JOURNAL OF VIROLOGY, Sept. 2007, p. 10047–10054 Vol. 81, No. 18
0022-538X/07/$08.00+0 doi:10.1128/JVI.02496-06

Mutational Analysis of the C-Terminal Gag Cleavage Sites in Human Immunodeficiency Virus Type 1▽†

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Received 13 November 2006/Accepted 7 July 2007

Human immunodeficiency virus type 1 (HIV-1) Gag is expressed as a polyprotein that is cleaved into six proteins by the viral protease in a maturation process that begins during assembly and budding. While processing of the N terminus of Gag is strictly required for virion maturation and infectivity, the necessity for the C-terminal cleavages of Gag is less well defined. To examine the importance of this process, we introduced a series of mutations into the C terminus of Gag that interrupted the cleavage sites that normally produce in the nucleocapsid (NC), spacer 2 (SP2), or p6^{Gag} proteins. Protein analysis showed that all of the mutant constructs produced virions efficiently upon transfection of cells and appropriately processed Gag polyprotein at the nonmutated sites. Mutants that produced a p9$^{NC/SP2}$ protein exhibited only minor effects on HIV-1 infectivity and replication. In contrast, mutants that produced only the p8$^{SP2/p6}$ or p15$^{NC/SP2/p6}$ protein had severe defects in infectivity and replication. To identify the key defective step, we quantified reverse transcription and integration products isolated from infected cells by PCR. All mutants tested produced levels of reverse transcription products either similar to or only somewhat lower than that of wild type. In contrast, mutants that failed to cleave the SP2-p6^{Gag} site produced drastically less provirus than the wild type. Together, our results show that processing of the SP2-p6^{Gag} and not the NC-SP2 cleavage site is important for efficient viral DNA integration during infection in vitro. In turn, this finding suggests an important role for the p9$^{NC/SP2}$ species in some aspect of integration.

The Gag polyprotein is the major structural component of retroviruses and is the only viral protein strictly required for particle formation by orthoretroviruses (reviewed in references 8 and 13). During assembly and budding, human immunodeficiency virus type 1 (HIV-1) Gag is cleaved by the viral protease, liberating six mature proteins from the Gag polyprotein (13, 45), p15^{MA}, p24^{CA}, SP1 (spacer peptide 1, also known as p2), p7^{NC}, SP2 (also known as p1), and p6^{Gag}. The organization of HIV-1 Gag and its mature cleavage products is presented in Fig. 1. The newly liberated mature Gag proteins then complete the maturation process by rearranging the interior core structure of the virion, observable by electron microscopy as a transition from the doughnut-shaped immature core morphology to a cone-shaped core of the mature virus (13, 15, 42, 45). By this maturation process, the proteins within Gag and Gag-Pol shift roles from parts of a polyprotein that is dedicated to assembly to individual proteins that function in the infection process.

Gag processing occurs in an ordered fashion (37, 41, 48). The initial cleavage of Gag appears to take place between SP1 and NC, liberating MA-CA-SP1 (p40^{Gag}) and NC-SP2-p6^{Gag} (p15$^{NC/SP2/p6}$) as intermediary products. Processing at this site and at the others in the p40^{Gag} partial cleavage product is essential for proper maturation and virion infectivity (19, 26, 36). In contrast, the importance of processing at the C-terminal sites in Gag, NC-SP2-p6^{Gag}, is not altogether clear. In vitro, processing at the SP2-p6^{Gag} site can be dependent on the RNA-binding sequences in the NC protein (44). Alteration of the SP2-p6^{Gag} site by mutating both the P_1 and P_1' positions produced virions with mostly p8$^{SP2/p6}$ with some p15$^{NC/SP2/p6}$ that exhibited a replication defect in T cells (54). Mutations altering the two proline residues in the middle of SP2 eliminated viral replication (23). Nevertheless, p9$^{NC/SP2}$ has been found to be more efficient than p7^{NC} at promoting reverse transcriptase (RT)-associated functions in vitro (3, 27). Still, it is not clear how important the processing of NC, SP2, and p6^{Gag} is for the infection process.

To better understand the requirement for proteolytic processing of the C-terminal p15$^{NC/SP2/p6}$ region of Gag, we produced a series of NL4-3 constructs with mutations designed to block processing at these cleavage sites. The results showed that while processing of the NC-SP2 site is dispensable for infectivity, cleavage at the SP2-p6^{Gag} site is required for efficient integration by the infecting virus.

MATERIALS AND METHODS

DNA mutagenesis. The pNL4-3 infectious molecular clone of HIV-1 (1) (GenBank accession no. AF324493) was used for these studies and altered by site-directed mutagenesis using PCR-based methods, either by direct amplification with a mutagenic primer or by two rounds of amplification using the overlap extension procedure (24). The NL4-3 mutants constructed included the following: NC-N55D, with nucleotide 2083 changed from A to G, resulting in an asparagine-to-aspartic acid substitution at NC position 55; NC-N55I, with nucleotide 2084 changed from A to T, resulting in an asparagine-to-isoleucine substitution at NC position 55; NC-SP2, with nucleotide 2084 changed from A to G, resulting in an asparagine-to-serine substitution at NC position 55 that was combined with another mutation, nucleotide 2089 changed from T to C, resulting

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† Supplemental material for this article may be found at http://jvi.asm.org/.

▽ Published ahead of print on 18 July 2007.

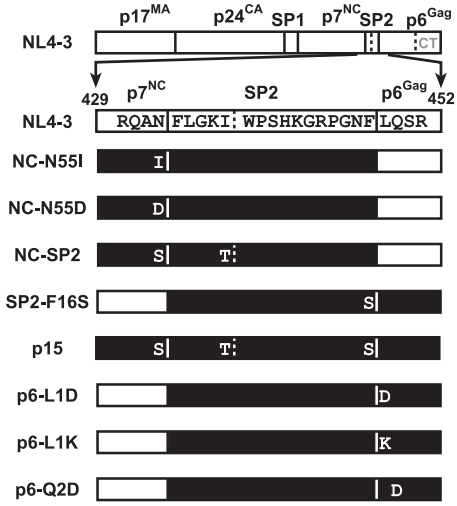

FIG. 1. Proviral constructs. Diagrams of the Gag regions of the various pNL4-3 constructs used in this study are presented. The mature proteins within Gag and the minor cleavage sites (denoted by dotted lines in SP2 and $p6^{Gag}$) are indicated on the NL4-3 wild-type construct map. The region mutated is presented for each mutant with the expected product highlighted in black. A dotted line indicates the C-terminal partial protease cleavage site (after position 36) in $p6^{Gag}$.

in an isoleucine-to-threonine substitution at SP2 position 5; SP2-F16S, with nucleotide 2131 changed from T to C, resulting in a phenylalanine-to-serine substitution at SP2 position 16; p15, a combination of the NC-SP2 and SP2-$p6^{Gag}$ mutants; p6-L1D, with nucleotides 2133 to 2136 changed from TCTT to CGAT, resulting in an aspartic acid-to-leucine substitution at $p6^{Gag}$ position 1 and a serine-to-arginine substitution at polymerase (Pol) position 17; p6-L1K, with nucleotides 2133 to 2136 changed from TCTT to CAAA, resulting in an aspartic acid-to-lysine substitution at $p6^{Gag}$ position 1 and an alteration of Pol codons serine 17 to glutamine and serine 18 to threonine; p6-Q2D, with nucleotides 2136 to 2139 changed from TCAG to CGAC, resulting in a glutamine-to-aspartic acid substitution at $p6^{Gag}$ position 2 and an alteration of Pol serine 17 to leucine and serine 18 to arginine. After construction, the regions of DNA that were PCR amplified were sequenced to confirm the mutations and to rule out the possibility of any additional changes introduced during the PCR-mediated mutagenesis process.

Cell culture. 293T human embryonic kidney, HeLa-CD4-LTR-*lacZ* (HCLZ) (18), JC53BL-13 (also known as TZM-bl) (49), and HOS human osteosarcoma cell line cells were cultured in Dulbecco's modified Eagle's medium, while H9 human T-cell leukemia cells were cultured in RPMI 1640. All media were supplemented with 2 mM L-glutamine, 100 U per ml penicillin, 100 μg per ml streptomycin, and either 10% (vol/vol) fetal bovine serum (293T, JC53BL-13, HCLZ, and H9) or 5% (vol/vol) fetal bovine serum and 5% (vol/vol) calf serum (HOS). All cell culture products were obtained from Invitrogen, Inc. (Carlsbad, CA). Transient transfections of 293T cells were carried out by using calcium phosphate transfection (20) or TransIT-293 (Mirus Bio Corp., Madison, WI). The HIV-1 infection assays using either HCLZ or JC53BL-13 cells were carried out as previously described (18). Briefly, the cells were infected with dilutions of virus and the assay was developed for β-galactosidase activity by staining with 5-bromo-4-chloro-3-indolyl-β-galactoside 48 h postinfection. Infected cells, those staining blue, were observed by light microscopy and counted to score infection events. The ability of viruses to replicate on H9 cells was carried out as previously described (18). Briefly, H9 cells were exposed to 10-fold dilutions of virus and the cultures were monitored periodically for infection by the presence of and increase in RT activity in the culture medium over a 6-week culture period. Samples that were 10 times the background level and greater than 1/100 of the peak level of the undiluted wild-type control were considered positive for infection.

Protein analysis. Isolation of virions by density centrifugation and immunoblot analysis were performed as previously described (33). Primary goat antiserum against $p7^{NC}$ (goat 77), $p24^{CA}$ (goat 81), and $p17^{MA}$ (goat 83), as well as rabbit antiserum against $p6^{Gag}$ (DJ-30552), were obtained from the AIDS Vaccine Program, NCI-Frederick, Frederick, MD. Mouse antibody against RT, NEA-9304001EA, was obtained from Perkin-Elmer (Wellesley, MA). Proteins were detected by developing blots with either horseradish peroxidase-conjugated anti-goat, anti-rabbit (both from Biochain Institute, Hayward, CA), or anti-mouse (Life Technologies, Inc., Gaithersburg, MD) secondary antibody and the Immun-star horseradish peroxidase substrate kit (Bio-Rad, Hercules, CA) on LumiFilm (Roche Applied Science, Indianapolis, IN). Reversed-phase high-pressure liquid chromatography (HPLC) and matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry were carried out as previously described (33).

Real-time PCR of reverse transcription products and proviral DNA. Assays for reverse transcription and integration products within HOS cells after infection were carried out as previously described (25). Briefly, wild-type and mutant viruses pseudotyped with the vesicular stomatitis virus G surface glycoprotein (VSV-G) were generated by calcium phosphate cotransfection of 293T cells followed by a repetitive washing procedure to remove plasmid DNA. The virus stocks were then used to infect HOS cells, and total DNA was isolated from the cells 24 h postinfection. For HIV-1 Env-mediated infections, virus stocks were prepared by calcium phosphate transfection and then treated with DNase to remove carryover plasmid from transfections: supernatants were exposed to 10 U/ml of DNase (Sigma-Aldrich, St. Louis, MO) in 4 mM $MgCl_2$ at 37°C for 1 h. H9 cells (3×10^6) were infected in a 24-well plate using Virofect R/L Magnetofection reagent (OZ Biosciences, Inc., Marseilles, France) according to the manufacturer's instructions using the recommended magnetic plate (OZ Biosciences). Analyses of early (strong stop; R-U5) and late reverse transcription DNA products (plus-strand transfer; R-5′ untranslated region [UTR]) and 2-LTR junction products were carried out as previously described (5). The proviral DNA assay (Alu-LTR) was performed as described elsewhere (46). Negative control infections with heat-inactivated (65°C for 20 min) aliquots of the virus stocks were used to assess the levels of carryover plasmid DNA from the transfection. The quantities of carryover plasmid DNA were typically less than 0.1% of that obtained from live virus.

RESULTS

To examine the importance of the processing of the $p15^{NC/SP2/p6}$ precursor, we produced a series of mutant constructs in full-length pNL4-3 designed to eliminate Gag proteolytic processing in the C terminus of $p7^{NC}$ and the N terminus of SP2 to generate viruses that contain $p9^{NC/SP2}$, $p8^{SP2/p6}$, or $p15^{NC/SP2/p6}$ (Fig. 1). One potential complication to this approach is that, in addition to these primary cleavage sites, there are minor secondary sites in this region of Gag (Fig. 1) that can be cleaved in mature virions (21, 22, 33). One of these minor cleavages occurs after SP2 amino acid 5, potentially cleaving within a $p9^{NC/SP2}$ or $p15^{NC/SP2/p6}$ species produced by our cleavage site mutants. To eliminate this possibility, we mutated the P_1 amino acid of this site (SP2 amino acid 5, Gag position 437) (Fig. 1) in constructs that had mutations either at the NC-SP2 site (NC-SP2 in Fig. 1) or at both the NC-SP2 and SP2-$p6^{Gag}$ sites (p15 in Fig. 1).

A complication in genetically blocking protease cleavage is the difficulty in predicting what sequence substitutions at the protease cleavage site will eliminate protease recognition in the viral mutants. General selection rules based on protease crystal structure and studies with peptides suggest that insertion of hydrophilic and charged residues at the cleavage site interrupts processing (39, 52, 53). Nevertheless, our experience has shown that some of the mutations suggested by these rules do not always prevent cleavage when placed in the context of the virus. Using these rules as well as our previous observations, we introduced a series of mutations into the C terminus of Gag. Contrary to expectations, some of the mutated cleavage sites were either completely or mostly cleaved in virions despite a poor predicted substrate site: preliminary immunoblotting with $p7^{NC}$ antiserum showed that NC-SP2 site mutants containing isoleucine, histidine, and lysine in the P_1 position

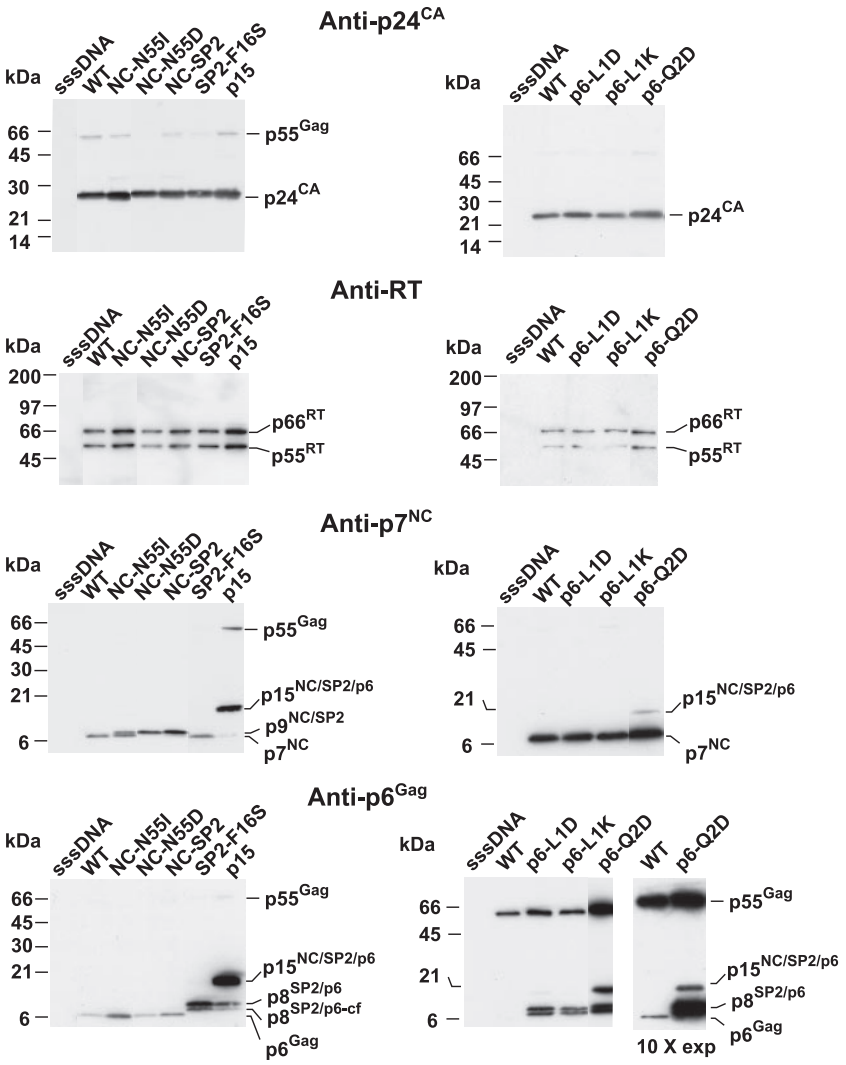

FIG. 2. Immunoblots of mutant virion preparations. $p24^{CA}$, RT, $p7^{NC}$, and $p6^{Gag}$ immunoblots of equal volumes of virion preparations are presented. Samples are identified above the respective lanes. sssDNA, a virus preparation produced from transfected, sheared salmon sperm DNA as a mock control. Molecular masses, as calculated by relative mobilities, and identities of bands are indicated at the margins of the blots. Due to the poor retention of wild-type $p6^{Gag}$, a 10-fold-longer exposure is provided to the right of the second $p6^{Gag}$ immunoblot to show the migration of wild-type $p6^{Gag}$.

were processed by protease, while those containing serine and aspartic acid were not cleaved (data not shown). Also, an initial $p6^{Gag}$ immunoblot assay revealed that a serine in the P_1' position of SP2-$p6^{Gag}$ was also processed, while aspartic acid and lysine substitutions were not cleaved.

The NL4-3-based constructs selected for further study are displayed in Fig. 1. All of the NC and SP2 mutants were constructed so that the Gag mutations did not alter the overlapping Pol reading frame or alter the frameshift slippery site (which overlaps the wobble codon of the asparagine at P_1 of the NC-SP2 site). The $p6^{Gag}$ mutants had changes in the Pol frame at the beginning of the $p6^{Pol}$ protein (also referred to as the preprotease or transframe protein). These secondary mutations did not alter processing of Gag at other sites or reverse transcriptase incorporation (presented below).

To study the mutant virions, preparations were produced by transfecting each molecular clone into 293T cells and then isolating virions by density centrifugation for protein analyses. To examine any impact the mutations might have on virus production and processing of the N-terminal Gag proteins (i.e., those in the $p40^{Gag}$ partial cleavage product), equal amounts of virion preparations (by volume) were analyzed by immunoblotting with $p24^{CA}$ antiserum. The results showed that the mutants produced nearly equivalent levels of virions, indicating that they exhibit no overt assembly defects. The virion samples also did not contain appreciable amounts of $p55^{Gag}$ or partially processed products; thus, the mutations in the C terminus of Gag did not alter the overall processing of $p24^{CA}$ or $p17^{MA}$. Since changes to the middle of SP2 can alter Gag processing and Gag-Pol incorporation (23), we detected the Pol protein product in the samples by stripping the blot and exposing it to an antibody against RT. The blot showed that the relative intensities of the RT signals present in the mutant samples compared to those of Gag were similar to the wild-type sample (Fig. 2). Hence, the amount of Pol incorporation does not appear to be significantly altered by these mutations.

A $p7^{NC}$ immunoblot analysis of the virions revealed that mutating the NC-SP2 cleavage site had various effects on processing. The NC-N55I sample contained a band that was similar to the wild type, $p7^{NC}$, as well as a readily detectable minor band that migrated higher in the gel, likely $p9^{NC+SP2}$. Therefore, processing apparently still occurred, though inefficiently, at the NC-SP2 site in this mutant. Only one band was present in the NC-N55D and NC-SP2 virion samples, migrating at a position higher than the wild-type band, consistent with the presence of the $p9^{NC/SP2}$ species that would be produced by blocking the NC-SP2 cleavage site. As expected, the SP2-F16S, p6-L1D, p6-L1K, and p6-Q2D mutants contained a normal-sized $p7^{NC}$ band (Fig. 2), as the NC-SP2 site was not mutated in these constructs. The p15 mutant sample contained a band that migrated much higher, consistent with the expected $p15^{NC/SP2/p6}$ product.

To examine the status of $p6^{Gag}$ in the mutants, the blot was stripped and exposed to $p6^{Gag}$ antiserum. This revealed that the samples of the NC-N55I, NC-N55D, and NC-SP2 mutants contained a wild-type-sized $p6^{Gag}$ band. (The wild-type p6 bands are less intense due to the nature of $p6^{Gag}$, a protein that is relatively hydrophilic and does not efficiently transfer from the gel onto polyvinylidene difluoride membranes [33].) The SP2-F16S, p6-L1D, p6-L1K, and p6-Q2D mutant samples contained two higher-migrating bands on the blots. Since the C terminus of $p6^{Gag}$ is alternatively processed after $p6^{Gag}$ residue 36 (Gag position 483) (Fig. 1) (21, 22, 33), the former is likely an SP2-$p6^{Gag}$ product while the latter is an apparent SP2-$p6^{Gag}$ product missing its extreme C terminus. The p15 mutant contained a much higher migrating band that coincided with the band in the $p7^{NC}$ immunoblot, confirming the presence of both $p7^{NC}$ and $p6^{Gag}$ in this species. In addition to the $p15^{NC/SP2/p6}$ product, there were weaker bands that corresponded to those observed for the SP2-$p6^{Gag}$ mutants, indicating that there was a small amount of processing at the NC-SP2 site. Together, these results indicate that we have produced a functional series of cleavage site mutants.

HPLC and mass spectrometry analysis. Considering the relatively small size of the mutant proteins and the potential for minor cleavages in this region, we confirmed the identities of the $p7^{NC}$, SP2, and $p6^{Gag}$ products by isolating them from virion preparations with reversed-phase HPLC and identified them with a subsequent MALDI-TOF mass spectrometry analysis (Fig. 3; Table 1). These data generally confirmed the immunoblot data (Fig. 2). Nearly all of the NC in the NC-N55D mutant was present as a $p9^{NC/SP2}$ product (Fig. 3; Table 1), though these virions did contain a small amount of $p7^{NC}$ (less than approximately 5%, based on relative absorbance at 206 nm) (Fig. 3; Table 1). (There is also another peak in the HPLC that elutes at a similar time as the $p9^{NC/SP2}$ peak that corresponds to ubiquitin [Ub] [Fig. 3], which is incorporated into virions [34, 35, 43].) The NC-SP2 mutant contained only $p9^{NC/SP2}$ with no detectable $p7^{NC}$ (Fig. 3; Table 1). Thus, NC-SP2 processing appears to be completely blocked in this mutant. NC-N55I virions displayed an intermediate phenotype (Fig. 3), containing a mixture of $p7^{NC}$ and $p9^{NC/SP2}$ (approximately 40% based on relative absorbance) (Fig. 3; Table 1), in agreement with our prior blotting data (Fig. 2). Mass spectrometry revealed that the $p7^{NC}$ in this mutant virus, while appearing normal-sized by immunoblotting, contained additional mass that is consistent with the addition of a phenylalanine from the N terminus of SP2. Likewise, the SP2 protein was missing mass that would account for this residue. Thus, the mutation appears to have altered processing at this site in two ways, either moving the cleavage site over one residue, making $p7^{NC+F}$, or preventing cleavage entirely (Fig. 3). This underscores the important of biochemical analyses of cleavage site mutants.

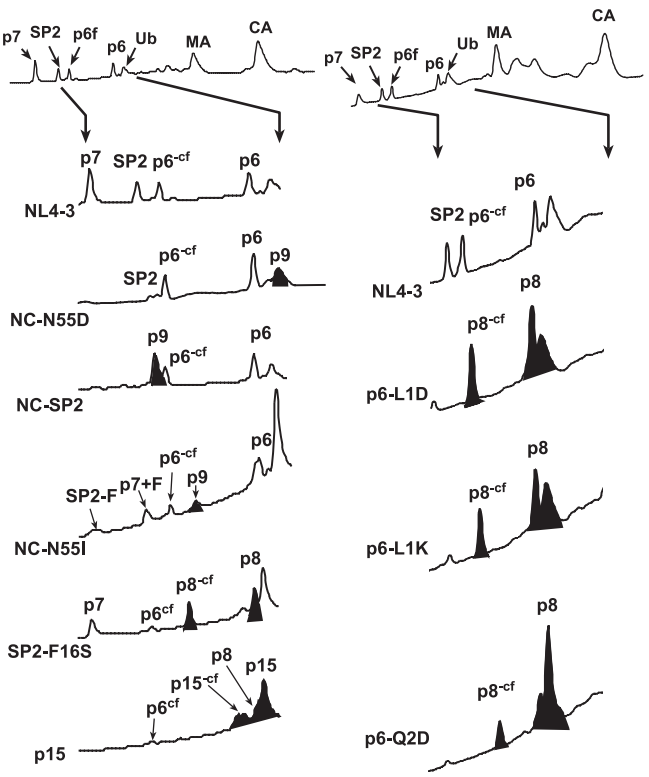

FIG. 3. HPLC analysis of mutants. The complete HPLC chromatogram for wild-type NL4-3 virions is shown at the top of the figure with the region that is presented for the mutants indicated by arrows. The identities of the Gag proteins, determined by Coomassie brilliant blue-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis, immunoblotting, protein sequencing, and mass spectrometry, are identified above each respective peak. The mutant proteins as identified by MALDI-TOF mass spectrometry are shaded in black. The $p6^{Gag}$-containing species that are missing the C-terminal 16 amino acids due to cleavage after residue 36 are indicated by a "-cf" notation, e.g., $p6^{-cf}$, in the identifying superscript. The C-terminal fragment itself was labeled $p6^{cf}$ when observed.

As expected, the SP2-$p6^{Gag}$ cleavage site mutants, SP2-F16S, p6-L1D, p6-L1K, and p6-Q2D, contained only the $p8^{SP2/p6}$ product with the expected mass (Table 1), though we did observe a truncated form (Fig. 3, $p8^{-cf}$) that matched the expected size for a $p8^{SP2/p6}$ product with a C-terminal cleavage at p6 residue 36. This species was previously observed in our immunoblot data (Fig. 2). Additionally, we also could detect the C-terminal 16-amino-acid protein of $p6^{Gag}$ in this sample (Fig. 3, cf).

Analysis of the p15 mutant virions detected the $p15^{NC/SP2/p6}$ species (Fig. 3; Table 1) and two proteins with masses consistent with the expected N- and C-terminal fragments for a cleavage in the C-terminal tail (Fig. 3, $p15^{-cf}$ and cf, respec-

TABLE 1. Mass spectrometry data for cleavage site products

Virus/protein detected	Observed mass (Da)	% Difference from theoretical
NL4-3/NC	6,352.1	0.01
NL4-3/SP2	1,841.9	0.04
NL4-3/p6^{Gag}	5,803.6	0.06
NL4-3/p6$^{Gag\text{-}cf}$	4,135.7	0.04
NC-N55D/p9$^{NC/SP2}$	8,178.2	0.03
NC-N55D/p7^{NC}	6,354.7	0.05
NC-N55I/p9$^{NC/SP2}$	8,199.5	0.3
NC-N55I/p7^{NC+F}	6,497.4	0.3
NC-N55I/SP2^{-F}	1,693.93	0.1
NC-SP/p9$^{NC/SP2}$	8,140.5	0.06
SP2-F16S/p8$^{SP2/p6}$	7,577.5	0.09
SP2-F16S/p8$^{SP2/p6\text{-}cf}$	5,927.5	0.1
p6-L1D/p8$^{SP2/p6}$	7,640.4	0.1
p6-L1D/p8$^{SP2/p6\text{-}cf}$	5,977.2	0.1
p6-L1K/p8$^{SP2/p6}$	7,653.5	0.1
p6-L1K/p8$^{SP2/p6\text{-}cf}$	6,002.9	0.02
p6-Q2D/p8$^{SP2/p6}$	7,617.39	0.05
p6-Q2D/p8$^{SP2/p6\text{-}cf}$	5,975.0	0.02
p15	13,908.4	0.3

TABLE 2. Infectivity and replication of mutants

Virus	Single-round titer[a]	Relative titer[b]	H9 replication[c]
sssDNA	4	2×10^{-6}	<1
NL4-3	3.6×10^{7}	1	10^{6}
NC-N55D	6.9×10^{7}	2	10^{5}
NC-N55I	1.1×10^{7}[d]	0.3	10^{4}
NC-SP2	3.9×10^{7}	1	10^{6}
SP2-F16S	4.0×10^{5}	0.01	1
P6-L1D	7.5×10^{4}	2×10^{-3}	1
P6-L1K	2.9×10^{5}	8×10^{-3}	<1
P6-Q2D	5.9×10^{4}	2×10^{-3}	<1
p15	8.1×10^{4}	2×10^{-3}	<1

[a] Single-round infectivity was based on β-galactosidase activity and is reported as blue cell-forming units per ml.
[b] Relative to wild-type titer.
[c] Tissue culture infective dose per milliliter.
[d] Value normalized from HCLZ data.

tively). In addition, we did find a slight amount of p8$^{SP2/p6}$ (Fig. 3). Together, these data confirm the immunoblot data and further demonstrate that there was no unexpected processing at any minor sites.

Mutant infectivity and replication. The ability of the HIV-1 mutants examined above to carry out a single round of infection was tested by using JC53bl-13 cells in an LTR-*lacZ* Tat complementation assay (18). Their ability to perform multiple rounds of replication in H9 cells was determined using an endpoint titer method. The results (Table 2) showed that the NC mutants NC-N55D, NC-N55I, and NC-SP2 produced single-round titers and H9 endpoint tissue culture infective doses that were only somewhat lower than those containing wild-type NC. Thus, even though both the NC-N55D and NC-SP2 mutants contain mostly uncleaved p9^{NC+SP2}, they had only modest reductions in the infectivity and replication assays. Also, the NC-N55I virions, which contain partially or miscleaved p7^{NC}, maintained high relative infectivities. These results show that the cleavage of p7^{NC} from SP2 is not essential for replication.

In contrast, the single-round infectivities of the SP2-p6^{Gag} cleavage site mutants, SP2-F16S, p6-L1D, p6-L1K, and p6-Q2D, were at least 2 logs lower than wild-type levels. The effect on replication was even more dramatic: the titers for these mutants were $\leq 10^{-6}$ of the titer of the wild type. Similar to the SP2-p6^{Gag} cleavage site mutants, the infectivity and replication competence of the p15 mutant were also reduced to levels near those of the SP2-p6^{Gag} mutants. Therefore, these data indicate that cleavage of SP2 from p6^{Gag} is important in HIV-1 replication.

Analysis of infection products. To determine the replication block for the SP2-p6^{Gag} and p15 mutants, we examined the ability of VSV-G-pseudotyped mutants (NC-N55D, NC-SP2, SP2-F16S, and p15) to carry out reverse transcription and integration steps in HOS cells by detecting key DNA products inside the cell with real-time PCR 24 h postinfection. Processivity of reverse transcription, measured by conversion of early (strong stop; R-U5) to late (plus-strand transfer; R-5′ untranslated region [UTR]) reverse transcription products (RT^{N}), was only slightly reduced for the NC-ND, SP2-F16S, and p15 mutants when compared to the wild-type samples (a representative experiment is shown in Fig. 4, early-late RT^{N}; full data sets are presented in Tables S1 to S3 of the supplemental material). The NC-SP2 mutant produced reverse transcriptase products essentially at a wild-type efficiency, consistent with its infectivity phenotype (Fig. 4; see also Tables S1 to S3). Therefore, a gross failure in carrying out reverse transcription was not the major defect in the SP2-F16S and p15 mutants.

Another aspect assayed was the production of 2-LTR circles (representative data shown in Fig. 4, late RT^{N}-2-LTR circles). A small amount of the newly reverse transcribed DNA forms 2-LTR circles as a dead-end by-product that is produced during infection. This is commonly used as an indicator that plus-strand synthesis was completed and that the resulting viral DNA was exposed to nuclear enzymes. The PCR analysis showed that 2-LTR formation for the SP2-F16S and the p15 mutants was one-half to one-third of the wild-type value and that of the NC cleavage site mutants (Fig. 4; see also Tables S1 to S3 in the supplemental material). While this is somewhat reduced, the differences do not account for the greater than 6-log difference in replication observed between these viruses.

Unlike the two other steps, integration, as measured by an Alu-LTR-based PCR assay, was drastically reduced in the cells infected with the SP2-F16S and p15 mutants (representative data shown in Fig. 4, late RT^{N}-provirus). The analysis showed that the conversion of late reverse transcription product (R-5′UTR) to provirus (Alu-LTR) was greatly reduced for these mutants, to less than 3% of the wild-type levels (Fig. 4; see also Table S1 in the supplemental material). Thus, the primary defect in the SP2-F16S and p15 mutants appears to be a severe defect in integration.

Even though both VSV-G- and HIV-1 Env-mediated infection of cells with NL4-3 produces reverse transcription products at the same efficiency in unperturbed cells (47), VSV-G pseudotyping can overcome various blocks to HIV reverse transcription during infection that are induced by genetic mutants and cellular inhibitors (2, 40). Thus, the use of VSV-G could possibly mask a reverse transcription defect in our mutants. To address this possibility, we infected H9 cells using our mutants without VSV-G pseudotyping (NC-N55D, NC-SP2, SP2-F16S, p6-Q2D, and p15), thereby relying on CD4/gp120-

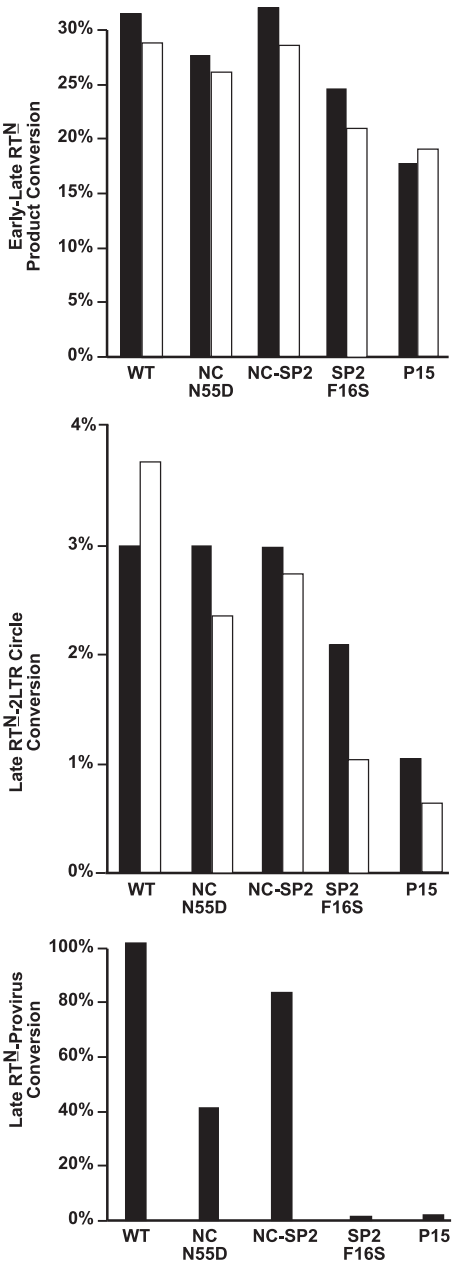

FIG. 4. Reverse transcription and integration products assayed by real-time PCR. Representative results of a quantitative real-time PCR analysis are presented as percent conversion between two representative steps in the infection process. Results for VSV-G-mediated infection are presented as black bars, and data from HIV-1 Env-mediated infections are in white. Parameters presented are indicated next to each graph. Typical samples analyzed contained extract from about 1.1×10^4 cells. For the wild type, the target quantities detected were 3.2×10^5 copies of R-U5, 1.33×10^5 copies of R-5′UTR, 3.3×10^4 copies of provirus, and 4.8×10^3 copies of 2-LTR circles. The amounts of provirus in H9 cells infected with HIV-1 Env-containing virus were below the level of detection and thus were omitted. Full data sets of experiments are presented in Tables S1 to S6 in the supplemental material.

mediated infection. The results essentially mirrored those obtained from the VSV-G/HOS cell experiments (Fig. 4; see also Tables S4 to S6 in the supplemental material): the mutants had only minor decreases in reverse transcription (early-late RT^N) and a modest defect in nuclear accessibility (late RT^N-2 LTR circle). Neither defect accounts for the gross defect in replication. The amounts of provirus for these experiments were below the level of detection due to the reduced number of infection events with the HIV-1 Env-mediated infections. Overall, these results demonstrated that the mutants carry out reverse transcription relatively efficiently. Thus, the defect in infection is at a later step, immediately prior to or during integration.

DISCUSSION

Our results show that removal of SP2 from $p7^{NC}$ is not required for efficient HIV-1 replication. In contrast, processing at the SP2-$p6^{Gag}$ cleavage site is required for efficient HIV-1 replication. These latter results are consistent with a replication defect observed by Yu et al. with a different SP2-$p6^{Gag}$ cleavage mutant (54). Our real-time PCR data revealed that the primary defect caused by blocking SP2-$p6^{Gag}$ processing was at or just before the integration step. It is unclear how blocking the SP2-$p6^{Gag}$ site could affect integration. The loss of free SP2 does not appear to play a direct role, as our $p9^{NC/SP2}$ mutants were infectious despite containing no detectable SP2. The Pol polyproteins appear to be incorporated into virions at near-wild-type levels (Fig. 2; immunoblotting results for protease are not shown), and so it is unlikely that there is a simple IN-related explanation. Therefore, the production of either $p6^{Gag}$ or $p9^{NC\text{-}SP2}$, both of which are not produced by the SP2-$p6^{Gag}$ or p15 mutants, could be required for integration. The $p6^{Gag}$ protein has not been implicated in any postentry/infection events and is located outside the mature core in virions; thus, it is unlikely to interact with the RNA or integrase (50). In contrast, the NC protein has already been implicated in promoting efficient integration (5, 16, 17, 46). Thus, it is possible that a block in the production of $p9^{NC\text{-}SP2}$ is responsible for the defect in integration. Similar to the results presented here, our previous results from genetic studies support some role for NC at or just before the integration step: HIV-1 mutants with CCHC NC zinc finger substitutions to other nonretroviral motifs, CCCC and CCHH, maintain genomic RNA incorporation and exhibit only somewhat reduced levels of reverse transcription, yet have a severe block in integration (5, 16, 17, 46).

How NC, potentially as a $p9^{NC/SP2}$ species, could be required for integration is not clear. NC could be acting as a somewhat labile component of the integration complex or as a protective factor for the cDNA ends, presumably by binding to them (5, 17, 46). In vitro, $p7^{NC}$ and especially $p9^{NC/SP2}$ have been found to increase the efficiency of concerted integration (7, 14, 38). Interestingly, $p9^{NC/SP2}$ can condense double-stranded DNA into dense protein/DNA complexes much more efficiently than either $p7^{NC}$ or $p15^{NC/SP2/p6}$ (32), suggesting that $p9^{NC/SP2}$ might specifically associate with the viral cDNA. Alternatively, $p9^{NC/SP2}$ could play a role in nuclear localization of the preintegration complex, although our experiments were conducted on dividing cell cultures. Despite all of these indirect supporting data, neither NC nor $p9^{NC/SP2}$, also called NCp7 or NC[1-72] (11), is routinely observed in isolated preintegration complexes (30). Detectable amounts of $p9^{NC/SP2}$ do not appear to be present in HIV-1 cores (50): nearly all of the NC appears

to be $p7^{NC}$ with a very minute amount of $p15^{NC/SP2/p6}$. Also, in our years of biochemical analyses of wild-type HIV-1 virions, we have not found much evidence for $p9^{NC/SP2}$ in virions (21) (data not shown). However, it is possible that only a small amount, two to four molecules per virion bound to the cDNA ends, might be present, levels below the sensitivity of our past and current protein analyses.

Alternatively, a $p9^{NC/SP2}$ species could act transiently at the assembly/maturation step by assisting the correct formation of the mature virus, perhaps placing IN in the correct position within the viral core. Recent reports of in vitro experiments have implicated $p9^{NC/SP2}$ in the maturation process (31, 32). Theoretically, $p9^{NC/SP2}$ would not have to be present in the core to assist the integration process in this way.

From our data and other processing site mutation studies (19, 26, 42, 51), it appears that the only Gag proteolyic cleavage site that is not strictly required for replication in vitro is the NC-SP2 junction. Unlike NC, the proteins in the N-terminal region of Gag play mostly structural roles. Therefore, having additional sequences attached to them due to incomplete processing likely causes defects in their fairly constrained protein-protein interactions. However, NC primarily functions in RNA binding and chaperone activity (4, 10, 28), roles that apparently can tolerate additional sequences attached to $p7^{NC}$.

One question that remains unanswered is the following: why is $p7^{NC}$ produced if $p9^{NC/SP2}$ is sufficient for replication? It is important to consider that in cell culture with limited rounds of replication, we may not detect minor replication inefficiencies that would apply a selective pressure for the maintenance of this site over the many cycles of replication that occur in an infected individual (9). Indeed, sequential passaging of the NC-N55D and NC-SP2 mutants results in a restoration of the NC-SP2 site within four passages (4 weeks) (data not shown), supporting this hypothesis. Furthermore, some protease inhibitor-resistant viruses isolated from patients have sequence changes at both the NC-SP2 and the $SP2\text{-}p6^{Gag}$ sites that appear to restore normal levels of processing by the mutant protease (6, 12, 29, 55). Thus, processing at both sites appears to be important for long-term viral fitness in vivo.

Overall, our findings are consistent with a role for NC in promoting the integration process, possibly as a $p9^{NC/SP2}$ species. The potential involvement of NC in integration suggests further studies to clarify its role in this process, e.g., either a direct or indirect interaction of $p9^{NC/SP2}$ with the preintegration complex or its providing an organizing function during the maturation phase of assembly.

ACKNOWLEDGMENTS

We thank Laurie Queen and Teresa Shatzer for technical assistance and Lou Henderson for the inspiration to examine this topic.

This project has been funded in whole or in part with federal funds from the National Cancer Institute, National Institutes of Health, under contract no. NO1-CO-12400.

The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organization imply endorsement by the U.S. Government.

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