

Alteration of the Proline at Position 7 of the HIV-1 Spacer Peptide p1 Suppresses Viral Infectivity in a Strain Dependent Manner

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Abstract: The HIV-1 spacer peptide p1 is located in the C-terminus of the Gag polyprotein and separates the nucleocapsid (NC) and p6^{Gag}. Research centered on p1 has been limited and as yet no function has been ascribed to this spacer peptide. We have previously found that the conserved p1 proline residues (position 7 and 13) are critical for replication in the HIV-1 strain HXB2-BH10. In this study we have focused on the proline rich p1-p6^{Gag} C-terminus of HIV-1. We individually examined the role of p1 proline's in multiple strains of HIV-1 and investigated the role of three proline residues in p6^{Gag} (P24, P25 and P30). Assessment of the HXB2-BH10 based mutants revealed that Gag-Pol incorporation relative to Gag decreased in the p1 mutant virions, with the double proline mutant the most impaired. Mutating both p1 proline residues was found to abolish infectivity in multiple strains of HIV-1. Independent mutation of the p1 proline at position 7 resulted in a strain-dependent suppression of viral infectivity. This defect correlates with the presence of a tyrosine residue at position 9 of p1 and occurs in the early phase of the HIV-1 replication cycle. The p1 proline residues were found to be functionally distinct from P24, P25 and P30 in p6^{Gag}. This work affords novel insights into our understanding of the role of p1 in HIV-1 replication.

Keywords: HIV, spacer peptide, p1, p6^{Gag}, Gag.

INTRODUCTION

The Gag polyprotein of human immunodeficiency virus type 1 (HIV-1) is the fundamental driving force behind viral assembly, encoding the viral structural proteins and harbouring key assembly domains. Cleavage of Gag by the viral protease (PR) releases the mature proteins matrix (MA), capsid (CA) nucleocapsid (NC) and p6^{Gag}. Two small spacer peptides, p2 and p1, are also found within HIV-1 Gag. The 14 amino acid p2 spacer peptide separates CA and NC and the 16 amino acid p1 spacer peptide separates NC and p6^{Gag} (Fig. 1A).

Numerous studies have explored the role of the p2 spacer peptide in HIV-1 replication, demonstrating that p2 transiently supports: 1) the proteolytic processing of viral precursor proteins; 2) assembly of virion particles; and 3) the selective virion packaging of HIV-1 RNA genomes [1, 18, 19, 25, 27, 28, 29, 34, 35]. Moreover, it has been shown that the cleavage of p2 acts as a regulatory switch for the morphological conversion of newly assembled immature virions

to mature HIV-1 particles [18]. The function of p2 is reliant on protein structure, wherein the formation of an alpha-helix that spans the CA-p2-NC junction influences the folding of CA and NC to increase the helix forming tendency of both proteins during Gag/Gag interactions and Gag/HIV-RNA interactions [28]. Accordingly, changes to p2 structure directly inhibit the production of infectious HIV-1 [1].

Comparatively, investigations into the function of p1 have been rare. The p1 spacer peptide is located at the point where the critical RNA structure of the frameshift stem-loop overlaps with both the Gag p1 and the Gag-Pol transframe (TF) open reading frames. Consequently, mutagenesis of this region has the potential to interfere with the functioning of multiple elements. A systematic mutagenesis strategy to isolate p1 function enabled us to demonstrate a critical role for p1 in HIV-1 replication that is clearly independent of the roles of the RNA frameshift stem-loop and the Gag-Pol TF protein [20]. We further determined that simultaneous alteration of p1's two conserved proline residues (position 7 and 13) to leucines influenced protein processing, reduced genomic RNA dimer stability and abolished viral infectivity in peripheral blood mononuclear cells (PBMCs) [20].

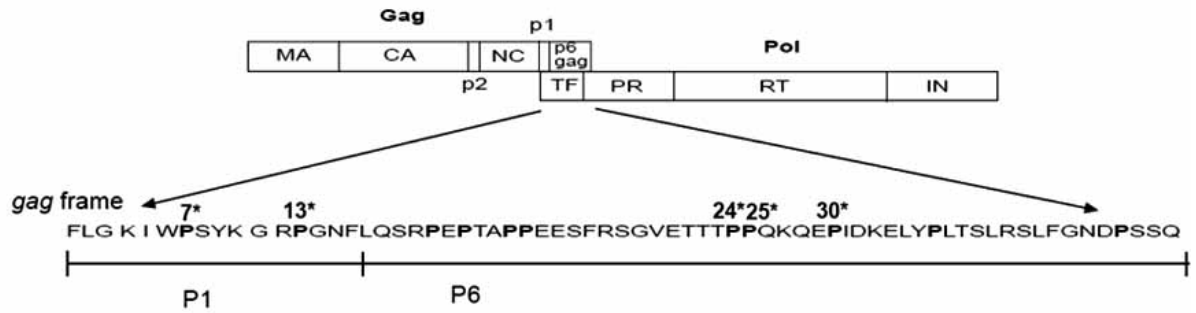
The nature of the involvement of p1 in HIV-1 function remains unclear. As proline residues can confer unique conformational constraints on a peptide, our results suggest that like the analogous p2, the structure of p1 is critical. The impact of p1 on HIV-1 biology is anticipated to be derived from a contribution to the overall protein folding of one of the precursor proteins that encompass p1, such as Gag (MA-

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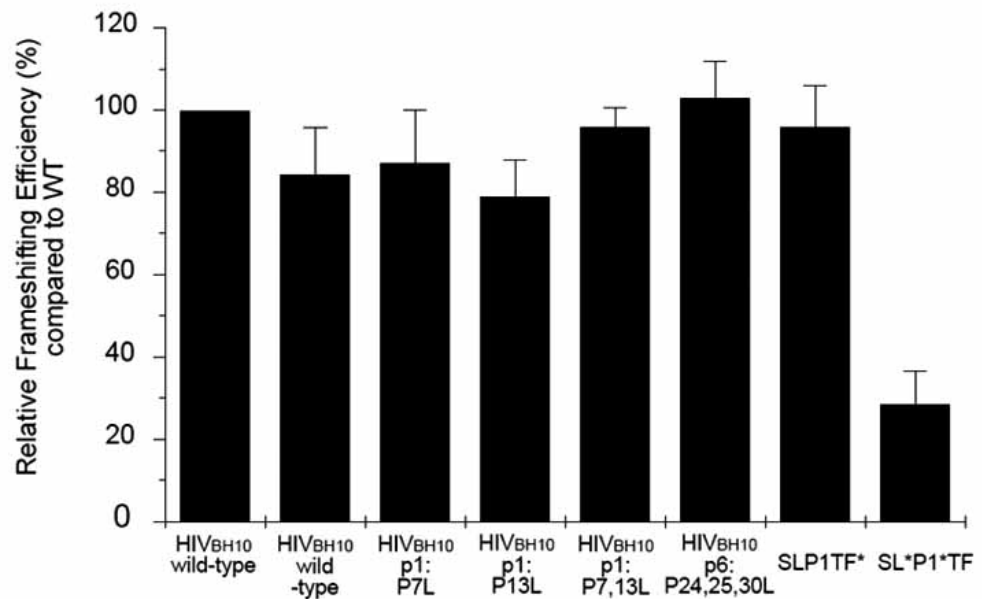
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A.



B.



C.

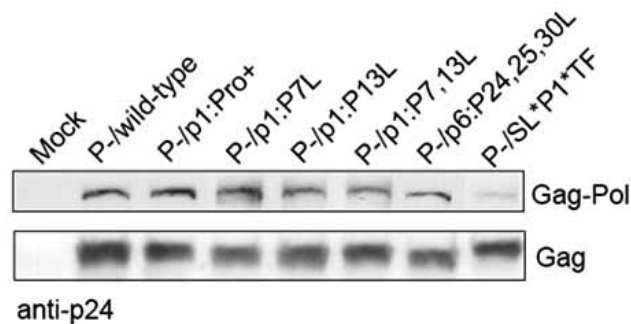


Fig. (1). The p1 and p6^{Gag} mutations do not affect the function of the frameshift stem-loop. (A) Schematic representation of the Gag and Gag-Pol polyproteins showing the major domains and the corresponding amino acid sequence for the P1 and P6^{Gag} region from HXB2-BH10. The proline residues are in bold and those mutated in this study are indicated with an asterisk (*). (B) Frameshifting activity was assessed using the dual luciferase reporter system [17]. The wild-type frameshifting level was set at 100% for comparison to the mutants. The graph depicts the average values from three distinct experiments done in duplicate. (C) Gag-Pol expression was measured in cell lysates obtained from transfection of 293T cells with PR-defective constructs (P-). The lysates were immunoprecipitated with HIV-1-infected patient sera and subsequently assayed by Western blot analysis using a monoclonal anti-P24 antibody. The figure is representative of four experiments.

CA-p2-NC-p1-p6^{Gag}), p15 (NC-p1-p6^{Gag}) or p9 (NC-p1). The p1-p6^{Gag} region at the C-terminus of HIV-1 Gag is unusually proline rich [12] (Fig. 1A), making it important to delineate whether comparable proline mutations in p6^{Gag} can emulate the phenotype of the p1 proline mutations. Few studies have included an investigation into the role of the eight proline residues in p6^{Gag} that lie outside the late domain motif (PTAPP). Single mutations of prolines at position 5 [9] and 24 [21] of p6^{Gag} were shown to have little or no effect on viral replication in T-cell lines. While alteration of prolines at position 37 and 49 of p6^{Gag} led to reduced infectivity in both H9 cells and in PBMCs [30]. Interestingly, two highly conserved prolines in p6^{Gag} (position 24 and 30) are separated by the same number of amino acids as those located in p1, but their contributions to HIV-1 replication have not been clearly established. The individual role of p1's proline residues has not been determined and it is unknown whether the p1 prolines are functionally important across different strains of HIV-1.

METHODS

Construction of full-length HIV-1 DNA plasmids. The HIV-1 DNA constructs used in this study were derived from the full-length wild-type HIV-1 plasmids, HXB2-BH10 [41], NL4.3 [2] AD8 [42], LAI [31] and MAL [3]. The PR defective constructs have the active site mutation D25R. All mutants were created using PCR stitch mutagenesis and subsequent cloning into the requisite HIV-1 backbone *via* the restriction sites *ApaI* and *BclI* as previously described [20]. All mutant constructs were verified by sequencing.

Assessment of frameshifting activity. The thermodynamic stability of the predicted stemloop structures for each of the p1 mutants was determined with the *mfold* program by M. Zuker of Washington University School of Medicine [13, 24, 36] using the server located at The Macfarlane Burnet Institute for Medical Research and Public Health (<http://mfold.burnet.edu.au/>) (Table 1).

Table 1. Mutations Within p1 in HXB2-BH10

Construct Name	Energy $\Delta G^{\circ a}$
Wild-type	-21.4
p1:pro+	-21.2
p1:P7L	-18.9
p1:P13L	-19.1
p1:P7,13L	-16.9
^b SLP1TF*	-16.2
^b SL*P1*TF	-6.4

^aThe thermodynamic stability (ΔG°) of each stem-loop structure was estimated using the *mfold* program by M. Zuker of Washington University School of Medicine [13, 24, 36]. Values are given in kcal/mol⁻¹.

^bTwo mutants were included as controls for frameshifting; SLP1TF* has altered stem-loop stability and replicates at wild-type levels. SL*P1*TF is a stem-loop defective mutant that was non-infectious in PBMCs [20].

Frameshifting activity was assessed using the dual luciferase reporter system [17]. The p2luc vector used in this system was a kind gift from S. Goff at Columbia University New York. The entire overlapping region of Gag and Gag-Pol (307bp) from the wild-type and mutant viruses was inserted into the p2Luc vector between the 5' *Renilla* (rluc)

and 3' firefly (fluc) luciferase genes, such that expression of fluc was dependent on a -1 frameshift [17]. Lipofectamine (Invitrogen) was used according to the manufacturers' instructions for the transient transfection of 293T cells with the dual luciferase reporter constructs. The cells were seeded at 1.5×10^5 per well of a 24 well tissue culture plate 24 h prior to transfection. Routinely, 0.8 μ g of DNA was used for transfection. Luciferase activity was measured 24 h after transfection using the Dual-LuciferaseTM reporter assay (Promega) that was read on a Triathler LSC luminometer (LabLogic). The recoding efficiency of wild-type in this system was approximately 2.4-3%, which was consistent with other reports of 2.8% [17], 2-4% [23] and 1-2% [6]. The frameshifting level of the wild-type construct was set at 100% and used to determine the relative frameshifting levels of the mutants.

Virus production. The calcium phosphate co-precipitation method was utilized for the transient transfection of 293T cells. The cells were maintained in Dulbecco's Modified Eagle's Medium (Gibco BRL) containing 10% heat-inactivated foetal bovine serum (P. A. Biological Co.) and 1% penicillin-streptomycin. Ten micrograms of DNA from each of the HIV-1 constructs were routinely used for transfection. Supernatants were collected at 36 h post-transfection and centrifuged for 30 min at 3000 rpm (Beckman Model GS-6) to remove cellular debris. The RT activity of cell culture supernatants was measured utilizing a micro RT assay as previously described [16]. Virions were sedimented by ultracentrifugation (Beckman model L-90, SW 41 rotor) of the transfection supernatants through a 20% sucrose cushion at 26,500 rpm for 1 h at 4°C.

Immunoprecipitation and Western blot analysis. Intracellular viral protein and virion protein derived from transfected 293T cells were assessed by Western blot analysis of total HIV-1 proteins using pooled sera from HIV-1 infected patients as previously described [20, 37].

Gag-Pol expression was measured in cell lysates obtained from transfection of 293T cells with PR-defective wild-type and mutant constructs. The lysates were immunoprecipitated with HIV-1-infected patient sera and subsequently assayed by Western blot analysis using a monoclonal anti-p24 antibody (NEN) as previously described [20].

The relative levels of virion-associated Gag and Gag-Pol proteins were assessed using PRdefective wild-type and mutant constructs. Following transfection in 293T cells, the viral particles were lysed and assessed by Western blot analysis using an anti-p24 monoclonal antibody (NEN) in conjunction with a goat anti-mouse secondary antibody conjugated to Alexa Fluor 680 (Molecular Probes). The Gag and Gag-Pol proteins were detected and quantified using the Odyssey infrared imaging system (LI-COR).

Replication kinetics in PBMCs. PBMCs were isolated from HIV-seronegative buffy coats (supplied by the Red Cross Blood Bank, Melbourne, Victoria, Australia) as previously described [8]. PBMCs were stimulated with 10 μ g/ml of phytohemagglutinin (Murex Diagnostics) and maintained in RPMI (Roswell Park Memorial Institute) 1640 medium (Gibco) containing 10% fetal bovine serum, gentamicin, glutamine and 5% interleukin-2 (Boehringer) for 3 days. Viral supernatants, which were normalized for RT activity, were then mixed with 10^5 PBMCs in a 96-well tissue culture

plate. Eight 10-fold dilutions of each virus were tested in triplicate. A half media change was carried out every 3-4 days. Viral infectivity was assessed by monitoring RT activity, as described above, with supernatants collected on day 3, 7, 10 and 14 post infection.

Single round infectivity assay. The TZM-bl indicator cell line [10, 33, 44] was obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health, from J. C. Kappes, X. Wu and Tranzyme (Research Triangle Park, NC). Wild-type and mutant virions were normalized for RT activity and used to infect the TZM-bl indicator cell line. Cells were lysed at 48 h post-infection and the infectious titer of the viruses was determined by counting blue foci as previously described [10]. Wild-type infectivity was set at 100% for comparison to the mutants.

RESULTS

The p1 and p6^{Gag} mutants maintain the functional stability of the frameshift stem loop. Our initial study was undertaken using HXB2-BH10 [41] HIV-1 constructs. The two proline residues in p1 (position 7 and 13) were changed to leucines to create HIV_{BH10} p1:P7L and HIV_{BH10} p1:P13L. HIV_{BH10} p1:P7,13L and HIV_{BH10} p1:Pro+ [19] were also included in the panel. In addition, proline residues at positions 24, 25 and 30 of p6^{Gag} were altered to leucines to create the triple proline mutant HIV_{BH10} p6:P24,25,30L and all mutational combinations. The amino acid sequence of TF was unchanged in all mutants except HIV_{BH10} p1:Pro+, where lysine was altered to arginine at position 12 of TF to maintain stem-loop stability [20]. When compared to wild-type, the predicted thermodynamic stability of the frameshift stem-loop for each of the p1 mutants (Table 1) was shown to fall within a 5 kcal/mol deviation, which is sufficient to support viral replication at wild-type levels [20].

The impact of these mutations on both frameshifting efficiency and Gag-Pol synthesis was monitored. Frameshifting efficiency was assessed using the dual-luciferase reporter system [17]. The overlapping region of Gag and Gag-Pol from the wild-type and mutant viruses was inserted between the 5' *Renilla* luciferase (*rluc*) and 3' firefly luciferase (*fluc*) genes, so that the expression of *fluc* was dependent on a -1 frameshift. The frameshifting efficiency of the p1 and p6^{Gag} mutants and the replication competent SLP1TF* mutant [20] were comparable to wild-type (Fig. 1B), while, as anticipated, frameshifting in the stem-loop defective mutant SL*P1*TF [20] was greatly reduced. Furthermore, analysis of Gag-Pol synthesis using PRdefective constructs (P-) [20] revealed similar levels of Gag-Pol in the transfected cells expressing wild-type p1 and p6^{Gag} constructs (Fig. 1C, Lane 1-7), while the cells expressing stem-loop defective mutant SL*P1*TF [20] had reduced levels of Gag-Pol (Fig. 1C, Lane 8). Our results demonstrated that the p1 and p6^{Gag} mutants do not affect the function of the frameshift stem-loop. Consequently, any observed defects can be solely attributed to the amino acid changes in p1 and p6^{Gag}.

The incorporation of Gag-Pol relative to Gag is decreased in the p1 double proline mutant. Gag-Pol packaging is believed to be primarily dependent on interactions between the CA domains of Gag and Gag-Pol [22, 40, 45]. Other regions of Gag can also impact upon Gag-Pol packag-

ing, as the replacement of NC by a yeast leucine zipper domain inhibits Gag-Pol incorporation into assembling VLPs [7]. The ratio of virion-associated Gag-Pol to Gag was determined in PR-defective wild-type and mutant constructs (Fig. 2A). The ratio of Gag-Pol to Gag was set at 1 for wild-type for comparison to the mutants. Five independent experiments were performed and statistical analysis was undertaken *via* a paired *t* test using a 2-sided hypothesis with the significance level set at 0.05. Compared to wild-type, the incorporation of Gag-Pol in HIV_{BH10} p1:P7,13L was found to be significantly decreased (*P* = 0.016). Analysis of the remaining p1 mutants revealed a trend toward lower levels of Gag-Pol incorporation compared to wild-type that did not reach statistical significance.

Alterations to cellular or virion protein processing profiles were not readily observed in the majority of p1 or p6^{Gag} mutants (Figs. 2B, 2C). The HIV_{BH10} p1:P7,13L virion lysate was the exception with an observable decrease in the levels of p66-RT (mature protein) and an increase in Pr55-Gag (precursor protein) (Fig. 2C). The stability of the genomic RNA dimers was also assessed in these mutants. As previously reported, dimer stability was dramatically reduced in HIV_{BH10} p1:P7,13L [20]. The genomic RNA stability of the p1 single proline mutants and the p6^{Gag} mutants was not detectably different from wild-type (data not shown). Further, the alterations to Gag-Pol packaging seen in these mutants did not translate into changes in virion morphology as assessed by thin section electron microscopy analysis (data not shown).

The p1 and p6^{Gag} mutants display a range of viral infectivity levels in PBMCs. HIV_{BH10} p1:P7L and HIV_{BH10} p1:P7,13L were non-infectious in PBMCs, while HIV_{BH10} p1:pro+, HIV_{BH10} p1:P13L and HIV_{BH10} p6:P24,25,30L replicated at a low level (Fig. 3). Mutant HIV-1 containing 1, 2 or all 3 proline to leucine mutations at p6:P24, 25 and 30 exhibited defects in replication kinetics (data not shown), with the triple proline mutant HIV_{BH10} p6:P24,25,30L consistently the most impaired.

Viral infectivity of p1:P7L is strain dependent. To determine whether the p1 prolines were important for viral replication in other HIV-1 strains, the p1:P7L, p1:P13L and p1:P7,13L mutations were introduced into NL4.3 [2] AD8 [42], LAI [31] and MAL [3]. A comparison of the p1 sequences of each of these HIV-1 strains is shown in Fig. 4A. In all strains, p1:P7,13L was non-infectious (Fig. 4B-E), demonstrating that the proline residues are critical for viral infectivity in multiple strains of HIV-1. The p1:P13L mutants replicated at a low level in all strains (Fig. 4B-E). The replication of the p1:P7L mutants, however, appeared to be strain dependent. The p1:P7L mutant was non-infectious in HXB2-BH10 (Fig. 3) and displayed greatly reduced infectivity in the context of LAI (Fig. 4C), yet replicated at a level similar to wild-type in NL4.3 (Fig. 4B), MAL (Fig. 4D) and AD8 (Fig. 4E). Unlike the other HIV-1 strains, HXB2-BH10 and LAI did not encode a functional Nef or Vpr. NL4.3 based p1 mutants with a functionally deleted Nef and Vpr were created for analysis. These mutants did not emulate the HXB2-BH10 or LAI phenotypes (data not shown), making it unlikely that the presence of a functional Nef or Vpr may have an impact upon the strain dependent differences seen with the p1:P7L mutation.

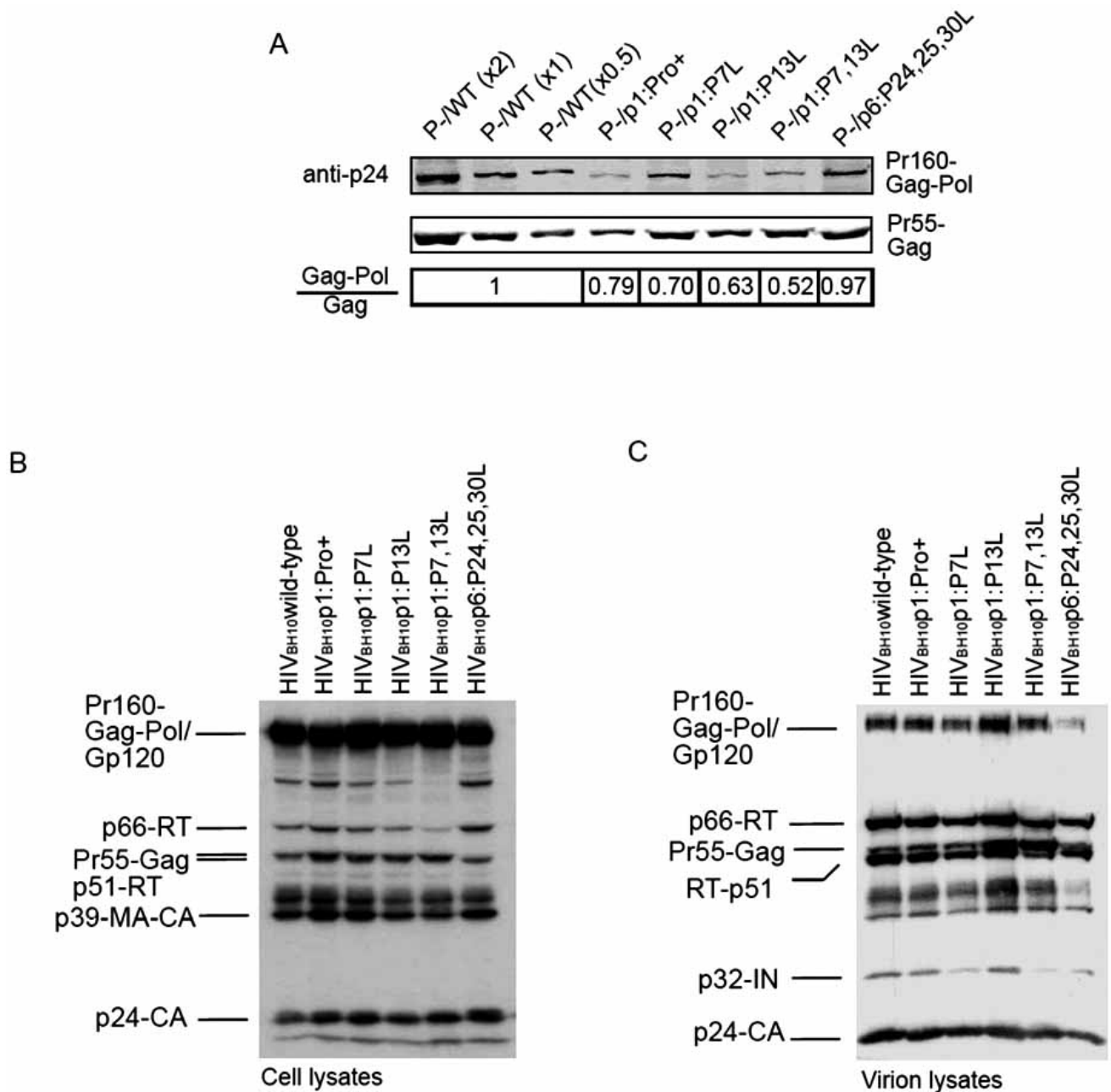


Fig. (2). Impact of the p1 and p6^{Gag} mutations on Gag-Pol incorporation into virions and HIV-1 protein profiles. (A) The relative levels of virion-associated Gag and Gag-Pol proteins were assessed by transfecting 293T cells with protease-defective (P-) wild-type and mutant constructs. The resultant viral lysates were assessed by Western blot analysis using a monoclonal anti-P24 antibody. The intensities of the Gag and Gag-Pol protein bands were quantified using the Odyssey infrared imaging system (LI-COR) and the relative ratio of Gag-Pol to Gag was determined with wild-type (1X) set at 1. This figure is representative of five experiments. (B) Cell and (C) virion lysates were produced from transfection of wild-type and mutant constructs in 293T cells, and subjected to Western blot analysis using sera from HIV-1 infected individuals.

Another conspicuous difference between these strains of HIV-1 was the identity of the amino acid at position 9 of p1 (Fig. 4A). The two strains where the infectivity of p1:P7L was dramatically reduced or totally abrogated (HXB2-BH10 and LAI) had a tyrosine at position 9 of p1 (p1:Y9), whereas the strains where p1:P7L replicated more robustly (NL4.3, AD8 and MAL) had a histidine at position 9 (p1:H9). To determine the involvement of p1:Y9 in p1 function, a series

of p1 mutants were created: HIV_{BH10} p1:Y9H, HIV_{BH10} p1:Y9H-P7L, HIV_{NL4.3} p1:H9Y and HIV_{NL4.3} p1:H9Y-P7L. The Y9H mutation in HIV_{BH10} p1:P7L rescued the replication defects of HIV_{BH10} p1:P7L emulating the pattern of replication seen in HIV_{NL4.3} p1:P7L (Fig. 4F). Furthermore, the H9Y mutation in HIV_{NL4.3} p1:H9Y-P7L abolished the infectivity observed in HIV_{NL4.3} p1:P7L to reproduce the HIV_{BH10} p1:P7L replication pattern (Fig. 4G). Overall these results

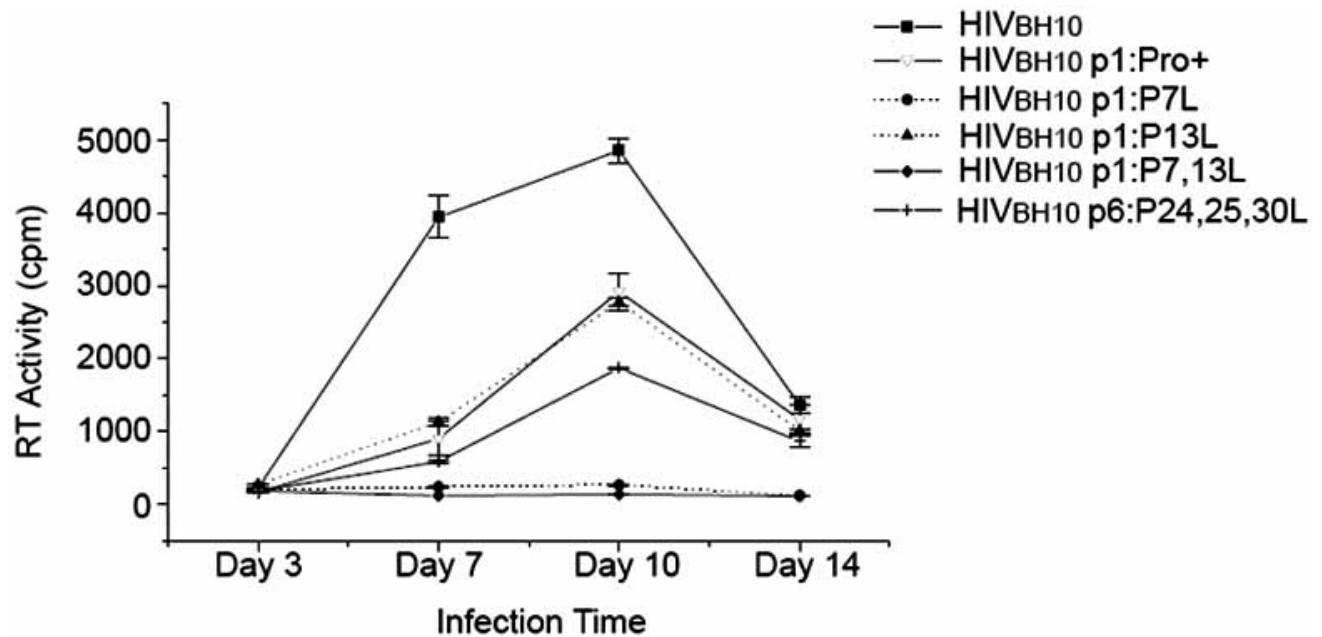


Fig. (3). Replication kinetics of the HXB2-BH10 p1 and p6^{Gag} mutants. Newly isolated PBMCs were stimulated with phytohemagglutinin for 3 days and then infected with either wild-type or mutant virus that had been normalized for RT activity. Viral supernatants were collected 3, 7, 10 and 14 days post-infection and the RT activity in each sample was measured. The results depicted represent the mean and standard deviation of duplicate samples. The graph is representative of five separate infections.

support the notion that the impact of the P7L mutation is context dependent and suggests that the suppressive activity of the p1:P7L mutation is associated with the presence of a tyrosine residue at position 9 of p1.

The replication defect in HIV_{BH10} p1:P7L occurs in the early phase of viral replication. A single cycle infection assay using the TZM-bl indicator cell line [10, 33, 44] was used to assess the replication defect observed in the p1 proline mutants and compared two strains with differing p1:P7L phenotypes (HXB2-BH10 and NL4.3) (Fig. 5). The pattern of viral infectivity reflected the results of the replication kinetics assay in PBMCs, wherein p1:P7,13L was non-infectious, p1:P13L replicated at a moderate level in both strains and the replication of p1:P7L was strain dependent. As successful infection of the TZM-bl cell line is dependent upon Tat expression, the defects observed in HIV_{BH10} p1:P7,13L, HIV_{NL4.3} p1:P7,13L and HIV_{BH10} p1:P7L may act at an early stage in the replication cycle. In addition, the clear changes to protein processing and Gag-Pol packaging that are evident in the double proline mutant suggest a defect that emanates through to the late stages of replication.

DISCUSSION

In this study we investigated the role of proline residues in the proline-rich P1-P6^{Gag} Cterminus of Gag, focusing on the two highly conserved proline residues in p1 (position P7 and P13) and three proline residues in p6^{Gag} (P24, P25 and P30). Mutating both p1 proline residues was found to abolish infectivity in multiple strains of HIV-1, highlighting the critical role of these residues in HIV-1 replication. Intriguingly, independent mutation of the p1 proline at position 7 resulted in a strain-dependent suppression of viral infectivity.

In addition, we have shown that the p1 proline residues are functionally distinct from P24, P25 and P30 in p6^{Gag}.

The spacer peptide p1 resides within the region of HIV-1 that encompasses the critical secondary structure of the frameshift stem-loop and encodes for two distinct proteins (p1 in Gag and TF in Gag-Pol). To ensure that the introduced mutations did not affect the function of the frameshift stem-loop, we monitored both frameshifting efficiency and Gag-Pol synthesis and found that both were maintained at a level similar to that of wild-type in each of the mutants (Fig. 1A and 1B). Consequently, it is appropriate to ascribe any observed defects in the mutant constructs to changes in the protein sequences of p1 and p6^{Gag}.

The HXB2-BH10 p1 and p6^{Gag} mutants were analyzed for Gag-Pol incorporation, protein processing and viral infectivity in PBMCs. Notably, Gag-Pol incorporation relative to Gag was consistently reduced in each of the p1 mutant virions, with the levels of Gag-Pol in the double proline mutant significantly decreased (Fig. 2A). Given the proximity of p1 to NC, the hypothesized change to protein folding introduced by these proline mutations could in fact have had an affect on NC's capacity to support Gag-Pol packaging [7]. As immature HIV-1 was used in this work, our findings are independent from studies suggesting that p6^{Gag} has a role in the packaging of the mature Pol proteins [7, 11, 46].

Across the full series of mutants analyzed in this study, protein processing was only detectably altered in the double proline mutant HIV_{BH10} p1:P7,13L (Fig. 2B and 2C). This may be the result of the observed reduction in Gag-Pol (and therefore PR) incorporation seen in HIV_{BH10} p1:P7,13L. The reduction in Gag-Pol and PR may also contribute to the de-

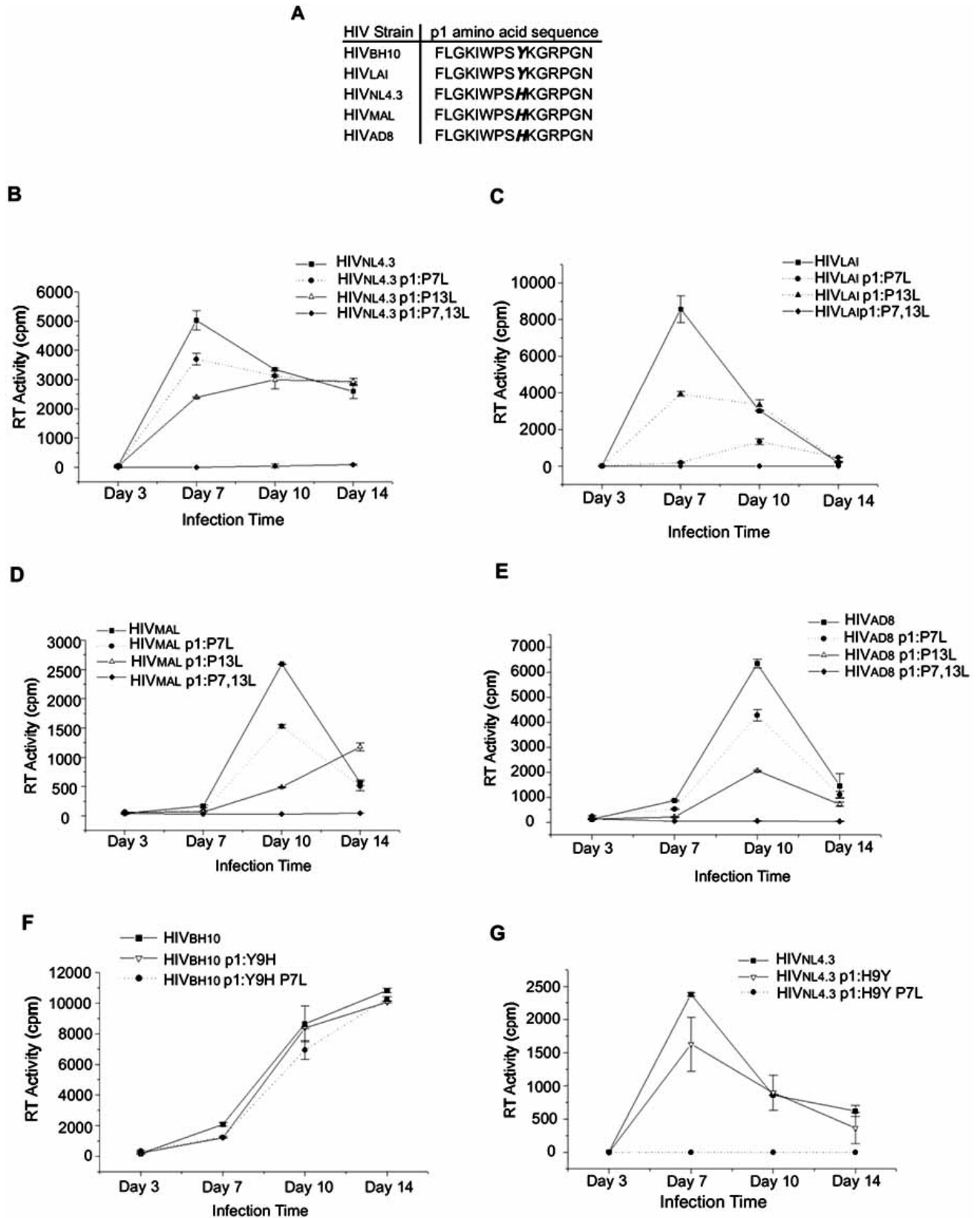


Fig. (4). Replication kinetics of the p1 mutants in multiple strains of HIV-1 (A) Comparison of p1 sequences from multiple strains of HIV-1. (B-E) Replication kinetics of the p1 proline mutants introduced into the HIV-1 strains; NL4.3 (B), Lai (C), Mal (D) and AD8 (E). (F-G) The effect on replication kinetics of altering p1 residue 9 in BH10 (F) and NL4.3 (G) in the wild-type and P7L constructs. Each graph is representative of three separate PBMC infections.

crease in the genomic RNA stability of HIV_{BH10} p1:P7,13L [20], as proteolytic processing is required for correct genomic RNA dimer formation [14]. Interestingly, more modest reductions of virion Gag-Pol incorporation, such as those seen in HIV_{BH10} p1:P13L, do not substantially impact upon the processing of viral proteins.

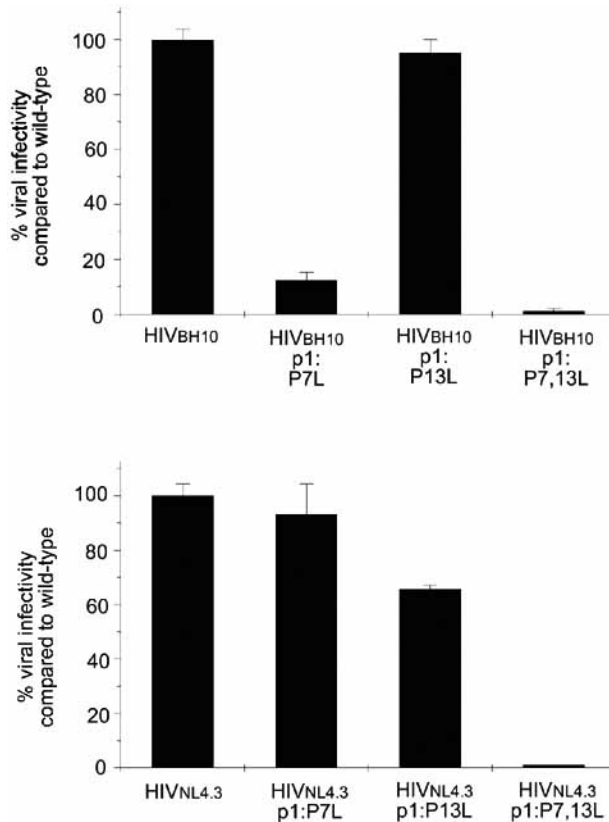


Fig. (5). Single round infectivity assay with the p1 proline mutants. Wild-type and mutant virions were normalized for RT activity and used to infect the TZM-bl indicator cell line. Cells were lysed at 48h postinfection and the infectious titer of the viruses was determined by counting of blue foci as previously described [10]. Wild-type infectivity was set at 100% for comparison to the mutants.

Each of the p1 mutants displayed a decrease in viral replication in PBMCs with HIV_{BH10} p1:P7L and HIV_{BH10} p1:P7,13L rendered non-infectious (Fig. 3). While the p6^{Gag} proline mutant HIV_{BH10} p6:P24,25,30L also displayed reduced infectivity, the defect was not as dramatic as that seen for the p1 proline mutants. We speculated that the proline residues in p1 and the similarly spaced P6^{Gag} proline residues P24 and P30 may impact upon HIV-1 replication in a comparable manner. It appeared that the p1 proline residues are in fact functionally distinct to those we investigated in p6^{Gag}, as the double proline mutations in p1 resulted in the abrogation of viral infectivity and defects in protein processing that were not apparent in any of the p6^{Gag} proline mutants. Taken together with previous studies altering p6^{Gag} proline residues outside the PTAPP motif that found the replication to be either similar to wild-type [9, 21] or impaired but not abrogated [30], it appeared that the p6^{Gag} prolines may be important but not critical for viral function. In contrast to our findings, Blieder *et al.* [4] have shown in NL4.3 that it is possi-

ble to delete the p6^{Gag} region S14-I31, which includes the prolines at position 24 and 30, without major consequences for viral replication. This may reflect a difference between HIV-1 strains, or alternatively, it may be that altering proline residues and consequently changing protein folding are more detrimental to p6^{Gag} function than actually deleting the region.

The p1 amino acid sequence is highly conserved, with the notable exception of residue 9 which can be arginine, serine, tyrosine or histidine. Remarkably, mutation of the p1 proline at position 7 resulted in a strain-dependent suppression of viral infectivity that was correlated with the presence of a tyrosine residue at position 9 of p1 (Fig. 4). At present, the mechanism generating this strain dependent phenotypic difference is unknown. We demonstrated that the disparity is contingent on whether a histidine or tyrosine residue is present at position 9 of p1. One explanation may be that the identity of the amino acid at position 9 generates a difference in the post-translational modification of p1. While tyrosine, serine and threonine are considered to be the classical amino acid targets for phosphorylation in eukaryotes, there are numerous studies which demonstrate that phosphohistidine is crucial in mammalian systems (for review see [26]). Notably, the mechanism of phosphorylation differs between tyrosine and histidine with tyrosine phosphorylated on its hydroxyl group, while histidine is phosphorylated on both its imidazole nitrogens. As the presence of histidine at position 9 of p1 (NL4.3, MAL and AD8) highlights the fact the replication capacity of these strains is not abolished by the p1:P7L mutations, it is possible that the ability of histidine to be phosphorylated on more than one site allows it to functionally compensate for the p1:P7L mutation.

Using a single cycle replication assay, we observed that the defects in HIV_{BH10} p1:P7,13L, HIV_{NL4.3} p1:P7,13L and HIV_{BH10} p1:P7L acted at an early stage in the replication cycle (Fig. 5). One possibility was that the p1 mutations had an impact on NC function, as the NC protein is critical in the early phase processes of reverse transcription [43] and integration [5]. This suggestion is consistent with Gao *et al.* [15] proposal that p1 may have a role in the direction of viral integration by NC. It is currently unclear whether mature NC, or one of the p1 containing NC precursors, such as NC-p9 or NC-p15 carries out the roles of NC in viral replication. The cleavage of p9 to release NC is the final cleavage step in virion proteolysis [32], allowing p1 to impact upon NC in all precursor forms. It has been suggested that it is the kinetics of NC precursor processing that in fact controls the functions of the various forms of NC [38, 39]. Accordingly, p1 could influence NC function by directly contributing to NC structure or alternatively, the cleavage of p1 may be important for the regulation of the various functions of NC. One possibility is that the post-translational modification of p1 could give increased control over the cleavage of the p1 containing precursor proteins, which would in turn suggest a regulatory role for p1 being not dissimilar to that of the spacer peptide p2.

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