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Synonymous mutations in stem-loop III of Rev responsive elements enhance HIV-1 replication impaired by primary mutations for resistance to enfuvirtide

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ABSTRACT

Primary mutations in HIV-1 that are directly involved in the resistance to enfuvirtide have been well documented. However, secondary mutations that are associated with primary mutations and contribute little to the resistance still remain to be elucidated. This study reveals that synonymous mutations at gp41 Q41 (CAG to CAA) or L44 (UUG to CUG) act as secondary mutations. Complementary mutations in the nucleotide level are located in the Rev responsive element (RRE) of the HIV-1 RNA-genome and maintain the replication kinetics of HIV-1 through increasing the structural stability of stem-loop III in the RRE. Therefore, synonymous mutations in the gp41/RRE sequence improve the viral replication impaired by the primary mutations and play a key role as secondary (complementary) mutations.

1. Introduction

Enfuvirtide (T-20), an HIV-1 fusion inhibitor which has been approved for the treatment of HIV-1 infected patients, successfully suppresses the replication of HIV-1 even in strains resistant to various reverse transcriptase and protease inhibitors (Fung and Guo, 2004; Lalezari et al., 2003; Lazzarin et al., 2003; Manfredi and Sabbatani, 2006). However, HIV-1 variants resistant to T-20 have emerged after prolonged T-20 therapy (Lu et al., 2006; Marcelin et al., 2004; Sista et al., 2004; Wei et al., 2002). The majority of resistant variants develop primary mutations to T-20, including V38A and N43D, in the consensus sequence, GIVQQQNNLL (DIVQQQNNLL for NL4-3) motif of the gp41 within amino acid positions 36–45 (Cabrera et al., 2006; Fikkert et al., 2002; Lu et al., 2006; Marcelin et al., 2004; Menzo et al., 2004; Mink et al., 2005; Poveda et al., 2002; Sista et al., 2004; Su et al., 2006; Wei et al., 2002). Few studies have so far addressed secondary mutations for resistance to T-20.

Primary and secondary mutations were recently well-defined for C34, a peptide derived from the gp41 carboxyl terminus heptad repeat (C-HR) in vitro (Nameki et al., 2005). A30V and D36G in the gp41 act as secondary mutations which enhance the replication kinetics impaired by primary mutations. Interestingly, these mutations are complementarily located in stem-loop IIA and C (Fig. 1) of the Rev responsive element (RRE) which is an essential RNA structure for transporting non- and singly spliced viral RNA to the cytoplasm from the nucleus (Olsen et al., 1990; Zapp and Green, 1989). Most recently, T18A and V38A complementarily located in the stem IIA of the RRE has been detected in clinical isolates treated with T-20 (Svicher et al., 2008). Co-presence of T18A and V38A is associated with high level of viral load. These data support our recent in vitro results that these secondary mutations in stem II maintain HIV-1 replication through substitutions at the nucleotide as well as the amino acid level (Nameki et al., 2005). This study analyzed the nucleotide sequences of T-20 resistant variants deposited in the GenBank (http://www.ncbi.nlm.nih.gov/Genbank) and revealed that synonymous mutations in stem-loop III of the RRE maintain HIV-1 replication impaired by N43D, one of primary mutations for T-20 resistance.

2. Materials and methods

2.1. Antiviral agents and cells

T-20 was synthesized as described previously (Otaka et al., 2002). 2′,3′-Dideoxycytidine (ddC) was purchased from Sigma (St.

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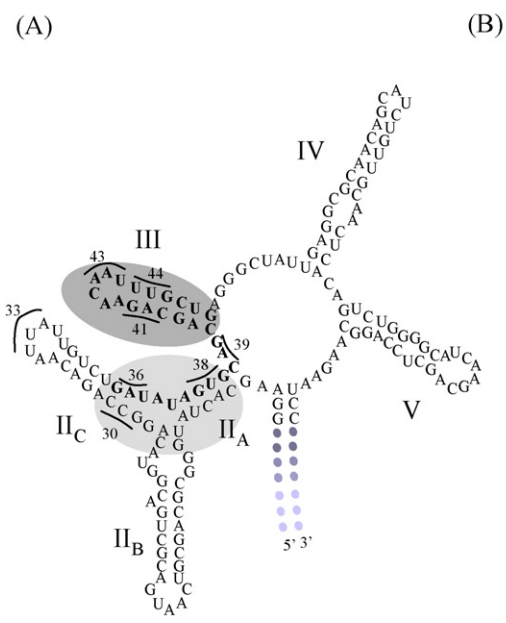
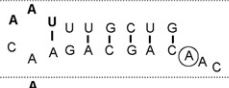
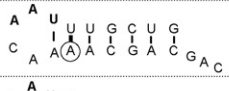
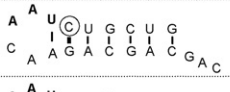
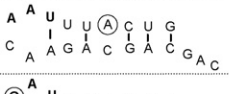
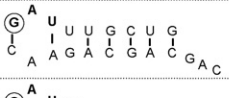
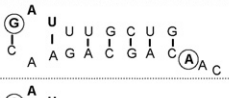
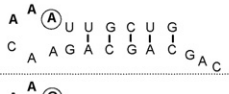
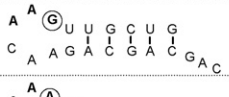
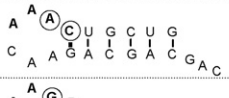
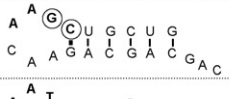
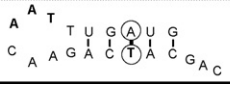


(B)

Mutations: Amino acid	Mutations: Nucleotide	Secondary structure of stem loop III	ΔG (kcal/mol)
None (WT: NL4-3)	None		- 6.5
Q39Q	CAG → CA**A**		- 6.8
Q41Q	CAG → CA**A**		- 6.4
L44L	UUG → **C**UG		- 9.1
L44L	UUG → **U**U**A**		0.1
N43D	AAU → **G**AU		- 6.5
N43D L44L	AAU UUG	**G**AU **C**UG		- 9.0
Q39Q N43D	CAG AAU	CA**A** **G**AU		- 6.8
Q41Q N43D	CAG AAU	CA**A** **G**AU		- 6.4
N43K	AAU → AA**A**		- 4.9
N43K	AAU → AA**G**		- 5.4
N43K L44L	AAU UUG	AA**A** **C**UG		- 7.7
N43K L44L	AAU UUG	AA**G** **C**UG		- 7.9
Q40H L45M	CAG CUG	CA**T** **A**UG		- 4.5

(C)

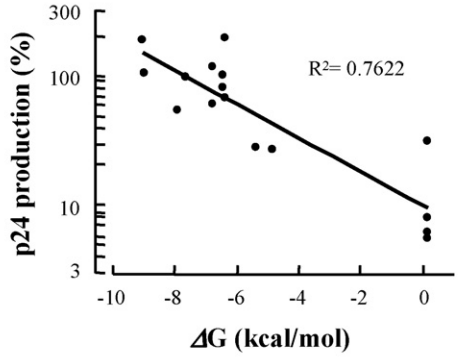


Fig. 1. (A) Secondary structure of RRE depicted based on references (Olsen et al., 1990; Zapp and Green, 1989). T-20-resistant mutations are accumulated between amino acid position from 36 to 45 of gp41 and nucleotides coding for these amino acids, are given in bold. Stem-loop II (light gray region) was examined as described previously (Nameki et al., 2005) and stem-loop III (dark gray region) was analyzed in this study. (B) Amino acid substitutions in the gp41 with their nucleotide changes and putative secondary structures and their stabilities of stem-loop III are shown. Nucleotide substitutions are indicated in bold and circle. Newly generated nucleotide pairs are indicated by the bold line. The stability of stem-loop III defined by ΔG value was calculated using the MFold program version 3.2 (Mathews et al., 1999; Zuker, 2003). (C) Correlation of the amounts of p24 production and stability of stem-loop III (ΔG value) is shown. Plots of Q41H and L45M were included (see text) but that of V38A was excluded since V38A is located in stem IIA. Since replication of N43D containing clones were extremely low, p24 productions obtained from D36G combined N43D or K containing clones, were used. When excluding data of D36G combined with N43D or N43K and including that of N43K without D36G, correlation coefficient (R^2) was 0.72.

Louis, MO). HeLa-CD4-LTR-β-gal cells were used for the drug susceptibility assay (MAGI assay) as described (Nameki et al., 2005). MT-2 and 293T cells were grown in RPMI1640- and Dulbecco's modified Eagle's medium (DMEM)-based culture medium, respectively.

2.2. Generation of recombinant viruses

An HIV-1 infectious clone, pNL4-3, which was kindly provided by Dr. H. Sakai, Institute for Virus Research, Kyoto University (Kyoto, Japan), was used for the construction and the production of HIV-1 variants. To generate major T-20-resistent molecular clones, desired mutations were introduced into the Nhe I-BamH I region (1221 bp) of pSLgp41WT, which encoded nucleotides 7250–8470 of pNL4-3, by site-directed mutagenesis as described (Nameki et al., 2005). The Nhe I-BamH I fragments were then inserted into pNL4-3, generating various molecular clones with the desired mutations. Viruses were recovered from the supernatant of the transfected 293T cells and stored at −80 °C until use. A wild type HIV-1, HIV-1WT, was generated by transfection of pNL4-3 into 293T cells.

2.3. Determination of titer and drug susceptibility of HIV-1

The titer and peptide-sensitivity of infectious clones was determined by the MAGI assay with some modifications (Kimpton and Emerman, 1992; Kodama et al., 2001; Maeda et al., 1998). Briefly, the target cells (HeLa-CD4-LTR-β-gal; 10^4 cells/well) were plated in 96-well flat microtiter culture plates. On the following day, the cells were inoculated with the supernatant of 293T cells transfected with HIV-1 clones and the titrated HIV-1 clones in the presence of various concentrations of drugs in fresh medium, for determination

of titer and drug susceptibility of HIV-1, respectively. Forty-eight hours after the viral exposure, all the blue cells stained with X-Gal were counted in each well. Viral titer was determined with the MAGI assay as blue cell forming units (BFUs). The activity of test compounds was determined as the concentration that blocked HIV-1 replication by 50% (EC_{50}).

2.4. Viral replication kinetics assay

MT-2 cells (10^5 cells) were infected with each virus preparation (500 BFUs) derived from molecular-constructed clone for 4 h. The infected cells were washed and cultured in a volume of 3 ml. The culture supernatants were harvested on day 4 after infection during the linear replication phase and p24 antigen production was determined (Hachiya et al., 2008). For competitive HIV-1 replication assays (CHRA), the two titrated infectious clones were mixed and added to MT-2 cells as described previously (Nameki et al., 2005). To ensure that the two infectious clones being compared were of approximately equal infectivity, a fixed amount (500 BFUs) of one infectious clone was mixed with three different amounts (250, 500 and 1000 BFUs) of the other infectious clone. On day 1, one-third of the infected MT-2 cells were harvested, and subjected to DNA extraction. The purified DNA was used for nested PCR and then direct sequencing. Every 4–5 days, the viral population change was also determined, and the cell-free supernatant of the virus coculture (1 ml) was transmitted to new uninfected MT-2 cells. The cells harvested at the end of each passage were subjected to direct sequencing, and the viral population change was determined.

2.5. GenBank accession numbers

All sequences of clinical isolates referred in this study are available under GenBank accession nos. AF500084 to AF500093, AJ964904 to AJ964940, AY185366 to AY185492, AY436381 to AY436401, AY523979 to AY523991, AY750998 to AY751078, AY768582 to AY768660, and AY785131 to AY785135.

3. Results

3.1. T-20 susceptibility of HIV-1 clones

V38A, N43D, and a combination of Q40H and L45M conferred strong resistance to T-20 (more than 48-fold) while the Q40H, N43K, and L45M substitutions conferred moderate resistance (5.4–13-fold) in comparison to NL4-3 (wild type, WT) (Table 1). These results are consistent with those of a previous report (Labrosse et al., 2006; Lu et al., 2004; Menzo et al., 2004; Pérez-Alvarez et al., 2006; Wei et al., 2002) and demonstrate that these substitutions act as a primary mutation for T-20 resistance. All synonymous mutations at 39, 41, and 44 conferred little T-20 resistance by themselves and even in combination with other substitutions, including N43D and N43K (Table 1). Since Q39, located adjacent to stem-loop III, has no complementary partner, Q39 was used for further experiments as a control for synonymous mutations. Therefore, the gp41 amino acid sequence solely confers T-20 resistance, while the RNA sequence or the structure itself may not markedly contribute to T-20 resistance.

Although the D36G substitution located in the stem-loop II is observed in the vast majority of HIV-1 strains, only NL4-3 strain contains D36 at this position. The introduction of D36G into NL4-3 based T-20 resistant clones enhanced T-20 susceptibility by approximately 10-fold (Table 1). The D36G partially or completely restored T-20 susceptibility attenuated by N43D or K in NL4-3 viruses, respectively. Therefore, D36 may actually enhance T-20 resistance caused by N43D or N43K in the vast majority of the HIV-1 strains. In fact, D36 is frequently detected in T-20 experienced patients and is associated with T-20 resistance (Cabrera et al., 2006). However, N43K containing variants with D36G background seem to be insufficient for resistance to T-20 (only 4-fold resistance), which is consistent with low frequency of emergence of N43K containing variants in T-20-experienced patients (Lu et al., 2006; Morozov et al., 2007; Si-Mohamed et al., 2007).

3.2. Replication kinetics

In D36 background (pNL4-3 derived virus), replication of HIV-1 with primary mutation(s) was much attenuated (Table 1). It is well consistent with rare frequency of simultaneous introduction of V38A and/or N43D with D36 in vivo (Cabrera et al., 2006). Synonymous substitutions at amino acids, Q41 and L44 were frequently observed in T-20-resistant clinical isolates with N43 substitutions deposited in the GenBank, suggesting that these synonymous mutations may work as secondary mutations. To prove this hypothesis, N43D mutants were generated with synonymous mutations, L44L-CUG: UUG → CUG, Q39Q-CAA: CAG → CAA and Q41Q-CAA: CAG → CAA, designated as HIV-$1_{N43D/L44L-CUG}$, HIV-$1_{Q39Q-CAA/N43D}$ and HIV-$1_{Q41Q-CAA/N43D}$, respectively, and their replication kinetics were determined. The p24 production of all of the mutants remained less than 2% in comparison to that of HIV-1WT (Table 1). Replication kinetics were compared based on CHRA, demonstrating that combination of synonymous and complementary mutations, Q41Q-CAA and L44L-CUG restored replication kinetics impaired by N43D, while that of a simple synonymous mutation (not complementary), Q39Q-CAA, did not (Table 2). However, the D43N (GAU → AAU) substitution, which reverts to WT sequence, was detected in the virus population as early as on day 10 during the CHRA, when HIV-1N43D was used (Fig. 2). N43D (GAU) is the putative target site of apobec3F or 3G deamination that is involved in innate immunity to HIV-1 infection (Bishop et al., 2004; Harris et al., 2003; Liddament et al., 2004; Mangeat et al., 2003; Wiegand et al., 2004; Zhang et al., 2003). The MT-2 cells used in this study express both apobec 3G and 3F as confirmed by reverse transcription 165 coupled PCR (data not shown). Therefore, N43D might be reverted to N43 (identical sequence of HIV-1WT) by the deaminases, thus resulting in the appearance of NL4-3 strains.

Since D36G increases the level of replication by controlling the fusogenic activity (Kinomoto et al., 2005) and stability of RRE structure (Nameki et al., 2005), the generated N43D mutants were combined with D36G (HIV-$1_{D36G/N43D}$, HIV-$1_{D36G/N43D/L44L-CUG}$, HIV-$1_{D36G/Q39Q-CAA/N43D}$ and HIV-$1_{D36G/Q41Q-CAA/N43D}$). These recombinant viruses showed comparable replication kinetics with HIV-1WT or HIV-1_{D36G} (Table 1) and revealed an identical order of replication observed in the N43D containing HIV-1s by the CHRA (Table 2). It is likely that N43D mainly impaired replication kinetics through altered fusion kinetics, since reduced replication kinetics by N43D can be partially restored by introduction of S138A (Cabrera et al., 2006; Marcial et al., 2006; Mink et al., 2005; Xu et al., 2005) and the ΔG value is identical to that of WT (Fig. 1B). It is also possible that nucleotide sequence of N43D-GAT may influence Rev binding to stem III.

In N43K mutants, the synonymous mutation, L44L-CTG, also enhanced the replication kinetics (Table 2). The L44L-CTG enhancement was greater in N43K-AAA mutant. Taken together, synonymous and complementary mutations restore HIV-1 replication impaired by introduced primary mutations, suggesting that these mutations act as secondary mutations.

3.3. Stability of stem-loop III

The structural stability of stem-loop III for N43K with codons AAA or AAG, were both comparable (Fig. 1) when calculated using

Table 1
Drug susceptibility[a] and viral replication[b] of HIV-1 clones with primary mutations or synonymous mutations.

Mutation(s)	EC_{50} [c] (μM)		p24(%)
	ddC	T-20	
WT[d]	0.51 ± 0.089	0.021 ± 0.0093	100
D36G	0.66 ± 0.22 (1.3)[e]	0.0026 ± 0.0012 (0.1)	95 ± 15
Primary mutations			
V38A	0.65 ± 0.12 (1.3)	>1.0 (>48)	3.3 ± 1.7
Q40H	0.47 ± 0.11 (0.9)	0.21 ± 0.087 (10)	31 ± 11
N43D	0.48 ± 0.13 (0.9)	>1.0 (>48)	<2
$N43K_{AAA}$	0.22 ± 0.005 (0.4)	0.28 ± 0.024 (13)	21 ± 11
$N43K_{AAG}$	0.32 ± 0.009 (0.6)	0.11 ± 0.019 (5.4)	30 ± 16
L45M	0.68 ± 0.12 (1.3)	0.27 ± 0.11 (13)	5.4 ± 0.8
Q40H/L45M	0.37 ± 0.2 (0.7)	>1.0 (>48)	7.7 ± 3.7
Synonymous mutations			
$Q_{CAG}39Q_{CAA}$	0.62 ± 0.077 (1.2)	0.015 ± 0.006 (0.7)	115 ± 16
$Q_{CAG}41Q_{CAA}$	0.58 ± 0.13 (1.1)	0.054 ± 0.011 (2.6)	192 ± 53
$L_{UUG}44L_{CUG}$	0.62 ± 0.089 (1.2)	0.030 ± 0.018 (1.5)	191 ± 34
$L_{UUG}44L_{UUA}$	0.64 ± 0.18 (1.3)	0.014 ± 0.0026 (0.7)	5.9 ± 1.7
N43D series			
N43D[f]	0.48 ± 0.13 (0.9)	>1.0 (>48)	<2[g]
$N43D/L44L_{CUG}$	0.35 ± 0.050 (0.7)	>1.0 (>48)	<2
$Q39Q_{CAA}/N43D$	0.34 ± 0.17 (0.7)	>1.0 (>48)	<2
$Q41Q_{CAA}/N43D$	0.62 ± 0.097 (1.2)	>1.0 (>48)	<2
D36G/N43D	0.56 ± 0.18 (1.1)	0.13 ± 0.056 (6.1)	81 ± 10
$D36G/N43D/L44L_{CUG}$	0.63 ± 0.19 (1.2)	0.10 ± 0.045 (4.9)	103 ± 34
$D36G/Q39Q_{CAA}/N43D$	0.76 ± 0.12 (1.5)	0.14 ± 0.057 (6.8)	59 ± 23
$D36G/Q41Q_{CAA}/N43D$	0.74 ± 0.14 (1.5)	0.14 ± 0.066 (6.8)	67 ± 16
N43K series			
$N43K_{AAA}$ [f]	0.22 ± 0.005 (0.4)	0.28 ± 0.024 (13)	21 ± 11
$N43K_{AAG}$ [f]	0.32 ± 0.009 (0.6)	0.28 ± 0.077 (13)	30 ± 16
$N43K_{AAA}/L44L_{CUG}$	0.64 ± 0.087 (1.3)	0.22 ± 0.082 (11)	76 ± 18
$N43K_{AAG}/L44L_{CUG}$	0.59 ± 0.10 (1.2)	0.20 ± 0.067 (10)	37 ± 15
$D36G/N43K_{AAA}$	0.61 ± 0.086 (1.2)	0.0069 ± 0.0004 (0.3)	26 ± 18
$D36G/N43K_{AAG}$	0.60 ± 0.078 (1.2)	0.0087 ± 0.0008 (0.4)	27 ± 12
$D36G/N43K_{AAA}/L44_{CUG}$	0.28 ± 0.14 (0.6)	0.0057 ± 0.0016 (0.3)	96 ± 19
$D36G/N43K_{AAG}/L44L_{CUG}$	0.69 ± 0.14 (1.4)	0.0069 ± 0.0003 (0.3)	53 ± 8.1

[a] Antiviral activity was determined by the MAGI assay. The data shown are mean values and standard deviations obtained from the results of at least three independent experiments.

[b] Viral replication was determined by p24 ELISA. The data shown are mean values and standard deviations obtained from the results of at least three independent experiments. Absolute p24 value for $HIV\text{-}1_{WT}$ was 1.8 ± 0.2 ng/ml.

[c] 50% effective concentration was determined with the MAGI assay (Nameki et al., 2005).

[d] $HIV\text{-}1_{NL4\text{-}3}$ was used as a wild type virus.

[e] Fold change in EC_{50} of the gp41 recombinant molecular clone compared with that of $HIV\text{-}1_{WT}$.

[f] Also described in the *primary mutations* section of Table 1.

[g] Only insufficient replication of $HIV\text{-}1_{N43D}$ in MT-2 cells was observed. However, we could determine initial titer of the $HIV\text{-}1_{N43D}$ obtained from freshly transfected 293T cells in the MAGI cells, since the MAGI assay only detects first round of viral infection up to Tat expression. Therefore, it may be ideal for determination of accurate inhibitory effect compared to other assays that allow multiple replications.

the MFold program version 3.2 (http://frontend.bioinfo.rpi.edu/applications/mfold/) (Mathews et al., 1999; Zuker, 2003). The replication kinetics of HIV-1N43K-AAA and HIV-1N43K-AAG were comparable, in combination of L44L-CTG, however, that of HIV-1N43K-AAA was greater by the CHRA. It is possible that the nucleotide sequence itself may affect binding affinity of Rev to the RRE, although detailed mechanism of the difference between N43K-AAA and -AAG remains to be elucidated. Introduction of D36G to N43K-containing clones did not improve the replication kinetics of N43K-containing clones but restored their susceptibilities to T-20 (Table 1). These results indicate that synonymous mutations thus maintain HIV-1 replication.

A combination of primary mutations, namely Q40H and L45M (both ΔG values are 0.1 but in combination that is −4.5), which are complementarily located and stabilize stem III structure (Fig. 1B) and observed in vivo (Cabrera et al., 2006; Marcial et al., 2006; Mink et al., 2005; Xu et al., 2005), may alter the replication kinetics at either the nucleotide or amino acid level. Recent studies also highlight strong co-presence of Q40H and L45M in clinical isolates treated with T-20 (Svicher et al., 2008). These results sug-

Table 2
Effect of synonymous mutations on replication of HIV-1.

Mutation background	Order of replication
N43D	$HIV\text{-}1_{N43D/L44L\text{-}CUG} = HIV\text{-}1_{Q41Q\text{-}CAA/N43D} > HIV\text{-}1_{N43D} = HIV\text{-}1_{Q39Q\text{-}CAA/N43D}$
D36G/N43D	$HIV\text{-}1_{D36G/N43D/L44L\text{-}CUG} = HIV\text{-}1_{D36G/Q41Q\text{-}CAA/N43D} > HIV\text{-}1_{D36G/N43D} > HIV\text{-}1_{D36G/Q39Q\text{-}CAA/N43D}$
N43K	$HIV\text{-}1_{N43K\text{-}AAA/L44L\text{-}CUG} > HIV\text{-}1_{N43K\text{-}AAG/L44L\text{-}CUG} > HIV\text{-}1_{N43K\text{-}AAA} = HIV\text{-}1_{N43K\text{-}AAG}$
Synonymous mutations	$HIV\text{-}1_{L44L\text{-}CUG} = HIV\text{-}1_{Q41Q\text{-}CAA} > HIV\text{-}1_{WT} = HIV\text{-}1_{Q39Q\text{-}CAA}$

Competition of HIV-1 replication assay (CHRA) was performed in MT-2 cells. At least two independent CHRAs were performed.

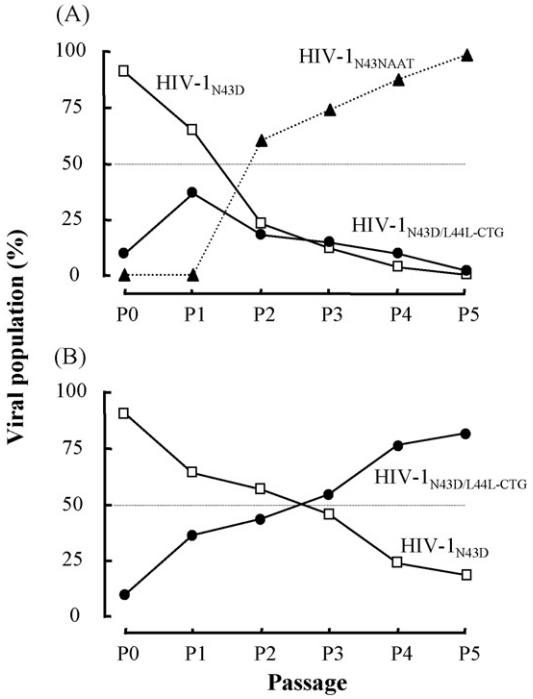


Fig. 2. Emergence of HIV-$1_{N43N\text{-}AAT}$ (HIV-1_{WT}) during CHRA for HIV-1_{N43D} and HIV-$1_{N43D/L44L\text{-}CTG}$ are shown. Open square, closed circle, and closed triangle represent HIV-1_{N43D}, HIV-$1_{N43D/L44L\text{-}CTG}$, and HIV-$1_{N43N\text{-}AAT}$, respectively. Absolute viral populations of each HIV-1s (A) and relative viral populations of HIV-1_{N43D} and HIV-$1_{N43D/L44L\text{-}CTG}$ (B) were shown.

gest that introduction of the primary mutations in the nucleotide level are affected by the RRE stability, indicating that amino acid and nucleotide substitutions in the gp41 and the RRE, respectively, co-operatively play a role.

3.4. Effect of synonymous mutations

As expected, the synonymous mutations (Q39Q-CAA, Q41Q-CAA, and L44L-CUG) solely affect viral replication but not T-20 susceptibility (0.7–2.6-fold in Table 1). They displayed an order of replication of HIV-1L44L-CUG = HIV-1Q41Q-CAA > HIV-1WT = HIV-1Q39Q-CAA, also demonstrating that only synonymous and complementary mutations, Q41Q-CAA and L44L-CTG, enhance replication kinetics. Finally, HIV-1L44L-UUA that is not detected in vivo was constructed and its replication kinetics was examined. As shown in Fig. 1, the third nucleotide for L44 raises the ΔG value, thus indicating that structure of the stem-loop III is unstable. The variants displayed impaired replication kinetics (Table 1). These results indicate that, in addition to the influence on gp41 function by amino acid substitutions, the structural stability of stem-loop III is one of the major determinants of the replication kinetics of mutated clones.

4. Discussion

This study demonstrated that synonymous mutations in the stem-loop III of RRE play an important role in the improvement of HIV-1 replication without affecting T-20 susceptibility. The structural stability of stem-loop III defined by ΔG value was strongly correlated with the replication kinetics ($R^2 = 0.76$, Fig. 1C), while the susceptibility, based on the EC_{50} value, was less ($R^2 < 0.3$ excluding clones that showed over 48-fold resistance, data not shown). Although it is likely that the nucleotide sequence of stem-loop III as well as the structural stability may also influence the RRE functions, including Rev binding, the current results indicate that the structural stability of RRE as well as gp41 amino acid substitutions seem to be a determinant for replication kinetics. At present, however, it is impossible to conclude that the pre-existence of such synonymous mutations in the RRE predicts on how T-20 resistance mutations are acquired.

Armand-Ugón et al initially isolated and reported resistance to C34 in vitro (Armand-Ugón et al., 2003), although we previously demonstrated that some of mutations for C34 resistance are involved in the RRE function as described (Nameki et al., 2005). The variants contained L33S or V38E mutations in the gp41 and both showed strong (more than 500-fold resistance) C34 resistance (Armand-Ugón et al., 2003). These mutations in the nucleotide level are also located in RRE (Fig. 1A); the nucleotide substitutions for L33S and V38E are located in the loop of stem IIB and the middle of stem IIC, respectively. Interestingly, V38E emerged in the HxB2-derived strain containing D36G polymorphism that stabilizes stem IIB structure, while L33S, which might have little effect on stem IIC stability due to its location, was observed in the NL4-3-derived resistant strain. These results also suggest that introduction of some mutations in the gp41 is restricted by RRE function. Armand-Ugón et al. (2003) failed to identify the secondary mutations for L33S or V38E. It is likely that the effect on RRE function may be tolerable for viral replication. Alternatively, relative short induction periods (maximum 17 passages) might also influence efficient introduction of the secondary mutations.

Functional analysis for Rev, RRE, and/or both seems to be important to reveal a detailed effect on viral replication. In this regard, we have previously demonstrated that effect of mutations for C34 resistance located in stem-loop II on binding of Rev to RRE was not apparent (less than 10% in the gel shift assay (Nameki et al., 2005)). It is possible that other factors including nuclear export and host factors, which may be influenced by the mutations, are involved in viral replication through interaction of Rev/RRE in HIV infected cells. Therefore, in the present study, we constructed an artificial mutant L44-TTA, which destabilizes or disrupts the stem III structure, and examined the effect on viral replication (Table 1). Replication of L44-TTA containing mutant showed much reduced replication kinetics even without gp41 amino acid substitutions, again indicating that stem III also plays an important role in viral replication.

So far, no information concerning Rev and Tat nucleotide substitutions is available in T-20 experienced patients. It is possible that the altered function of RRE may induce Rev mutation(s). In this regard, the entire sequence of Rev coding region of a C34 resistant variant was determined, however, no mutations were observed (Nameki et al., 2005). Most of the coding region of Rev also encodes Tat and gp41, thus indicating that Rev mutation(s) would alter these functions. This suggests that, even for single amino acid substitution, the genetic barrier for T-20 resistance seems to be relatively high when synonymous mutations are required to be introduced with the primary mutations and further fusion inhibitors that target the N-helical region thus appear to be promising.

In conclusion, this study provides valuable insight into the functional importance of RRE in HIV-1 with T-20 resistance for the replication kinetics. To reveal the function of gp41, experiments with artificial amino acid substitutions, *e.g.*, alanine scanning, which can be used to rapidly identify residues important for protein function by alanine substitution, should be carefully conducted. Further studies will reveal the functional significance of the RNA and protein function in this region.

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