayed in each sample. As a control, a construct mutated at the active center of the RT domain was also analyzed (pcHFVΔRT). Data obtained for the ΔRT mutant were subtracted from the

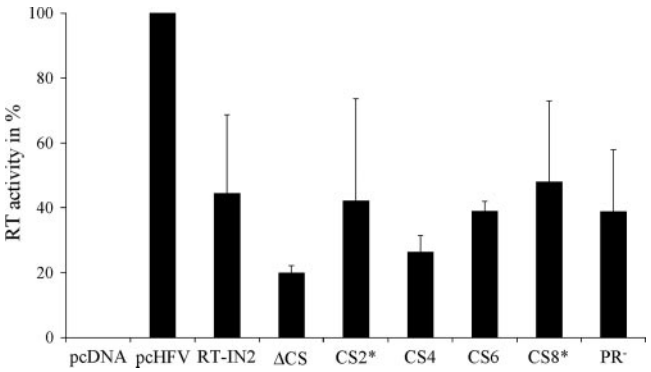

FIG. 5. Virion-associated RT assay. BHK cells were transfected, and viral supernatants were harvested 4 days posttransfection and concentrated. Aliquots of pelleted supernatants were normalized for Pol content, and the RT activity was determined for each of the concentrated samples by measuring the incorporation of a radiolabeled nucleotide as described in Materials and Methods. Incorporation was measured at 90 min and normalized to that of the wt sample (pcHFV). The mean values and standard deviations from three independent experiments are shown. The levels of RT activity were assessed by a paired *t* test, and all mutants showed statistically significant decreases ($P < 0.005$, except for mutant CS2, $P = 0.01$, and for mutant CS8, $P = 0.02$; indicated by $*$).

data for the wt virus and each of the CS mutants, and the wt level (pcHFV) was set to 100% (Fig. 5). All the mutants showed decreased RT activity relative to the activity in the wt virus. Mutant ΔCS showed the lowest level, 16% of the wt level. The decrease in RT activity for all mutants was statistically significant using a paired *t* test. These results indicate that blocking cleavage at the RT-IN site decreases RT activity, but does not eliminate it.

Infectivity of CS mutants. We next asked whether or not the CS mutants were able to replicate. Mutant or wt plasmids were transfected into 293T cells, and culture supernatants were used to infect FAB cells. These are BHK cells with an integrated copy of a plasmid containing the FV long terminal repeat (LTR) driving expression of the β-gal gene (43). No β-gal activity is detectable unless the FV Tas transactivator protein, which is absolutely required for transcription from the LTR promoter, is present to activate the viral LTR. Thus, these cells provide a quick and convenient assay for viral infectivity. In this single round of infection assay (Table 3), we found that the wt virus had a titer of 2.2 (± 0.8) × 10^6. All of the mutants had titers ranging from 63.6% of that of the wt in mutant CS2 to 0.8% of that of the wt in mutant ΔCS, which still retained a titer of greater than 10^4 infectious units per ml. However, the FAB assay measures only the ability of virus to infect cells and express Tas. Since FV has a DNA genome, it is possible that Tas could be transcribed and translated from the incoming genomic DNA. Indeed, DNA extracted from extracellular virions has been shown to be infectious (36, 44). However, induction of β-gal in the single round of infection assay does require the production of virions containing DNA. Thus, the FAB assay requires active PR and RT, but not necessarily IN. In fact, the IN^- mutant, lacking the IN active site, produced infectious viral particles and scored positive in the FAB assay (18.2% of the wt level).

TABLE 3. Titers of Pol CS mutants after transfection of 293T cells[a]

Virus or construct	Infectious units/ml[b]	% of wild type
pcDNA[c]	<0.1	<0.1
pcHFV	2.2 (± 0.8) × 10^6	100.0
RT-IN2	8.3 (± 1.4) × 10^5	37.7
ΔCS	1.8 (± 1.3) × 10^4	0.8
CS2	1.4 (± 0.2) × 10^6	63.6
CS4	4.1 (± 1.4) × 10^5	18.6
CS6	1.5 (± 0.1) × 10^5	6.8
CS8	1.7 (± 0.8) × 10^4	0.8
PR^-	<0.1	<0.1
IN^-	4.0 (± 6.9) × 10^5	18.2

[a] Assayed on FAB cells.
[b] Results are mean values ± standard deviations from three independent experiments.
[c] pcDNA, a plasmid containing a polylinker site (Invitrogen, Carlsbad, CA).

TABLE 4. Titers of Pol CS mutants after infection of HEL cells[a]

Virus or construct	Infectious units/ml[b]	% of wild type
pcDNA[c]	<0.1	<0.1
pcHFV	4.6 (± 7.5) × 10^5	100.0
RT-IN2	6.0 (± 9.5) × 10^3	1.3
ΔCS	<0.1	<0.1
CS2	5.1 (± 8.6) × 10^5	110.8
CS4	1.7 (± 2.2) × 10^3	0.4
CS6	2.1 (± 3.2) × 10^3	0.5
CS8	<0.1	<0.1
PR^-	<0.1	<0.1
IN^-	<0.1	<0.1

[a] Assayed on FAB cells.
[b] Results are mean values ± standard deviations from three independent experiments.
[c] pcDNA, a plasmid containing a polylinker site (Invitrogen, Carlsbad, CA).

In order to determine whether the mutant viruses were able to replicate in subsequent rounds of infection, supernatants from transfected cells were used to infect HEL cells. Two days postinfection, including one cell passage, the titers of supernatants from infected HEL cells were determined on FAB cells (Table 4). As previously reported (25), the IN^- control did not replicate in the second-round assay, confirming that IN is required for efficient viral replication. The difference in titer between the wt virus and all mutants except CS2 is greatly amplified in this assay. The mutants with the most severe mutations, ΔCS and CS8 (Table 1), did not replicate to detectable levels after a second round of infection, although infectious virus was produced after the first round of infection. Thus, the cleavage between RT and IN is required for efficient viral replication, although it is not absolutely required for PR or RT activities.

DISCUSSION

The present study characterizes the role of the FV RT/RH-IN CS in viral replication. Mutation of the CS does not affect the normal expression of full-length Pol, although cleavage into Pol subunits is disrupted. We found that Pol encapsidation is decreased in several Pol CS mutants. In addition, cleavage appears to play a role in the enzymatic activities of PR and RT and possibly the activity of IN. The severest mutations lead to profound decreases in viral replication.

FV Pol differs from Pol of the orthoretroviruses in many respects. It is the only known enzyme that contains both RT and PR (18, 29). Some cleavage between PR and RT could be demonstrated in vitro using bacterially expressed FV Pol proteins, although the cleavage is inefficient (33). For retroviral PRs, the P2 residue (see Table 1) in a given substrate is critical for cleavage, and in most FV isolates, the PR CSs contain a Val at P2 (33). However, substrates containing Ala or Cys instead of Val are cleaved equally well by FV PR in vitro (2). In order to define the tolerated changes at the RT-IN CS, we replaced different numbers of amino acids at the CS defined by Pfrepper et al. (33) with alanines (Table 1, CS mutants). Mutant RT-IN2 contains three amino acid changes at the CS, including a Gly at P2 which has been shown to be inefficiently recognized by retroviral PR (2). In all mutants except CS2, which still contains the Val at P2, the Pol precursor protein remains uncleaved, confirming the importance of the P2 position at the CS for substrate specificity (2).

The cleavage of the Gag protein is affected in most of the CS mutants. It has been shown that only the Pol precursor protein, but not the individual PR-RT or IN subunits, is packaged into virions (31). We have also found that Pol mutants lacking the IN domain do not process Gag (mutants RTstop1 and RTstop2). Thus, this lack of Gag processing probably reflects the failure of the mutant Pol proteins to be packaged into virions where processing is believed to take place. Two PES in the viral genomic RNA are essential for Pol protein encapsidation (10, 13–16, 41), implying that Pol binds to RNA. However, it is also possible that Pol first interacts with Gag, so that a Gag-Pol complex binds to RNA and is packaged into virions through Gag determinants (E. G. Lee and M. L. Linial, unpublished data). Sequences in IN could be responsible for such an interaction with Gag.

We unexpectedly found that the levels of Pol in extracellular virions from cells transfected with most of the CS mutants are reduced more than would be expected from the intracellular Pol levels (Fig. 2 and 3). Some of these Pol mutants contain only small changes in the amino acid sequence. One explanation is that the mutations lead to a major conformational change in the structure of the Pol protein, so that it cannot bind to RNA and/or Gag. A second possibility is that the mutated Pol proteins localize incorrectly in the cell. Both Pol and Gag bear nuclear localization signals (NLSs) and are imported into the nucleus, where they might interact (17, 37). The NLS in Gag is thought to be in the C-terminal part of Gag (37). It has been proposed that at least two NLSs are present in Pol, one located in the PR-RT domain and the other one in the IN domain. However, the exact position and composition of the Pol NLSs are unknown and one could overlap the CS (17, 37). However, our results do not support incorrect localization, as Gag is cleaved in the mutants, indicating that Gag and Pol interact at some point during assembly, despite the fact that less Pol is found in mutant particles. A third possibility is that the Pol CS itself is an important domain required for interaction with the assembly complex.

Our main goal in mutating the RT-IN CS was to determine the effect on Pol enzymatic activities and infectivity. Very little is known about the effect of eliminating the PR CSs in Pol of FVs or other retroviruses. One study showed that mutations at the HIV-1 RT-RH PR CS (which does not exist in FV) resulted in greatly decreased RT activity relative to the activity in the wt virus (1). We found that elimination of the CS between RT and IN led to significant decreases in the levels of RT activity (Fig. 5). Mutants RT-IN2, CS2, and CS8 retained about half of the wt RT activity, but the deletion of 8 aa in mutant ΔCS appeared to lead to a sixfold loss of virion RT activity. Since FV virions contain fewer Pol molecules than other retroviruses, small negative effects on RT activity have a profound effect on viral replication (35).

As in all retroviruses, FV IN contains a zinc finger domain, a DNA binding domain, and a $DD_{35}E$ motif, which is the active center of the enzyme (30). Recombinant FV IN contains the expected site-specific endonuclease, IN, and disintegrase activities (30). While efficient FV replication requires IN (9, 25), the FAB assay is designed to measure only a single round of infection. Reverse transcription is a late event in FV morphogenesis, and infectious particles consist of a DNA genome instead of RNA (27, 42, 44). This means that, to score positive in the FAB assay, active PR and RT must both be made in the producer cell; however, integration appears not to be necessary. The IN^- mutant lacking the active site scores positive in the FAB assay, but cannot replicate in subsequent rounds (Tables 3 and 4) (25). Most of the CS mutants behave like the IN^- mutant, in that virions can score positive in the FAB assay but either fail to replicate after a second round of infection or do so at a greatly reduced titer. It is probable that genomic DNA introduced after infection of FAB cells can be used as a template for transcription and translation of enough Tas to activate the LTR and produce β-gal. Our results lead to the hypothesis that failure to cleave IN from the Pol precursor prevents at least one of the enzymatic activities of IN. Testing this hypothesis awaits the development of an in vitro integration assay for FVs. However, some of the CS mutants are more defective than the IN^- mutant in a single-round assay (Table 2). This suggests that IN activity as well as the reduced level of Pol packaging and PR and RT activity leads to the defects in replication of CS mutant viruses.

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