**ORIGINAL**

# Virological characterization of HIV-1 CA-NTD mutants constructed in a virus-lineage reflected manner

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**Abstract : Capsid (CA) protein is a major virion-constituent of all retroviruses including human immunodeficiency virus type 1 (HIV-1), and is essential for early and late phases in viral replication cycle through interaction with numerous cellular factors. In particular, N-terminal domain (NTD) of HIV-1 CA has been frequently and well reported to bind to various host cell proteins that considerably affect viral replication potential. In this study, in order to better define biological bases of the CA-NTD for HIV-1 replication, we performed an extensive mutational analysis in an unprecedented manner. By aligning CA-NTD sequences derived from representative infectious molecular clones of HIV-1, HIV-2, and simian immunodeficiency virus isolated from the rhesus macaque (SIVmac), a number of amino acids specific to HIV-1 were selected, and were replaced with those from SIVmac at the corresponding sites. Mutant viruses thus generated were then examined for multi-cycle infectivity, single-cycle infectivity, and ability to produce progeny virions. While some CA-NTD mutations affected viral replication ability to varying degrees, those in helix 7 abolished viral growth potential without exception. These results highlight functional importance of non-conserved amino acids in helix 7, and give new insights into functionality of HIV-1 CA-NTD. J. Med. Invest. 65 : 110-115, February, 2018**

***Keywords :*** *HIV-1, HIV-2, SIVmac, CA-NTD, mutational analysis*

## INTRODUCTION

Gag capsid (CA) protein, a major virion component of all retroviruses including human and simian immunodeficiency viruses (HIV/SIVs), plays an essential role for virus life cycle at both early and late replication phases (1-3). HIV-1 Gag is intracellularly synthesized as a precursor, and subsequently cleaved into matrix (MA), CA, nucleocapsid (NC), and p6 as functional mature products during the virion maturation process (2, 4-7). The CA N-terminal domain (NTD) of HIV-1 are composed of N-terminal β-hairpin, seven α-helices, and loop structures, and constitutes the exposed outer surface. Indeed, CA-NTD is known to interact with various cellular factors that regulate virus replication (2, 6-8). Of note, macaque α-isoform of tripartite motif-containing protein 5 (TRIM5α) and related proteins, major cellular determinants for HIV-1 species tropism, eliminate HIV-1 infectivity by binding to its CA-NTD and by aberrantly promoting the post-entry disassembly process of CA (9, 10).

To clarify the functional details of HIV-1 Gag-CA, its biological and biochemical properties have been extensively analyzed by numerous mutational studies until now (reference 11 as representative). In those works on Gag-CA, deletion, insertion, proline-scanning, and/or alanine-scanning mutations were introduced into the amino acid residues conserved among HIV-1/SIV strains, and resultant mutants were examined for their viral phenotypes. In this study, we have adopted a different strategy to construct test mutants hoping to identify potential new CA activities. Eighteen amino acids specific to HIV-1 in the sequence alignment of HIV-1 (NL4-3), HIV-2 (GL-AN), and SIVmac (MA239) were replaced with those of SIVmac at the corresponding sites to generate HIV-1 CA mutants. In addition, a mutant I134Q was constructed, considering that there are two consecutive isoleucine residues at amino acid positions 134 and 135 in the NL4-3 sequence, and also that there is a glutamine residue at position 135 conserved in the sequences of GL-AN and MA239. To increase chances to obtain new findings, we have focused on the CA-NTD region here because (i) CA-NTD is more heterologous than CA C-terminal domain (CTD) with respect to the amino acid sequence, and (ii) various interactions between CA-NTD and cellular factors have been documented to date. Totally, nineteen site-specific point mutants of HIV-1 CA-NTD were generated, and were characterized for basic viral phenotypes, i.e., virus growth potential, early post-entry infectivity, and virion production level at final replication phase, in this study.

## MATERIALS AND METHODS

### *Construction of proviral mutant clones*

Four infectious molecular clones designated NL4-3 (HIV-1), GL-AN (HIV-2), MA239 (SIVmac239, virus clone from a rhesus macaque (mac)), and NL-DT5R (macaque-tropic HIV-1) have been previously described (12-16). Proviral CA-NTD mutant clones derived from HIV-1 NL4-3 and NL-DT5R in this study were generated by the QuickChange site-directed mutagenesis kit (Agilent Technologies Inc) as previously described (17-19). Nucleotide sequences of the mutant clones were confirmed by ABI Genetic Analyzer 3130xl (Thermo Fisher Scientific).

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*Cells*

A human kidney cell line 293T and a HeLa-derived reporter cell line TZM-bl carrying a luciferase gene under control of HIV-1 long terminal repeat (LTR) were cultured in Eagle's minimal essential containing 10% heat-inactivated fetal bovine serum (20). Lymphocyte cell lines H9 (human) and HSC-F (cynomolgus macaque) were maintained in RPMI1640 medium containing 10% heat-inactivated fetal bovine serum (19, 21).

*Transfection*

Input viruses for infection experiments were prepared from 293T cells transfected with various proviral clones by the calcium-phosphate co-precipitation method (12, 17). Virus amounts were determined by virion-associated reverse transcriptase (RT) assays as previously described (17, 22). To determine the virus production level in lymphocyte cells, proviral clones (2 μg) and pGL3 (luciferase reporter vector) (2 μg) were cotransfected into H9 cells by Nucleofector Ⅱ using program X-005 and Nucleofector kit V (Lonza Ltd.). H9 cells were then cultured in the presence of AMD 3100 (antagonist against CXCR4-tropic HIV-1) that prevents the re-infection of cells with progeny viruses produced by transfection. On day 2 post-transfection, culture supernatants were collected, and virion production was monitored by HIV-1 Gag-p24 ELISA kit (ZeptoMetrix Corporation). Virion production level in H9 cells cannot be determined by the RT assay due to its relatively low sensitivity. Luciferase activity in cell lysates was used to normalize the transfection efficiency.

*Assays for viral infectivity*

To monitor multi-cycle infectivity (virus growth ability/potential), equal RT units ($10^5$ for H9 cells and $10^6$ for HSC-F cells) of virus samples were infected into H9 ($10^5$) or HSC-F cells (2 x $10^5$), and virus growth kinetics were determined by RT assays as previously described (17, 20). H9 and HSC-F cell lines show phenotypes very similar to that of their corresponding primary cells such as peripheral blood mononuclear cells and lymphocytes with respect to the susceptibility to infections with various HIV/SIVs and/or their mutant viruses. Progeny viruses produced in infected cells were monitored at intervals (every 3 days) during 15 days following infection. To determine single-cycle infectivity, equal RT units ($10^4$) of virus samples were inoculated into TZM-bl cells (4 x $10^3$ cells were seeded into a well of a 96-well plate one day before infection), and on day 1 post-infection, cells were lysed for luciferase assays (Promega Corporation) as described previously (20). Luciferase activity in cell lysates relative to that of NL4-3 was considered as viral single-cycle infectivity. This infectivity represents "viral infectivity at the early replication phase", because the luciferase activity in TZM-bl cells is induced by viral early protein Tat through the transcriptional activation.

## RESULTS

*Generation of HIV-1 CA-NTD mutants*

HIV-1 Gag-CA consists of two independent domains NTD and CTD connected by a flexible linker region (Fig. 1). There are a β-hairpin and seven helical structures in CA-NTD, whereas four

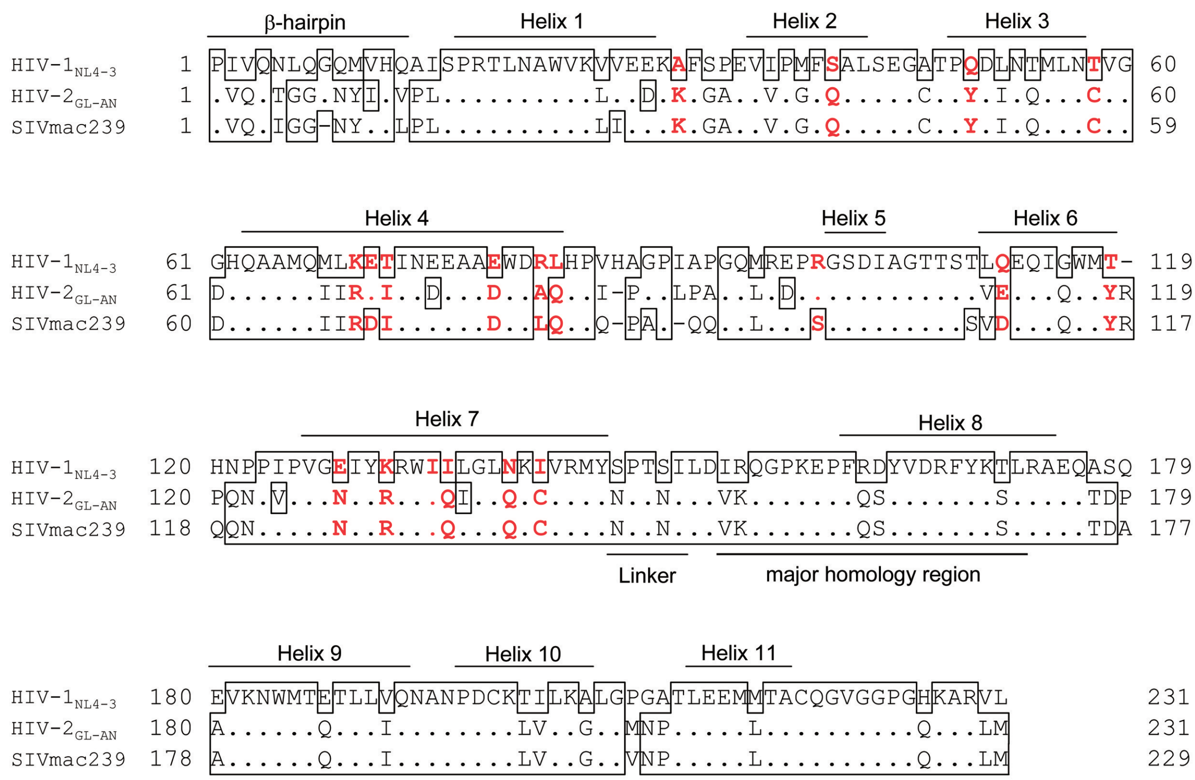


Figure 1 Alignment of Gag-CA NTD sequences. Amino acid sequences of HIV-1 NL4-3 (GenBank accession number : AF324493), HIV-2 GL-AN (GenBank accession number : M30895), and SIVmac MA239 (GenBank Accession number : M33262) CA-NTD proteins are comparatively presented. Structural domains/regions are indicated above (helices) or below (linker domain and major homology region (MHR)) the sequence alignment. HIV-1 CA-NTD is from the N-terminus to the end of helix 7, and CA-CTD is from I150 or L151 to the C-terminus (34). Amino acid sites for mutational analysis in this study are represented by red letters.

helices in CA-CTD. Although these structural characteristics are conserved, amino acid sequences in Gag-CA are quite different between viruses of HIV-1 and HIV-2/SIVmac groups. According to Genetyx version 11, as for CA-NTD amino acid sequences, 62% identity (85% similarity) for NL4-3 (HIV-1) vs. GL-AN (HIV-2), 62% identity (86% similarity) for NL4-3 vs. MA239 (SIVmac), and 85% identity (97% similarity) for GL-AN vs. MA239. Regarding CA-CTD amino acid sequences, 74% identity (97% similarity) for NL4-3 vs. GL-AN, 74% identity (97% similarity) for NL4-3 vs. MA239, and 98% identity (100% similarity) for GL-AN vs. MA239. Thus, CA-CTD is more conserved than CA-NTD in terms of amino acid sequences, and is almost indistinguishable between GL-AN and MA 239.

Based on the above consideration, we constructed a new series of CA-NTD mutants in a unique way, i.e., introduction of mutations into non-conserved amino acids as indicated in Fig. 1, to obtain potentially useful information for future studies. Exceptionally, we constructed a mutant I134Q side by side with the I135Q mutant (Fig. 1). We mainly introduced mutations into helical domains. It is already known that the loop structure between helices 4 and 5 (4-5 loop) serves as binding site for cellular proline isomerase cyclophilin A, regulating viral replication (2, 7, 8). The 4-5 and 6-7 loops in CA-NTD are shown to be critical by us for human specific nature of HIV-1 as viral determinants for narrow HIV-1 species tropism (16, 23). Moreover, amino acid mutations M94L and R98S in 4-5 loop and G114Q in helix 6 were demonstrated also by us to be important for the species tropism (17). On balance, as summarized in Table 1, we finally constructed 19 proviral mutants carrying a site-specific point mutation in CA-NTD.

*Virological characterization of HIV-1 CA-NTD mutants*

Various HIV-1 clones carrying a site-specific mutation in CA-NTD (Fig. 1 and Table 1) were analyzed for their viral properties by transfection and infection experiments. First, each mutant virus was examined for its ability to grow in H9 cells. Input viruses for infection prepared from transfected 293T cells were inoculated into target H9 cells, and virus growth properties were determined by RT assays. Representative results are shown in Fig. 2, and all the data obtained were summarized in Table 1. Based upon growth potentials in a lymphocyte cell line H9, mutant virus clones can be classified into four groups as readily recognizable in Fig. 2 : i) clones with a similar growth ability to parent NL4-3 (E79D and R 100S) ; ii) clone with a poor growth ability relative to NL4-3 (E71 D) ; iii) clone with a severely impaired growth ability (Q112D) ; iv) clone with undetectable growth ability (T119Y). Another important point to be mentioned here is that all helix 7 mutants lost infectivity as a result of the point mutation (Table 1). This is in contrast with the helix 4 mutants, which showed various growth phenotypes (from i) to iv)) as described above.

Next, in order to infer defective sites of non-infectious mutants in H9 cells (12 mutants in Table 1), single-cycle replication assays, i.e., TZM-bl and virion production assays, were performed to examine their early and late replication phases. Upon transfection into 293T cells, these mutants did produce virions, albeit to various degrees, as monitored by RT assays. Fig. 3 shows the early infectivity of the mutants derived from transfected 293T cells relative to that of NL4-3 as determined by TZM-bl assays. It is clearly observed that all the mutants examined exhibit significantly low infectivity compared to NL4-3. Particularly, the infectivity of A31K, Q50Y, K70R, R82L, T119Y, E128N, and I134Q was extremely low or even undetectable, indicating the functional importance of the authentic amino acids at early replication phase. Examination of the late replication phase by virion production in transfected H9 cells (Fig. 4) revealed that the non-infectious mutants tested were generally not so much defective as observed in Fig. 3. Of note, although data fluctuations were somewhat large, mutations in the 1-2 loop and helices 2/3 (A31K, S41Q, and Q50Y) did not affect much the late replication phase (compare the average values obtained for the three mutants and the others in Fig. 4). Because transfected 293T cells could produce virions at a considerable level as judged by RT assays as described above, the negative results for K70R and I134Q (perhaps for I135Q also) in Fig. 4 probably came from the inability of the anti-Gag-p24 (CA) antibodies in the ELISA kit to detect the mutant CA proteins. Table 1 summarizes all the quantitative data obtained for the early and late replication efficiencies of the mutants (Figs. 3 and 4).

Finally, the helix 7 mutants (I134Q and I135Q) were evaluated for their replication potentials in macaque cells. Based on striking results on the helix 7 mutants that none were infectious for H9 cells, we speculated that authentic amino acids there are somehow involved in the narrow host range of HIV-1. We generated mutants in a virus-lineage reflected way (Fig. 1), and could assume that there were some amino acids and/or regions in CA-NTD relevant to or responsible for efficient HIV-1 replication only in human cells. To test this hypothesis, I134Q and I135Q mutations were individually introduced into a macaque cell-tropic NL-DT5R clone (16). Input viruses were then prepared from 293T cells transfected with test samples or negative (NL4-3) and positive (NL-DT5R) control clones, and inoculated into cynomolgus macaque HSC-F cells (21). As clearly seen in Fig. 5, while NL4-3 did not grow at all in HSC-F cells, NL-DT5R grew well with a peak on day 9. No virus replication was detected during 15 days observation period for the two mutants of duplicate samples.

Table 1 Virological characteristics of CA-NTD mutants in this study

| Parent and mutant clones | Mutated domain | Virus growth in H9 cells | Infectivity in TZM-bl cells | Virion production in H9 cells |
|---|---|---|---|---|
| NL4-3 | None | +++ | 1.00±0.00 | 1.00±0.00 |
| **A31K** | H1/2 | – | 0.01±0.00 | 0.86±0.33 |
| **S41Q** | Helix 2 | – | 0.19±0.04 | 1.04±0.36 |
| **Q50Y** | Helix 3 | – | 0.06±0.01 | 0.93±0.24 |
| T58C | H3/4 | + | ND | ND |
| **K70R** | Helix 4 | – | 0.04±0.02 | NA |
| E71D | Helix 4 | ++ | ND | ND |
| T72I | Helix 4 | + | ND | ND |
| E79D | Helix 4 | +++ | ND | ND |
| **R82L** | Helix 4 | – | 0.01±0.01 | 0.35±0.09 |
| L83Q | Helix 4 | + | ND | ND |
| R100S | H4/5 | +++ | ND | ND |
| Q112D | Helix 6 | + | ND | ND |
| **T119Y** | Helix 6 | – | 0.03±0.01 | 0.31±0.05 |
| **E128N** | Helix 7 | – | 0.02±0.01 | 0.39±0.09 |
| **K131R** | Helix 7 | – | 0.38±0.11 | 0.46±0.08 |
| **I134Q** | Helix 7 | – | 0.00±0.00 | NA |
| **I135Q** | Helix 7 | – | 0.12±0.04 | 0.09±0.07 |
| **N139Q** | Helix 7 | – | 0.34±0.12 | 0.46±0.16 |
| **I141C** | Helix 7 | – | 0.12±0.04 | 0.37±0.08 |

*Parent and mutant clones :* Bold letters indicate the mutant clones that lack growth ability in H9 cells.
*Mutated domain :* H1/2, between helices 1 and 2 ; H3/4, between helices 3 and 4 ; H4/5, between helices 4 and 5. See Fig. 1 for details.
*Virus growth :* –, no growth ;+, severely retarded growth (see Q112D in Fig. 2) ;++, retarded growth (see E71D in Fig. 2) ;+++, similar growth with NL4-3.
*Infectivity :* ND, not determined.
*Virion production :* ND, not determined ; NA, not applicable (see text for details).

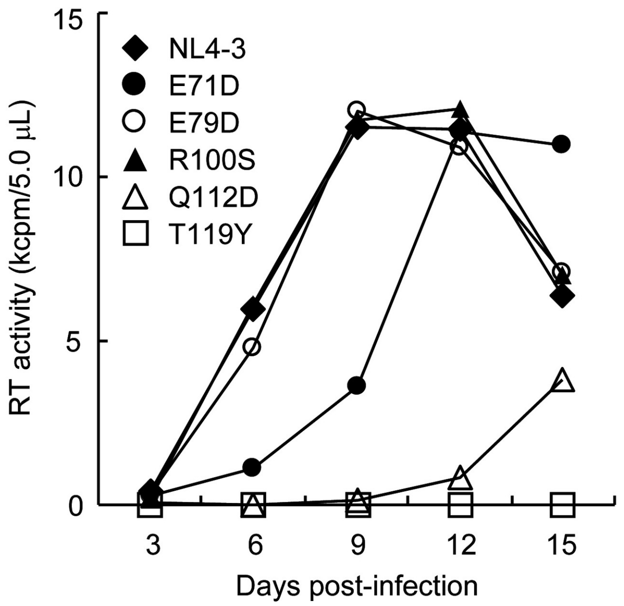


Figure 2 Growth kinetics in human H9 cells of CA-NTD mutants derived from HIV-1 NL4-3. Viruses were prepared from 293T cells transfected with proviral clones indicated, and equal amounts ($10^5$ RT units) were inoculated into H9 cells ($10^5$). RT activity in the culture supernatants was determined every 3 days up to 15 days post-infection. The experiment was repeated with similar results.

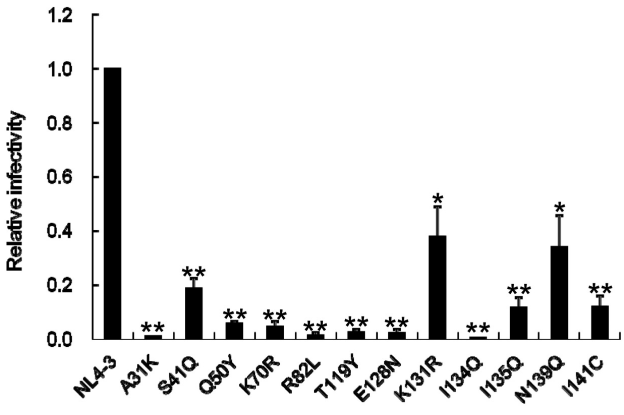


Figure 3 Single-cycle infectivity in TZM-bl cells of CA-NTD mutants derived from HIV-1 NL4-3. Viruses were prepared from 293T cells transfected with proviral clones indicated, and equal amounts ($10^4$ RT units) were inoculated into sub-confluent TZM-bl cells in wells of a 96-well plate. On day 1 post-infection, cells were lysed for luciferase assays. Infectivity of each mutant relative to that of NL4-3 is shown. Mean values ± SD from at least three independent experiments are presented. Significance relative to NL4-3 was determined by the Student *t* test (**, $p < 0.01$ ; *, $p < 0.05$).

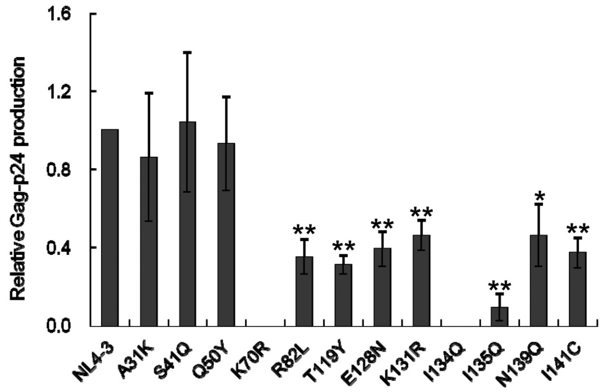


Figure 4 Ability of CA-NTD mutants derived from HIV-1 NL4-3 to produce progeny virions in H9 cells. Proviral clones indicated (2 μg) and pGL3 (luciferase reporter vector) (2 μg) were co-nucleofected into H9 cells, and on day 2 post-transfection, culture supernatants were prepared for Gag-p24 ELISA assays. Gag-p24 production by each mutant relative to that by NL4-3 is shown. Luciferase activity in cell lysates was used for normalization of the transfection efficiency. Mean values ± SD from at least three independent experiments are presented. Significance relative to NL4-3 was determined by the Student *t* test (**, $p < 0.01$ ; *, $p < 0.05$).

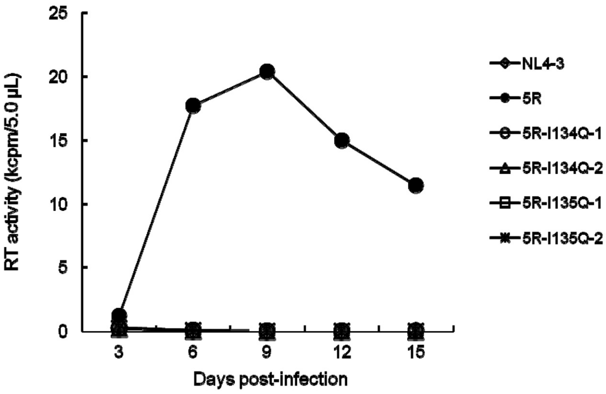


Figure 5 Growth in cynomolgus HSC-F cells of I134Q and I135Q mutants derived from macaque cell-tropic HIV-1 NL-DT5R. Viruses were prepared from 293T cells transfected with proviral clones indicated, and equal amounts ($10^6$ RT units) were inoculated into HSC-F cells (2 x $10^5$). RT activity in the culture supernatants was determined every 3 days up to 15 days post-infection. The infection experiment for the mutants was performed in duplicate using independently prepared virus samples (both DNA preparation for transfection and virus preparation from transfection were independently carried out). 5R, NL-DT5R (16).

## DISCUSSION

In this study, we have performed a systemic mutational analysis on HIV-1 CA-NTD (145 amino acids), and provide basic information on virological properties of the mutants with a site-specific point mutation throughout the domain (Table 1). Using a unique virus-lineage dependent mutagenesis, we totally constructed 19 mutants, and 12 mutants were found to be non-infectious for a human lymphocyte cell line H9. All the non-infectious mutants were defective at the early replication phase (A31K, S41Q, Q50Y, K70R, R82L, T119Y, E128, K131R, I134Q, I135Q, N139Q, and I141C), whereas three were certainly not defective at the late phase (A31K, S41Q, and Q50Y). Our results presented here are generally consistent with those previously reported (11). Of particular interest in this study is the unexceptional lethal effect by mutations in CA-NTD helix 7 (E128N, K131R, I134Q, I135Q, N139Q, and I141C).

HIV-1 Gag-CA is involved in a series of different interactions in the processes of viral core disassembly at the post-entry early replication phase, and virion assembly and maturation at the late final replication phase (2-8). It is mainly composed of helical domains NTD and CTD, and while NTD primarily forms a hexameric/pentameric structures, CTD interacts with the NTD of adjoining CA molecules (4-7). Although it has been well demonstrated structurally and functionally that amino acids in NTD helices 1-6 are important for core formation, Gag assembly, and virion production (11, 24, 25), structural and functional roles of the helix 7 in these processes are poorly understood as yet. Considering that all the non-infectious CA-NTD mutants including those of the helix 7 in this study are defective for the early replication phase (Table 1), it is pivotal to determine the underlying molecular basis. It is also necessary to define in detail how virion-Gag disassembles at the early post-entry stage. This subject is now under vigorous investigation by many researchers (26-31). For better understanding the HIV-1 virology and for therapeutic purpose, the above issues need to be precisely elucidated.

HIV-1 is believed to have evolved to its present form as a human specific pathogen following numerous mutations, recombinations, and adaptations from the ancestral virus(es) (32). Most evident virological property of HIV-1 is that it grows well almost exclusively in human cells and individuals. The determinants for this narrow host range are CA-NTD and Vif as demonstrated by us and others (16, 17, 33). Some amino acids responsible for the tropism are identified in CA-NTD as described above (16, 17). When selecting the target amino acids for mutation analysis in this study, we assumed a possibility that some, non-conserved among HIV-1, HIV-2, and SIVmac, might contribute to the efficient HIV-1 replication in human cells. We therefore introduced two mutations in helix 7 into macaque-tropic HIV-1 NL-DT5R, and verified the possibility by infecting macaque HSC-F cells with mutant NL-DT5R viruses. Although not completely excluded, our hypothesis is now unlikely from the results in Fig. 5. Further study is required to identify amino acids and/or regions relevant to or account for the efficient replication of HIV-1 specifically in human cells, if any.

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## CONFLICT OF INTEREST

The authors declare that no competing interests exist.

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