

ORIGINAL**Generation and characterization of new CCR5-tropic HIV-1rmt clones**Naoya Doi¹, Yosuke Sakai¹, Akio Adachi^{2*}, and Masako Nomaguchi^{1*}¹Department of Microbiology, Tokushima University Graduate School of Medical Science, Tokushima, Japan, ²Tokushima University, Tokushima, Japan

Abstract : To develop effective non-human primate models for coping with numerous HIV-1/AIDS studies, rhesus macaque-tropic HIV-1 (HIV-1rmt) clones with a variety of biological properties are required. Such clones, if available, are powerful tools to experimentally elucidate HIV-1 replication and pathogenicity in host individuals, and also to develop anti-HIV-1 drugs/vaccines. However, only limited numbers of HIV-1rmt clones have been currently reported. In the present study, we generated new HIV-1rmt clones carrying various CCR5-tropic *env* (envelope) genes by standard recombinant DNA and intracellular homologous recombination techniques. Resultant virus clones contain the *env* sequences derived from an AIDS-inducible laboratory or two clinically isolated viral strains. We further constructed their variant clones bearing N160K, S304G, or G310R mutation in Env that potentially can change the viruses to better grow. Newly generated clones were analyzed for their virological properties such as Env expression, single-cycle infectivity, and multi-cycle replication ability. Out of a number of new clones examined, two were found to grow better in macaque cells than the previously constructed clone used for comparison. Our study described here constitutes the initial and essential step towards obtaining CCR5-tropic HIV-1rmt clones useful for various basic and clinical research projects on infected individuals. *J. Med. Invest.* 64 : 272-279, August, 2017

Keywords : HIV-1rmt, CCR5-tropic, Env**INTRODUCTION**

Infection of rhesus monkeys with primate immunodeficiency viruses is considered to be best suited for *in vivo* model studies of HIV-1/AIDS (1). Extensive efforts to construct a novel class of HIV-1 clones that infect rhesus macaques and induce AIDS, i.e., pathogenic HIV-1rmt clones, have been made to date to establish an HIV-1 infection model system of rhesus macaques (1, 2). As ideal input viruses for the infection studies, HIV-1rmt clones with CCR5-tropic Env is required, since CCR5-tropic HIV-1 is strongly associated with virus transmission between individuals, and viral replication in an acute-infection phase (1). While some promising proviral clones which replicate fairly well in rhesus macaques and/or in primary rhesus peripheral blood mononuclear cells are presently available (1, 2, and our unpublished results), no HIV-1 rmt clones pathogenic for the animals have been reported as yet.

There have been two major approaches to generate pathogenic HIV-1rmt clones : (i) *in vitro* virus-construction by recombinant DNA technology and (ii) virus adaptation in cells/individuals (3,4). In stepwise processes of our constructing various HIV-1 clones infectious for rhesus macaque cells, we employed (i), (ii) and/or both strategies with the aid of computational predictive science (1, 3-7). During virus adaptation studies using macaque cells, we consistently observed that genetically engineered HIV-1 clones, macaque cell-tropic but with poor replication ability in macaque cells, readily acquired adaptive mutations in the *env* gene, and that the growth-enhancing effects were not compatible between CXCR4-tropic and CCR5-tropic viruses (5, 7). Thus, we could assume that

HIV-1 frequently adapts by Env-mutations to grow efficiently within macaque cell environments, and that the effects of the acquired mutations on viral growth may be specific for the HIV-1 clones.

In this study, in an attempt to improve the replication potentials of our HIV-1rmt clones, we newly generated various CCR5-tropic proviral clones, and also constructed their variants carrying N 160K, S304G, or G310R mutation in Env that were reported to have growth-promoting effects (7, 8). These new clones were analyzed for their single-cycle infectivity and multi-cycle replication ability. We have demonstrated here that two proviral clones grow better than a standard CCR5-tropic HIV-1rmt clone designated MN5/LSDQgtu (5gtu) (1, 9, 10). We have also shown, together with our previous results, that growth-promoting mutations in HIV-1 Env exert their positive effects in an Env-dependent manner.

MATERIALS AND METHODS*Proviral molecular clones*

Construction and characterization of a CCR5-tropic HIV-1rmt clone designated MN5/LSDQgtu (5gtu) have been previously described (1, 9, 10). New CCR5-tropic proviral clones were generated as outlined in Fig. 1. Mutations were introduced into the proviral clones by the QuickChange site-directed mutagenesis kit (Agilent Technologies Inc) as previously described (1, 11).

Nucleotide sequences

Nucleotide sequences were determined by ABI Genetic Analyzer 3130xl (Thermo Fisher Scientific).

Cells

Adherent cell lines, a human kidney 293T and a reporter cell line TZM-bl, were cultured in Eagle's minimal essential containing 10%

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heat-inactivated fetal bovine serum (10). A HeLa-derived cell line designated TZM-bl carries a luciferase gene in its genome driven by the HIV-1 long terminal repeat, and is widely used for HIV/SIV infectivity studies. M1.3S, a rhesus macaque lymphocyte cell line, was maintained in RPMI1640 medium containing 10% heat-inactivated fetal bovine serum and 50 units/ml of recombinant human interleukin-2 (Bio-Rad Laboratories Inc) (1, 10).

Transfection

Virus stocks for infection experiments were prepared from 293T cells transfected with various proviral clones by the calcium-phosphate co-precipitation method (1, 12). For determination of virus amounts, virion-associated reverse transcriptase (RT) activities were determined as previously described (1, 13).

Western blot analysis

Env proteins expressed in transfected cells and those in virions produced from transfected cells were examined by the Western blot technique as fully described previously (11). Virions were partially purified and concentrated by ultracentrifugation similarly as previously described (14, 15).

Single-cycle infectivity

Equal RT units of virus samples obtained from transfections were inoculated into TZM-bl cells, and on day 1 post-infection, cells were lysed with 1 x Cell Culture Lysis Reagent (Promega Corporation) and subjected to a standard 96 well plate luciferase assay (Promega Corporation) as instructed by the manufacturer (10). Luciferase activity in the cell lysates relative to that of 5gtu was determined, and considered as viral single-cycle infectivity.

Multi-cycle replication ability

Equal RT units of virus samples obtained from transfections were inoculated into M1.3S cells, and virus growth kinetics were determined by monitoring RT activity in the culture supernatants every 3 days as previously described (1, 10).

RESULTS

Generation of new HIV-1rmt clones

To our knowledge, pMN4/LSDQgtu is currently the best HIV-1 molecular clone to generate infectious viruses that replicate efficiently in rhesus macaque cells (1, 16). MN4/LSDQgtu is CXCR4-tropic and exhibits resistance to major intrinsic HIV-1 restriction factors in macaque cells, such as APOBEC3 proteins, TRIM5 proteins, and tetherin. In fact, the CXCR4-tropic virus derived from this molecular clone constantly grew to a considerable level in rhesus macaques (our unpublished data). However, any AIDS-related symptoms in rhesus macaques infected with MN4/LSDQgtu were not observed so far (our unpublished data). In addition, CCR5-tropic viruses are required to mimic well the disease progression in individuals (1). We, therefore, started generating a number of new CCR5-tropic HIV-1rmt clones to increase varieties of input viruses for rhesus experiments. In order to obtain new HIV-1rmt proviral clones, we used *in vitro* (DNAs) and *in vivo* (cells) methods as illustrated in Fig. 1. Briefly, *env* gene regions derived from a molecular clone (pSHIV_{AD8-EH}) of a pathogenic virus (17) and from two clinical isolates (CI) were inserted into 5gtu backbone by both standard DNA recombinant and intracellular homologous recombination (18) techniques. As a result, we successfully constructed five new clones designated gtu+AD8, gtu+CI1, gtu+A4CI1, gtu+CI3, and gtu+B3AD8 that produce infectious viruses upon transfection (Fig. 1). Clones gtu+AD8/gtu+B3AD8 and gtu+CI1/gtu+A4CI1/gtu+CI3 carry the *env* gene region derived from pSHIV_{AD8-EH} and from one of the two clinical isolates,

respectively.

Because gtu+AD8 and gtu+B3AD8 have the *env* gene region from pSHIV_{AD8-EH}, and the gtu+B3AD8 was generated by the intracellular recombination method, we were interested in potential sequence differences in the two clones. By the same reason, *env* gene regions of gtu+CI1 and gtu+A4CI1 were compared, and considerable sequence differences were noted (the sequences will be described elsewhere) (Table 1). Fig. 2 shows the sequence alignment of 5gtu, gtu+AD8, and gtu+B3AD8. Importantly, we found a 19-nucleotide deletion only in gtu+B3AD8 *vpu* (Fig. 2A and Table 1), which is very likely to inactivate the Vpu activity in cells by abolishing its expression or by expressing a defective protein. We also found 7 amino acid differences between Env proteins of gtu+AD8 and gtu+B3AD8 (Fig. 2B and Table 1). Finally, as expected, there were some amino acid differences among 5gtu, gtu+AD8, and gtu+B3AD8 (Fig. 2B). Parental clone 5gtu has the growth-enhancing N160K and S304G residues but not gtu+AD8 and gtu+B3AD8 (Table 1).

Virological characterization of HIV-1rmt clones

We then confirmed the Env expression of the five new CCR5-tropic clones in transfected 293T cells by Western blot analysis. HIV-1 Env is expressed in cells as a precursor gp160, which is composed of gp120 and gp41. Gp160 is cleaved at the final virus replication step (assembly/budding/maturation) to generate mature products gp120 (surface) and gp41 (transmembrane). Env-gp120 and Env-gp41 incorporated into virions subsequently work for the receptor/co-receptor binding and the virus/cell fusion in the next round of the infection cycle, respectively. As shown in Fig. 3, although heterogeneities were observed between clones in terms of the proteins size and amount, the five clones did express Env proteins both in cells and virions. While clones gtu+CI1 and gtu+A4CI1 gave a relatively uniform Env in virions, clones gtu+AD8 and gtu+B3AD8 particularly produced heterogeneous Env proteins at a relatively higher level.

We next comparatively determined replication potentials of the new clones relative to that of 5gtu. As shown in Fig. 4A, viral single-cycle infectivity in reporter TZM-bl cells was assessed for gtu+AD8, gtu+B3AD8, gtu+CI1, gtu+A4CI1, and gtu+CI3 (Fig. 1). All clones tested were found to exhibit significantly lower infectivity than that of 5gtu. However, of note, gtu+AD8 showed higher infectivity than gtu+B3AD8 ($P < 0.005$). We asked whether the amino acid substitutions, N160K and S304G (Fig. 2) that were reported to enhance viral infectivity (7, 8), improve replication potentials of the above five clones. Clones gtu+CI1 and gtu+A4CI1 natively have the G304 residue (Table 1). As shown in Figure 4B and 4C, no significant augmentation of viral infectivity by the mutations was observed.

Finally, we determined viral growth kinetics in macaque M1.3S cells of the five clones in Fig. 4A in comparison with 5gtu to see if there is any difference between results on single-cycle and multi-cycle infectivities. Notably, as shown in Fig. 5, gtu+CI1 and gtu+A4CI1 grew better than 5gtu as well as the other three clones (gtu+AD8, gtu+B3AD8, and gtu+CI3), in contrast to the results on single-cycle infectivity (Fig. 4A). The three clones (gtu+AD8, gtu+B3AD8, and gtu+CI3) grew more poorly than 5gtu in agreement with the single-cycle infectivity data (Fig. 4A). There are two modes of virus spreading infection, cell-free infection and cell-to-cell infection. The former is the process that infectious virions released into culture supernatants from cells are transmitted to uninfected cells. The latter is the mode that viruses spread via cell to cell interaction without infectious virions release. Therefore, even if infection efficiency of initial input virus is quite low, the virus with a higher capability of cell-to-cell infection may exhibit an enhanced growth ability in the multi-cycle replication analysis. The remarkable results on gtu+CI1 and gtu+A4CI1 could be explained by their

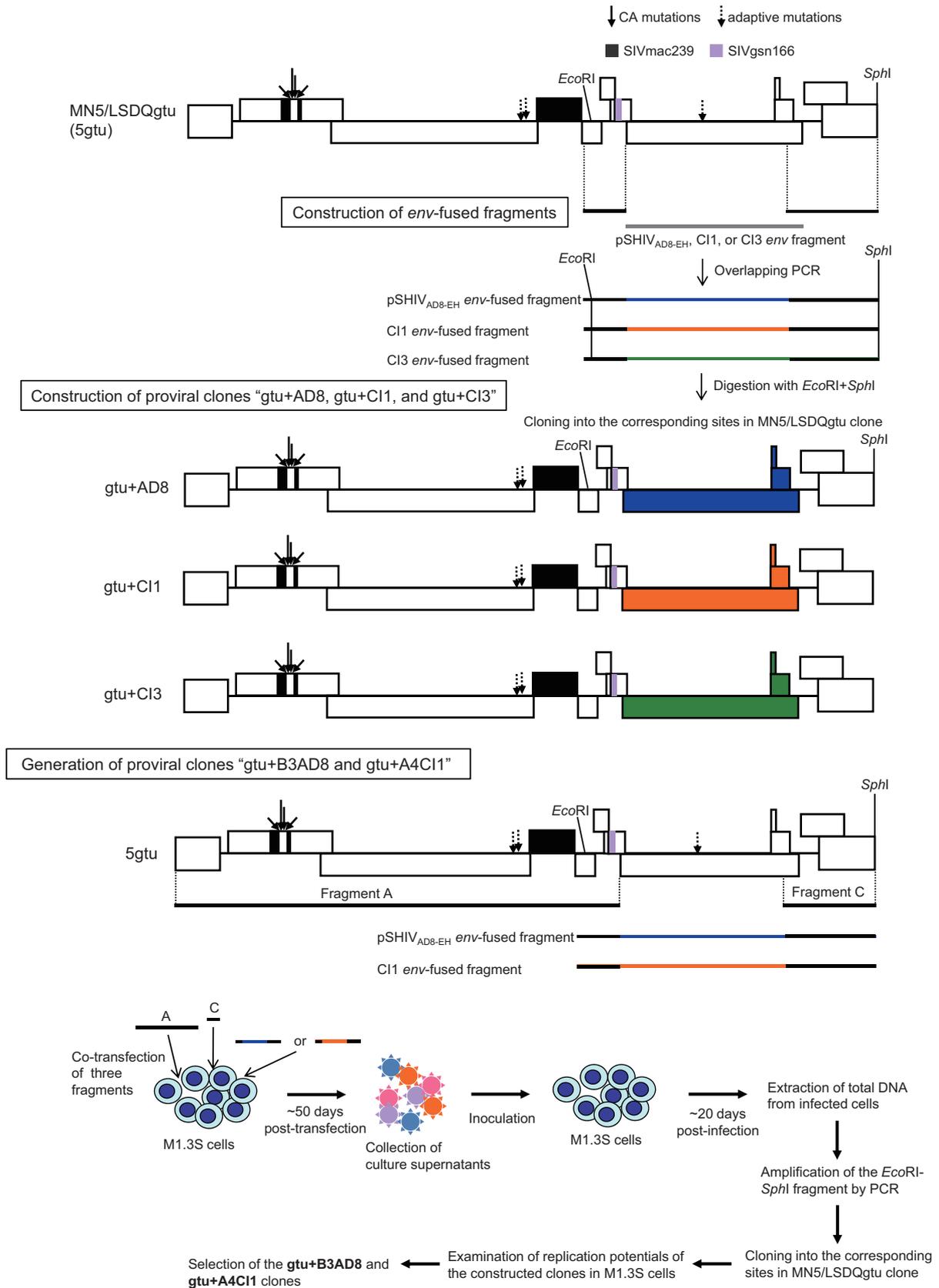


Figure 1 Generation of new full-length proviral clones. Using pMN5/LSDQgtu (5gtu) (1, 9, 10), pSHIV_{AD8-EH} (17), and two *env* clones from clinical isolates (CI) (unpublished), five proviral clones designated gtu+AD8, gtu+B3AD8, gtu+C11, gtu+A4C11, and gtu+C13 were generated as detailed in this figure. See reference 18 for the intracellular homologous recombination used to generate gtu+B3AD8 and gtu+A4C11. Locations of Gag-capsid (CA) mutations (1) and adaptive mutations in Pol (two mutations)/Env-S304G (7) are represented by arrows and dotted arrows, respectively. Regions derived from SIVmac239 (simian immunodeficiency virus clone 239 isolated from a rhesus macaque) and SIVgsn166 (SIV clone 166 from a greater spot-nosed monkey) are indicated by black and light purple, respectively.

accelerated cell-to-cell spread ability mediated by their Env proteins. Efficient cell-to-cell infection may have advantages in virus spread *in vivo*, because this mode of infection is in favor of evasion from host immune system. Apparent differences in single-/multi-cycle infectivity observed among gtu+AD8, gtu+B3AD8, and gtu+CI3 (compare the results in Fig. 4A and Fig. 5) may also be explainable with their different cell-to-cell spread abilities. We further examined the effect of G310R mutation in Env (Fig. 2) on the growth kinetics of gtu+AD8, gtu+B3AD8, gtu+CI1, gtu+A4CI1, and gtu+CI3 (Fig. 1). We recently showed that a mutant virus carrying a growth-enhancing mutation G310R exhibited distinct sensitivity to CCR5 antagonist TAK-779 from that of a mutant virus bearing S304G (19). Fig. 6 shows the results for gtu+B3AD8/gtu+CI3 and their mutant viruses as representatives. Neither of the mutants grew better than parental viruses like the other three mutants.

DISCUSSION

In this study, in order to increase viral replication potential and variation for infections of rhesus macaques, we generated five new

CCR5-tropic proviral clones (gtu+AD8, gtu+B3AD8, gtu+CI1, gtu+A4CI1, and gtu+CI3), and also mutant clones derived from them. From these, we successfully identified two proviral clones (gtu+CI1 and gtu+A4CI1) that grow better in macaque M1.3S cells than 5gtu, the best CCR5-tropic HIV-1rmt currently available (1, 9, 10). Proviral clones designated gtu+CI1 and gtu+A4CI1 showed high replication potentials relative to that of the standard clone 5gtu (Fig. 5) despite their low single-cycle infectivity relative to that of 5gtu (Fig. 4A). The unique and neat Env expression pattern observed for the two clones (Fig. 3) is noteworthy. Env-gp120 proteins of gtu+CI1 and gtu+A4CI1 may be proper for highly infectious virions. Biological and molecular bases for high replication potentials of gtu+CI1 and gtu+A4CI1 clones need to be clarified. In contrast, gtu+CI3 display a relatively low level of Env-gp120 content in the virion, and this may be responsible for the lower replication ability compared to that of the other R5-tropic HIV-1rmt clones. But, for now, we cannot exclude the possibility that the observation by the immunoblotting is due to the antibody affinity to each Env protein of HIV-1rmt clones. On the other hand, the number of HIV-1 Env trimers incorporated into virions is unexpectedly small, and suitable numbers are different for the production of infectious virions among HIV-1 strains (20). A relatively

Table 1 Summary of HIV-1rmt Env proteins in this study

HIV-1rmt clones	Virus origin of CCR5-tropic Env proteins	Number of amino acids in Env proteins	Comparison of amino acid sequences of Env proteins*		Amino acids at the sites of growth-promoting mutations			Comments
			Identity	Similarity	160	304	310	
MN5/LSDQgtu	HIV-1 _{SF162}	847			K	G	G	
gtu+AD8	SHIV _{AD8-EH}	850	87%	97%	N	S	G	A 19-nucleotides deletion in the <i>vpu</i> gene of gtu+B3AD8 relative to that of gtu+AD8 is found. Seven amino acid differences are present in the two Env proteins.
gtu+B3AD8		850	86%	97%	N	S	G	
gtu+CI1	Clinical isolate	876	77%	93%	N	G	G	Seven amino acid differences are noted in the Tat/Rev/Env/Nef proteins of the two clones.
gtu+A4CI1		876	77%	93%	N	G	G	
gtu+CI3	Clinical isolate	869	71%	91%	N	S	G	

*Identity and similarity for Env amino acids relative to those of MN5/LSDQgtu as analyzed by Genetics version 11 are shown.

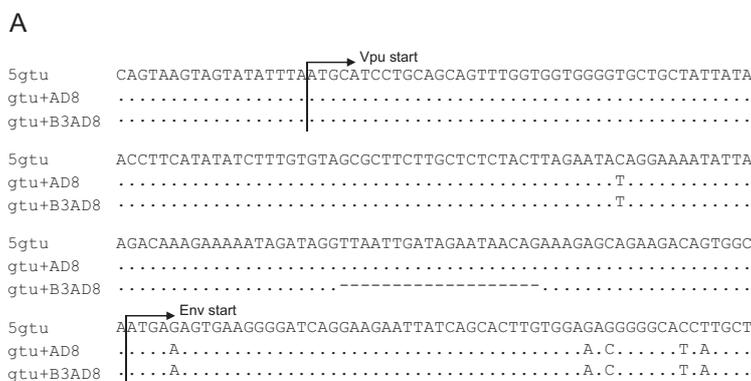


Figure 2 Sequence alignment of 5gtu, gtu+AD8, and gtu+B3AD8. (A) Nucleotide sequences of the *vpu-env* overlapping region. Dots and hyphens indicate identical nucleotides and nucleotide gaps, respectively. (B) Amino acid sequences of Env-signal peptide, Env-gp120, and Env-gp41. Dots and hyphens show identical amino acids and amino acid gaps, respectively. Env-gp120 domains (C1 to V5) are indicated above the sequences. Arrows represent different amino acids in Env proteins of gtu+AD8 and gtu+B3AD8. Sites for introduction of N160K, S304G, and G310R mutations are indicated (see text).

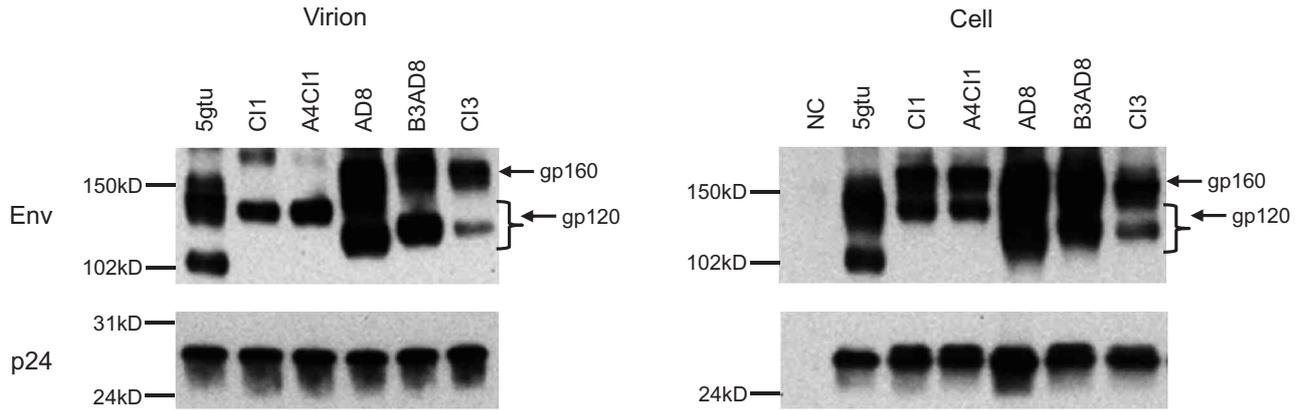


Figure 3 Western blot analysis of Env proteins in virions and cells. Proviral clones indicated were transfected into 293T cells, and on day 2 post-transfection, virion and cell lysates were prepared for Western blotting using anti-Env (#ADP409) and anti-Gag-p24 (#3537) antibodies. For detection, lysates containing 10 ng of p24 (Virion Env), 2 ng of p24 (Virion p24), and 1 ng of p24 (Cell Env and Cell p24) were used. NC, negative control (mock-transfected).

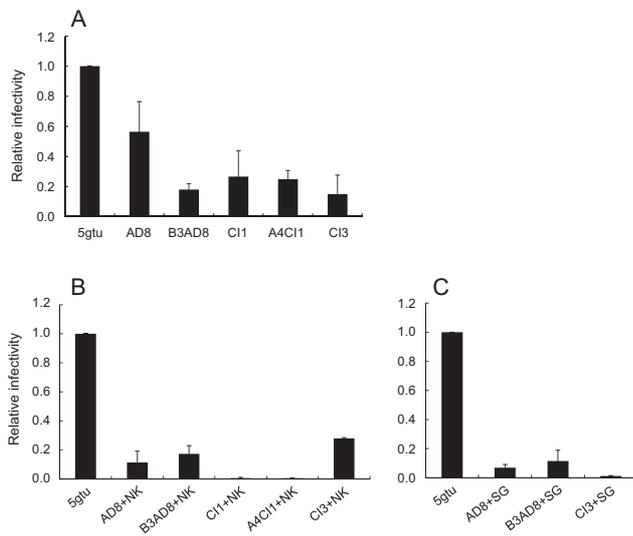


Figure 4 Single-cycle infectivity of new proviral clones. Virus samples were prepared from transfected 293T cells, and equal RT units (2×10^4 or 1×10^5) were inoculated into TZM-bl cells (5×10^3) for luciferase assays. Results are separately shown for new proviral clones (A) and their mutant clones (B and C). Luciferase activity relative to that by 5gtu is shown. Mean values \pm SD from at least three independent experiments for each panel are shown. Significance relative to 5gtu was determined by the Student's *t*-test (for all samples, $P < 0.005$). Clone names are indicated at the bottom. NK, N160K; SG, S304G.

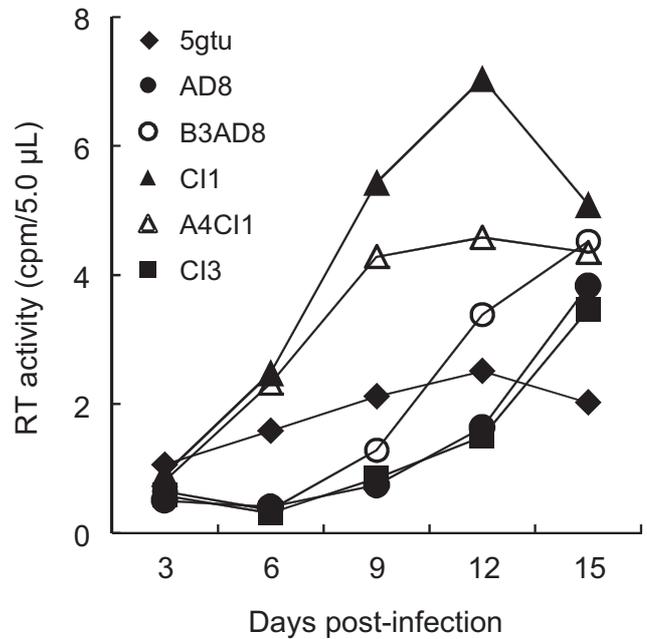


Figure 5 Growth kinetics of new viral clones in M1.3S cells. Viruses were prepared from 293T cells transfected with indicated proviral clones, and equal RT units (5×10^5) were inoculated into M1.3S cells (2×10^5). RT activity in the culture supernatants was determined every 3 days post-infection as shown. The experiment was repeated three times with consistent results.

high expression levels of Env observed for gtu+AD8 and gtu+B3AD8 can adversely affect the production of infectious virions and the subsequent replication process.

One of important points in the present study is regarding the receptive ability of HIV-1 Env proteins in amino acid substitutions for high functional activity. The N160K substitution was shown to influence viral infectivity positively (8) or negatively (20), which depends on the clones used. We previously reported that the S304G substitution enhances specifically the growth ability of CCR5-tropic NL-DT562 but not that of CXCR4-tropic NL-DT5R (7). Our results in this study are consistent with these results with respect to

the Env-dependency. The G310R mutation, another growth-enhancing adaptive mutation found to occur in NL-DT562 genome (7), impeded or abolished viral single-cycle infectivity of CCR5-tropic clones other than NL-DT562 (Fig. 6). In conclusion, we demonstrate here that the enhancing effects of Env mutations are applicable to some specific Env. Care must be taken if we are to generate better growing HIV-1rmt clones by Env-mutations.

Our goal would be the establishment of HIV-1rmt clone(s) that replicates well to a level sufficient enough for diversification of viruses to persist and cause AIDS/AIDS-related complex in

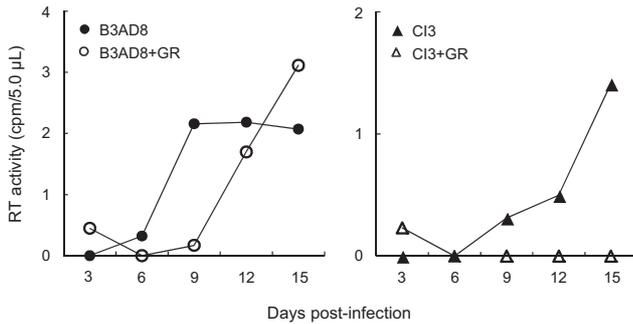


Figure 6 Growth kinetics of parental and mutant clones in M1.3S cells. M1.3S cells were infected with indicated viruses and monitored for virus growth as described in the legend to Figure 5. GR, G310R.

infected rhesus macaques. We have previously experienced that viruses with low replication potentials in macaque cells can frequently mutate to highly replicative viruses during numerous infection cycles in macaque cells (1, 3-7). This empirical “cell-adaptation” method is a powerful tool to generate virus variants with high replication potentials under some conditions. Indeed, based on the clones described here, we have now obtained some proviral clones by cell-adaptation promising for future use. Studies in this direction are in progress in our laboratory.

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CONFLICT OF INTEREST

The authors declare that no competing interests exist.

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