ORIGINAL

Growth properties of macaque-tropic HIV-1 clones carrying *vpr/vpx* genes derived from simian immunodeficiency viruses in place of their *vpr* regions

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Abstract : We have previously generated a macaque-tropic human immunodeficiency virus type 1 (HIV-1mt) clone designated MN4/LSDQgtu by genetic manipulation from a parental virus that replicates poorly in rhesus macaque cells. In rhesus cell line M1.3S and peripheral blood mononuclear cells (PBMCs), MN4/LSDQgtu grows comparably to a standard simian immunodeficiency virus clone derived from the rhesus macaque (SIVmac239) that can induce the acquired immunodeficiency syndrome (AIDS) in the animals. In this study, we further modified the Vpr-coding region of MN4/LSDQgtu genome by introducing *vpr* gene of an SIV clone from the greater spot-nosed monkey (SIVgsn166) or *vpx* gene of SIVmac239 to generate four new clones for determining functional importance of the central genomic area. Furthermore, two clones with an additional Gag-p6 mutation were made to ensure the virion-packaging of Vpx. In addition, accessory gene mutant clones of MN4/LSDQgtu with a frame-shift mutation, including a *vpr* mutant, were constructed and their growth properties were examined. Infection experiments showed that newly constructed viruses all grew poorly to various degrees in M1.3S cells, relative to MN4/LSDQgtu. Together with the previous data, our results here show that *vpr/vpx* gene in the appropriate context of HIV-1 genome is critical for viral growth ability. J. Med. Invest. 61 : 374-379, August, 2014

Keywords **:** *HIV-1mt, SIV, vpx, vpr*

INTRODUCTION

HIV-1 exhibits a uniquely narrow host range, and induces AIDS only in humans (1, 2). Due to this distinct property, animal models to study *in vivo* characteristics of HIV-1 are difficult to be established. Small animals are, of course, insusceptible to HIV-1, and even the primates frequently used for experimental infections such as cynomolgus and rhesus macaques can not be infected with HIV-1. Therefore, today, the experimental infection of rhesus macaques with SIVmac genetically related to HIV-1 are thought to be the best model system for various studies on HIV-1/AIDS. However, SIVmac is clearly distinct from HIV-1 by its genome composition and biological properties (2). For an attempt to establish an ideal animal infection model, we and others generated HIV-1mt clones that negotiate the intrinsic species barrier against HIV-1 (3, 4). Recently, we have obtained a much improved version of HIV-1mt designated MN4/LSDQgtu that grows comparably well to a standard pathogenic SIVmac clone SIVmac239 (5).

Of the five accessory proteins unique to HIV/SIV,

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Vpr and Vpx are least well studied and understood with respect to their functional roles in viral replication/pathogenicity. These two proteins are structurally closely related, and are found to display similar biological activities (6-8). Of note, Vpr and Vpx are demonstrated to be virion-associated, and are thought to exert their functions in the early phase of viral replication cycle (6-8). Although a variety of virus properties are attributable to Vpr/Vpx, their virological significances and underlying molecular bases remain to be elucidated (6-8). On one hand, while viruses of the HIV-2 group including SIVmac have Vpr and Vpx, those of the HIV-1 group encode Vpr only (Figure 1). Whether these viral proteins

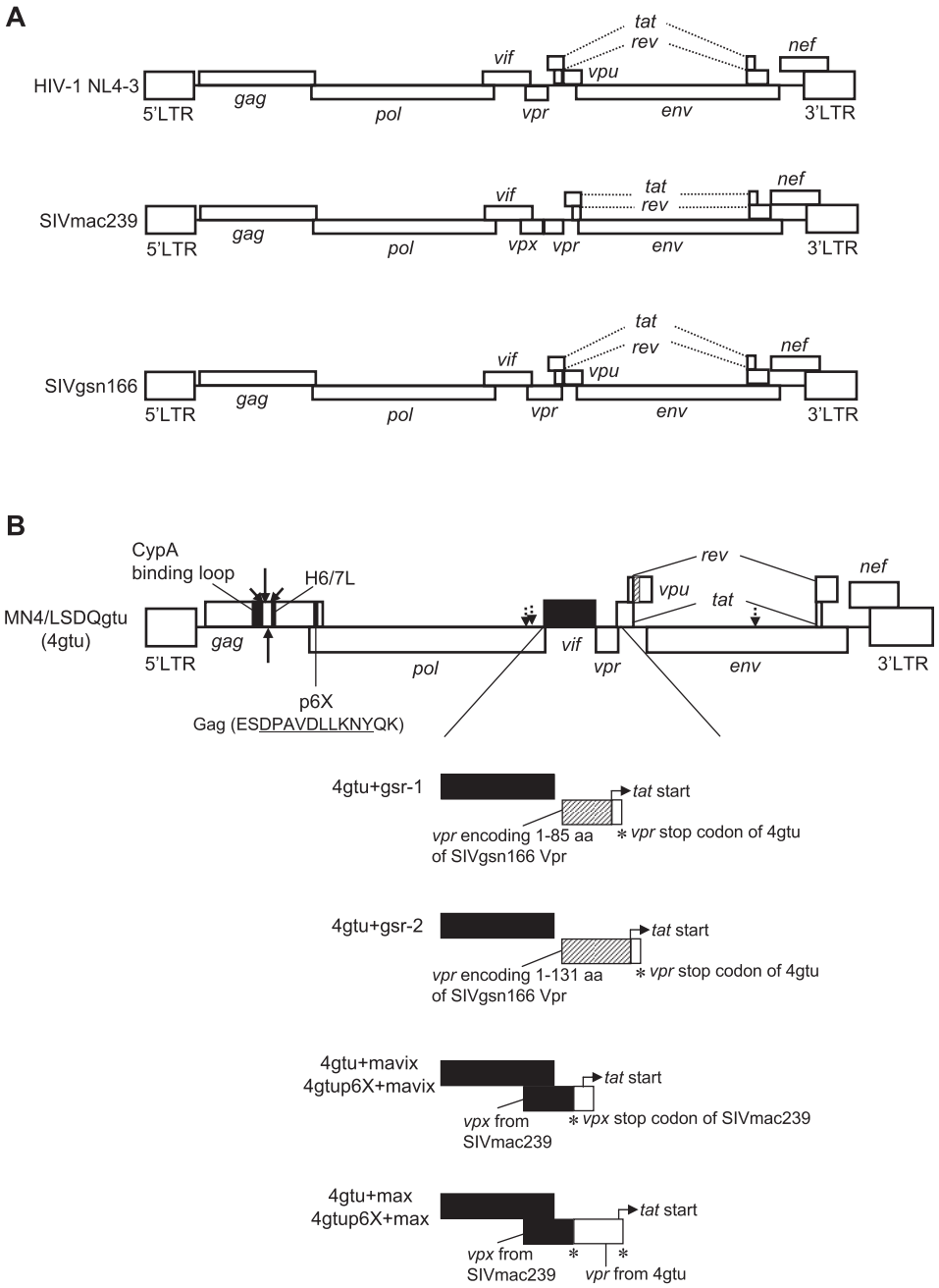

Figure 1. Proviral genome structures of various HIV/SIVs. (A) Genome comparison of HIV-1, SIVmac, and SIVgsn. GenBank accession numbers for HIV-1 NL4-3 (13), SIVmac239 (16), and SIVgsn166 (5) are AF324493, M33262, and AF468659, respectively. (B) Proviral genome structures of MN4/LSDQgtu derivatives. Six variants shown here were constructed from MN4/LSDQgtu as indicated. Clones 4gtup6X+mavix and 4gtup6X+max4 contain the p6X sequence (underlined amino acid sequence derived from SIVmac239 Gag-p6, shown just below the schema) in Gag-p6 in addition to alterations in clones 4gtu+mavix and 4gtu+max. White, black, and diagonal boxes represent sequence from HIV-1 NL4-3, SIVmac239, and SIVgsn166, respectively. Locations of four amino acids in Gag-CA critical for viral replication in rhesus cells (5) are indicated by arrows. Broken arrows show the sites of three adaptive mutations occurred during replication in macaque cells (17-19). CypA, cyclophilin A ; H6/7L, a loop structure between helices 6 and 7 of Gag-CA. For construction of frame-shift mutants of the four accessory genes (*vif*, *vpr*, *vpu*, and *nef*), see MATERIALS AND METHODS.

are functionally replaceable to each other is not determined as yet.

In this study, we constructed several proviral clones carrying *vpr/vpx* gene substitutions from the parental virus MN4/LSDQgtu. We also made accessory gene mutants of MN4/LSDQgtu to determine their necessity for replication in rhesus macaque cells. We report here the growth property in rhesus cells of the newly constructed HIV-1mt clones.

MATERIALS AND METHODS

Plasmid DNAs

Generation of an HIV-1mt clone designated MN4/LSDQgtu has been previously described (5). Substitution of the central genomic region of MN4/LSDQgtu with the sequence containing SIV *vpr/vpx* gene was performed by the overlapping PCR method to generate new proviral clones (4gtu+gsr-1, 4gtu+gsr-2, 4gtu+mavix, and 4gtu+max) as shown in Figure 1. The specific sequence of SIVmac239 in Gag-p6 required for the Vpx-packaging into virions was introduced as previously described (9, 10) into the corresponding region of MN4/LSDQgtu, 4gtu+mavix, and 4gtu+max by the QuikChange site-directed mutagenesis kit (Agilent Technologies Inc.) to generate additional new clones 4gtup6X, 4gtup6X+mavix, and 4gtup6X+max, respectively (Figure 1). Frame-shift (Δ) *vif*, *vpr*, and *nef* mutants of MN4/LSDQgtu was constructed by 2 base pairs-insertion at the *Nsp*V site in *vif*, 4 base pairs-insertion at the *Sal*I site in *vpr*, and 4 base pairs-insertion at the *Xho*I site in *nef*, respectively, using T4 DNA polymerase (TAKARA). Construction of a frame-shift *vpu* mutant of MN4/LSDQgtu was described previously (5).

Cells and virus infection experiments

Human 293T cells (11) were maintained in Eagle's minimal essential containing 10% heat-inactivated fetal bovine serum. Rhesus macaque M1.3S cells (12) were maintained RPMI1640 medium containing 10% heat-inactivated fetal bovine serum and 50 units/ml of recombinant human interleukin-2 (Bio-Rad Laboratories Inc.). Virus stocks for infection experiments were prepared from 293T cells transfected with various proviral clones by the calcium-phosphate co-precipitation method (13), and their virion-associated reverse transcriptase (RT) activities were determined as previously described (14). M1.3S cells were infected with equal virus amounts (RT units), and virus growth was monitored at intervals by RT activity in the culture supernatants.

RESULTS

Our previous study has shown that MN4/LSDQgtu grows similarly well to SIVmac239 in many but not all rhesus PBMC preparations examined (reference 5 and our unpublished data). In fact, resistance of MN4/LSDQgtu to rhesus TRIM5α protein, a potent restriction factor against HIV-1, is not complete (5). Increasing the TRIM5α resistance of MN4/LSDQgtu by extensive modifications of its Gag-capsid (CA) was unsuccessful so far (our unpublished results). Importantly, the functionality of MN4/LSDQgtu Vpr that may contribute to viral growth ability in macaque cells was not analyzed as yet. These considerations in mind, we here focused on constructing and characterizing variant clones of MN4/LSDQgtu with alterations in the central genomic region.

We first inserted the SIVgsn *vpr* or SIVmac *vpx* gene into MN4/LSDQgtu genome. SIVgsn (Figure 1A) is an ancestral virus of HIV-1 (15), and its *vpu* gene was demonstrated to be biologically active in rhesus macaque cells (5). SIVmac (Figure 1A) is a standard virus used for various model studies on HIV-1/AIDS (2). As shown in Figure 1B, while variants 4gtu+gsr-1 and 4gtu+gsr-2 carry sequences from SIVgsn166 *vpr*, the other four clones contain SIVmac239 *vpx* with/without a *gag*-p6 alteration, and with/without MN4/LSDQgtu *vpr*. Viruses of the HIV-2/SIVmac group carry both *vpx* and *vpr* genes (Figure 1A). Using these six clones, we examined the functionality of various *vpr/vpx* genes in different genomic contexts. For a control purpose, we also examined a *gag*-p6 mutant (4gtup6X) and a frame-shift *vpr* mutant along with the other three accessory gene mutants carrying a frame-shift mutation in *vif*, *vpu*, or *nef*.

We comparatively analyzed the growth potentials of various variant clones of MN4/LSDQgtu. Virus samples were prepared from transfected 293T cells, and inoculated into rhesus macaque M1.3S cells. As shown in Figure 2A, two clones encoding SIVgsn Vpr instead of HIV-1 Vpr (4gtu+gsr-1 and 4gtu+gsr-2) did not grow at all or did scarcely. In contrast, 4gtu+mavix carrying SIVmac239 *vpx* grew considerably relative to the parental clone MN4/LSDQgtu (Figure 2B). Similar result was obtained

for 4gtu+max (Figure 2C), which encodes two related proteins Vpx (from SIVmac239) and Vpr (from HIV-1 NL4-3) as viruses of HIV-2/SIVmac group (Figure 1A). Because clones 4gtu+mavix and 4gtu+max do not have sequence important for virion-packaging of Vpx (9, 10), it was possible that Vpx synthesized in cells were inefficiently or not incorporated into virions. Therefore, 4gtup6X+mavix and 4gtup6X+max carrying the p6X sequence in *gag*-p6 (Figure 1B) were constructed, and viruses derived from these clones were inoculated into M1.3S cells. As shown in Figure 2D, no enhancing effects of the *vpx* insertions on virus replication were noted. Finally, frame-shift accessory gene mutants (Δ mutants) of MN4/LSDQgtu were evaluated for their growth properties in M1.3S cells. Expectedly, ΔVif did not grow at all in M1.3S cells as reported for SIVmac239 ΔVif (12) (Figure 2E). Interestingly, while a slightly and modestly negative effect was seen for ΔNef and ΔVpu, respectively, ΔVpr grew clearly more poorly than parental MN4/LSDQgtu (Figure 2E).

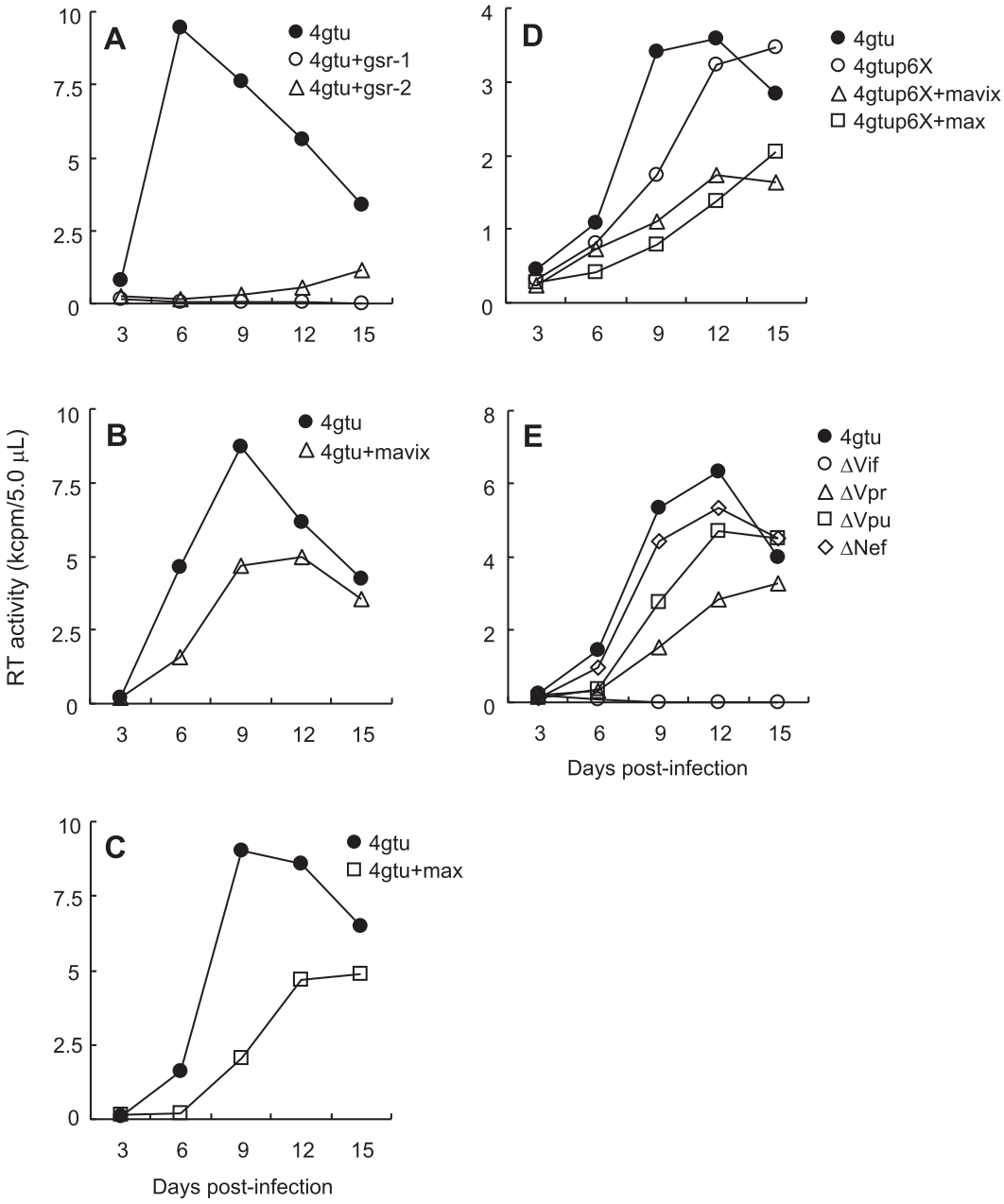

Figure 2. Growth properties of various HIV-1 derivative viruses in rhesus M1.3S cells. Input viruses were prepared from 293T cells transfected with proviral clones indicated, and inoculated into rhesus M1.3S cells. Virus growth was monitored at intervals by RT activity in the culture supernatants. Infection experiments were repeated, and the representative data are presented. For (A), (B), (C), and (E) experiments, 1×10^6 M1.3S cells were infected with equal virus amounts (5×10^6 RT units). For (D), 2×10^5 M1.3S cells were infected with equal virus amounts (5×10^5 RT units).

DISCUSSION

The functional importance of HIV-1 accessory proteins for virus replication in macaque cells is not yet determined to the best of our knowledge. In this study, eleven new proviral clones with genomic modifications were constructed from HIV-1mt MN4/LSDQgtu (Figures 1 and 2) to have a clue to understand the functional role of HIV-1 Vpr in macaque cells. For comparison, four accessory protein mutants (ΔVif, ΔVpr, ΔVpu, and ΔNef) were included in our study here. However, HIV-1 Vif can not be evaluated, since SIVmac Vif is essential for virus replication in macaque cells (Figure 1B) (3, 4). Various clones constructed were comparatively assessed for their growth potentials in rhesus M1.3S cells (Figure 2). A key finding in this study, as judged by previously published observations (6), is that Vpr appears to be more important for virus replication in macaque cells than Vpu and Nef (Figure 2). There may be some anti-HIV-1 factor(s) counteracted by HIV-1 Vpr in rhesus macaques.

All MN4/LSDQgtu derivative clones that contain the *vpr/vpx* from SIVs in place of the original *vpr* exhibited more or less retarded growth phenotype in M1.3S cells (Figure 2). Of note, clones 4gtu+mavix and 4gtu+max showed the ΔVpr phenotype. The reasons for these results are presently unclear. However, considering that each genome modification (Figure 1) gave negative effects to a different extent, it is evident that appropriate *vpr/vpx* in the right genome context is required for efficient virus replication in macaque cells. Further study is necessary to confer better replication ability in macaque cells on the HIV-1mt clone already established.

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REFERENCES

1. Nomaguchi M, Doi N, Kamada K, Adachi A : Species barrier of HIV-1 and its jumping by virus engineering. Rev Med Virol 18 : 261-275, 2008
2. Nomaguchi M, Doi N, Fujiwara S, Adachi A : Macaque-tropic HIV-1 derivatives : a novel experimental approach to understand viral replication and evolution *in vivo.* In : Chang T Y-L, ed. HIV-host interactions. InTech, Rijeka, Croatia, 2011, pp.325-348
3. Hatziioannou T, Princiotta M, Piatak M Jr, Yuan F, Zhang F, Lifson JD, Bieniasz PD : Generation of simian-tropic HIV-1 by restriction factor evasion. Science 314 : 95, 2006
4. Kamada K, Igarashi T, Martin MA, Khamsri B, Hatcho K, Yamashita T, Fujita M, Uchiyama T, Adachi A : Generation of HIV-1 derivatives that productively infect macaque monkey lymphoid cells. Proc Natl Acad Sci USA 103 : 16959-16964, 2006
5. Nomaguchi M, Yokoyama M, Kono K, Nakayama EE, Shioda T, Doi N, Fujiwara S, Saito A, Akari H, Miyakawa K, Ryo A, Ode H, Iwatani Y, Miura T, Igarashi T, Sato H, Adachi A : Generation of rhesus macaque-tropic HIV-1 clones that are resistant to major anti-HIV-1 restriction factors. J Virol 87 : 11447-11461, 2013
6. Fujita M, Otsuka M, Nomaguchi M, Adachi A : Multifaceted activity of HIV Vpr/Vpx proteins : the current view of their virological functions. Rev Med Virol 20 : 68-76, 2010
7. Schaller T, Bauby H, Hue S, Malim MH, Goujon C : New insights into an X-traordinary viral protein. Front Microbiol 5 : 126, 2014
8. Guenzel C, Herate C, Benichou S : HIV-1Vpr – a still "enigmatic multitasker". Front Microbiol 5 : 127, 2014
9. Sunseri N, O'Brien M, Bhardwaj N, Landau NR : Human immunodeficiency virus type 1 modified to package simian immunodeficiency virus Vpx efficiently infects macrophages and dendritic cells. J Virol 85 : 6263-6274, 2011
10. Bitzegeio J, Sampias M, Bieniasz PD, Hatziioannou T : Adaptation to the interferon-induced antiviral state by human and simian immunodeficiency viruses. J Virol 87 : 3549-3560, 2013
11. Lebkowski JS, Clancy S, Calos MP : Simian virus 40 replication in adenovirus-transformed human cells antagonizes gene expression. Nature 317 : 169-171, 1985
12. Doi N, Fujiwara S, Adachi A, Nomaguchi M : Rhesus M1.3S cells suitable for biological evaluation of macaque-tropic HIV/SIV clones. Front Microbiol 2 : 115, 2011
13. Adachi A, Gendelman HE, Koenig S, Folks T,

Willey R, Rabson A, Martin MA : Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone. J Virol 59 : 184-291, 1986
14. Willey RL, Smith DH, Lasky LA, Theodore TS, Earl PL, Moss B, Capon DJ, Martin MA : In vitro mutagenesis identifies a region within the envelope gene of the human immunodeficiency virus that is critical for infectivity. J Virol 62 : 139-147, 1988
15. Kirchhoff F : Is the high virulence of HIV-1 an unfortunate coincidence of primate lentiviral evolution? Nat Rev Microbiol 7 : 467-476, 2009
16. Shibata R, Kawamura M, Sakai H, Hayami M, Ishimoto A, Adachi A : Generation of a chimeric human and simian immunodeficiency virus infectious to monkey peripheral blood mononuclear cells. J Virol 65 : 3514-3520, 1991
17. Nomaguchi M, Yokoyama M, Kono K, Nakayama EE, Shioda T, Saito A, Akari H, Yasutomi Y, Matano T, Sato H, Adachi A : Gag-CA Q110D mutation elicits TRM5-independent enhancement of HIV-1mt replication. Microbes Infect 15 : 56-65, 2013
18. Nomaguchi M, Doi N, Fujiwara S, Saito A, Akari H, Nakayama EE, Shioda T, Yokoyama M, Sato H, Adachi A : Systemic biological analysis of the mutations in two distinct HIV-1mt genomes occurred during replication in macaque cells. Microbes Infect 15 : 319-328, 2013
19. Nomaguchi M, Miyake A, Doi N, Fujiwara S, Miyazaki Y, Tsunetsugu-Yokota Y, Yokoyama M, Sato H, Masuda T, Adachi A : Natural single-nucleotide polymorphisms in the 3' region of the HIV-1 *pol* gene modulate viral replication ability. J Virol 88 : 4145-4160, 2014