Prototype macaque cell-tropic HIV-1 (pHIV-1mt), NL-DT5R (5R; X4-tropic) and NL-DT562 (562; R5-tropic), carry a small portion of *gag* gene and an entire *vif* gene from SIVmac239 (Fig. 1). These viruses are insufficiently infectious for macaque cells by their partial resistance to CypA/TRIM5 proteins and evasion from APOBEC3G/F restriction, and their replication potentials were significantly lower than that of pathogenic SIVmac239 [14–16]. These results indicate that macaque cells still impose strong restrictive pressure on pHIV-1mt replication. Therefore, clones 5R and 562 are suitable materials to analyze what genetic changes they would acquire and how they would adapt themselves to environments within macaque cells. As experimental approach to do this, we have performed virus adaptation experiments by the long-term culture of 5R- or 562-infected macaque cells, and successfully obtained adapted viruses with enhanced growth ability [17]. In this study, we inquired into the biological relevance of mutations that appeared in the genomes of adapted viral clones by investigating thoroughly the effect of mutations on viral growth. Although a number of genetic substitutions were found in the genomes of adapted viruses, mutations in *pol*-integrase (IN) and *env*-gp120 only were found to be responsible for enhancement of viral replication. Extensive and repeated virus adaptation experiments revealed that viral clones with augmented growth potential are characterized by acquisition of adaptive mutations in *pol*-IN and in *env*-gp120. Our results suggest that pHIV-1mt evolves in a certain adaptation pathway under the restrictive and uniform environments imposed by macaque cells, and that genetic alterations do not always have biological significance for escaping selective pressure, supporting Kimura's neutral theory of molecular evolution [18].

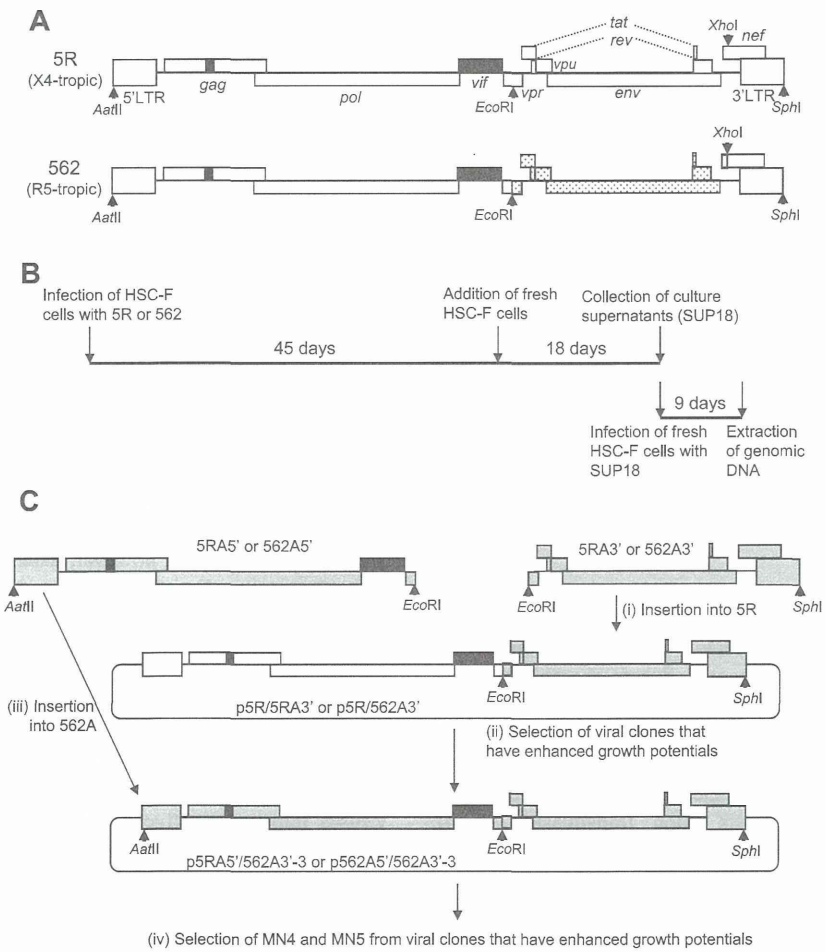


Fig. 1. Molecular cloning of viral genomes from macaque cell-adapted viruses. (A) Genome structure of 5R and 562 [14,16]. Sequence from NF462 [32] is shown by dotted areas. Black areas show the regions derived from SIVmac239. (B) Schedule of virus adaptation and the harvest of adapted viruses [17]. Genomic DNA prepared from infected cells for molecular cloning is indicated. (C) Generation and selection of proviral clones from adapted 5R and 562 viruses. The procedure starts with (i) and ends with (iv). For details, see Materials and methods. PCR amplified fragments derived from adapted 5R (5RA) or adapted 562 (562A) are shown by gray areas. White and black areas indicate the region from 5R.

2. Materials and methods

2.1. Cells

A human monolayer cell line 293T [19] was cultured in Eagle's MEM supplemented with 10% heat-inactivated FBS (hiFBS). A cynomolgus macaque (CyM) lymphocyte cell line HSC-F [20] and a rhesus monkey (RhM) lymphocyte cell line HSR5.4S1 [21] were maintained in RPMI1640 containing 10% hiFBS (for HSR5.4S1, 50 units/mL of IL-2 (AbD Serotec) were added). Human MT4/CCR5 cells (MT4 cells stably expressing CCR5) were maintained RPMI1640 containing 10% hiFBS and 200 μg/mL of hygromycin B (Sigma–Aldrich).

2.2. Transfection, RT assays and infection

Virus stocks were prepared by transfection of 293T cells with viral clones using the calcium phosphate co-precipitation method [22]. On day 2 post-transfection, culture supernatants were collected and stored at −80 °C until use. Virion-associated RT activity was measured as described previously [23]. HSC-F cells were infected with an equal amount of virus preparations in the presence of IL-2. For infection of MT4/CCR5 cells, the spinoculation method [24] was used. Virus replication was monitored by RT activity in the culture supernatants, and viral growth potentials were evaluated by the peak day of virus production or by the level of virus production on the peak day.

2.3. Plasmid DNAs

Construction of pHIV-1mt clones designated 5R and 562 has been previously described [14,16]. Proviral clones derived from adapted viruses were generated by PCR as described previously [14]. Two rounds of adaptation experiments independently performed are outlined in Figs. 1–4, and their details are described below. Introduction of genetic substitutions was performed by the QuickChange site-directed mutagenesis kit (Agilent Technologies).

2.4. First adaptation experiment

As indicated in Fig. 1, to construct molecular clones of adapted viruses from 5R and 562, CyM HSC-F cells were infected with the culture supernatants collected from long-term cultures. On day 9 post-infection, genomic DNA was extracted from cells, and integrated proviruses were amplified as two overlapping fragments by PCR as described previously [14]. To generate molecular clones that exhibit phenotypes of adapted viruses, we first introduced 3′ half genomes (*Eco*RI site in Vpr to *Sph*I site at 3′ end of the genome) from adapted 5R (5RA) and 562 (562A) viruses into 5R, and the resultant constructs were designated p5R/5RA3′ and p5R/562A3′, respectively. Virus stocks were prepared from 293T cells transfected with p5R/5RA3′ or p5R/562A3′, and inoculated into HSC-F cells. Virus replication was monitored by RT activity released into the culture supernatant. A molecular clone that grows best was selected for 5RA and 562A (p5R/5RA3′-14 and p5R/562A3′-3, respectively). Then, 5′ half genomes (*Aat*II site at the 5′ end of the genome to *Eco*RI site in Vpr) of adapted viruses (5RA5′ and 562A5′) were introduced into the selected clone (p5R/562A3′-3), and the resultant constructs were designated p5RA5′/562A3′-3 and p562A5′/562A3′-3, respectively. Proviral clones with the best replication potential were selected as described above. Finally, proviral clones that have a full-length viral genome derived from adapted 5R and 562 viruses were constructed, and designated MN4 and MN5, respectively (see Fig. 2 for their genome structures).

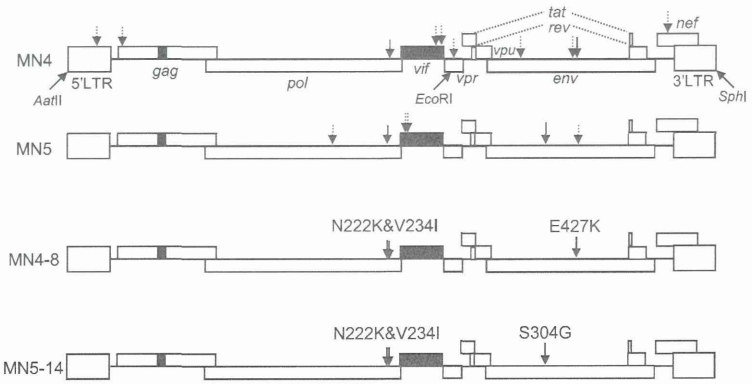
2.5. Second adaptation experiment

As indicated in Fig. 4, HSC-F (1×10^6) and RhM HSR5.4S1 cells (3×10^6) were infected with 5R or 562 viruses prepared from transfected 293T cells to obtain adapted viruses. Half of the culture medium (5 mL) was replaced every 3 days. Fresh HSC-F (1×10^6) and HSR5.4S1 cells (3×10^6) were added in each long-term culture on day 28 post-infection. Half of the culture medium (5 mL) was replaced every 3 days, and harvested supernatants were stored at −80 °C. HSC-F cells were infected with supernatants collected on day 33 post-infection from 5R- or 562-infected HSC-F long-term cultures. On day 9 and 12 post-infection, genomic DNA was extracted from infected cells. For HSR5.4S1 cultures, supernatants collected from 5R- and 562-infected cultures on day 39 and 42 post-infection, respectively, were inoculated into fresh HSR5.4S1 cells. On day 8 and 11 post-infection, genomic DNA was extracted from infected cultures. Amplification of integrated proviruses from genomic DNA and construction of proviral clones were carried out as described above. Viruses prepared from 293T cells transfected with these proviral clones were inoculated into HSC-F and HSR5.4S1 cells depending on which cells were used for long-term culture. Selection of viral clones with enhanced replication capability was carried out as described above.

3. Results

3.1. Mutations in Pol-IN or Env-gp120 are important for enhancement of pHIV-1mt replication in macaque cells

We previously reported that adapted viruses with enhanced growth potential emerged in prolonged cultures of 5R- or 562-infected macaque (CyM) cells [17]. Genomes of adapted viruses should have some genetic changes to augment their growth ability, and adapted viruses were expected to exist as a mass of viruses with distinct replication potential. To identify an adaptive mutation responsible for enhanced growth potential, it is necessary to construct proviral clones that exhibit a better-growing phenotype. Experimental details for construction are shown in Fig. 1. Of 72 proviral clones constructed, some of them (9 clones) did not produce virions in transfected 293T cells as determined by virion-associated RT activity or were not infectious for a CyM lymphocyte cell line HSC-F as well (16 clones). Finally, we have obtained molecular clones



Virus	Genomes from adapted virus	Nucleotide change	Region	Amino acid change in the region
MN4	5' half	t453c	LTR	None
(derived from adapted 5R)	(MN4-5')	g823a	Matrix	E12K
		g4923a	**Pol-IN**	**V234I**
		g5611a	Vif	R171K
		g5693a	Vif	None
	3' half	g5944a	Vpr	None
	(MN4-3')	g6915a	Env-gp120 (V2)	None
		g7606a	Env-gp120 (V4)	E401K
		g7684a	**Env-gp120 (C4)**	**E427K**
		g9191a	Nef	V74I
MN5	5' half	a4049c	Reverse transcriptase	None
(derived from adapted 562)	(MN5-5')	**t4889a**	**Pol-IN**	**N222K**
		g5156a	Vif	None
		t5195a	Vif	None
	3' half	**a7318g**	**Env-gp120 (V3 loop)**	**S304G**
	(MN5-3')	c7812t	Env-gp120 (C5)	None

Fig. 2. Various mutations found in the first adaptation experiment. (Upper) Proviral genome structures of MN4, MN5, and their derivatives. Black areas show the regions derived from SIVmac239. Broken arrows show mutations that appeared during viral adaptation. Solid arrows indicate adaptive mutations in Pol-IN and Env-gp120 that enhance viral growth (Fig. 3). Out of the mutations present in MN4 and MN5, clones MN4-8 and MN5-14 carry the adaptive (growth-enhancing) mutations only. (Lower) Mutations associated with adaptation of 5R and 562 viruses to HSC-F cells. Bold letters show adaptive mutations that are responsible for enhancement of viral replication in cells. 5′ half indicates a fragment from *Aat*II at the 5′ end to *Eco*RI in *vpr* of the viral genome. 3′ half indicates a fragment from *Eco*RI in *vpr* to *Sph*I at the 3′ end of the viral genome.

MN4 and MN5 from adapted 5R and 562 viruses, respectively (Fig. 2).

To identify genetic changes acquired in genomes during virus adaptation, the entire genomes of MN4 and MN5 were sequenced. As shown in Fig. 2, MN4 and MN5 contained ten and six nucleotide substitutions, respectively. To examine the effect of these mutations on viral replication, each mutation was introduced into parental clones 5R and 562 as follows: (i) mutations in MN4 were introduced into 5R; (ii) mutations in 5′ half of MN5 genome were introduced into 5R (5′ half of 562 genome is identical to that of 5R (see Fig. 1A)); (iii) mutations in 3′ half of MN5 genome were introduced into 562. To construct positive control clones for viral growth (Fig. 3), we inserted a half of MN4 and MN5 genomes into the corresponding regions of 5R to generate full-length clones MN4-5′/5R, 5R/MN4-3′, MN5-5′/5R, and 5R/MN5-3′ (Fig. 2). Viruses were then prepared from 293T cells transfected with parental clones, positive controls, or test clones carrying each mutation, and inoculated into HSC-F cells (Fig. 3). MN4-5′ contains five genetic mutations (Fig. 2). Of the five clones examined, only the 5R carrying g4923a (V234I in IN) exhibited a similar growth kinetics to MN4-5′/5R (Fig. 3A). Growth kinetics of 5R carrying the other mutations were similar or slower relative to the parental clone 5R (Fig. 3A). MN4-3′ and MN5-5′ carry five and four nucleotide substitutions, respectively (Fig. 2). A clone that exhibits similar growth kinetics to 5R/MN4-3′ was 5R carrying g7684a

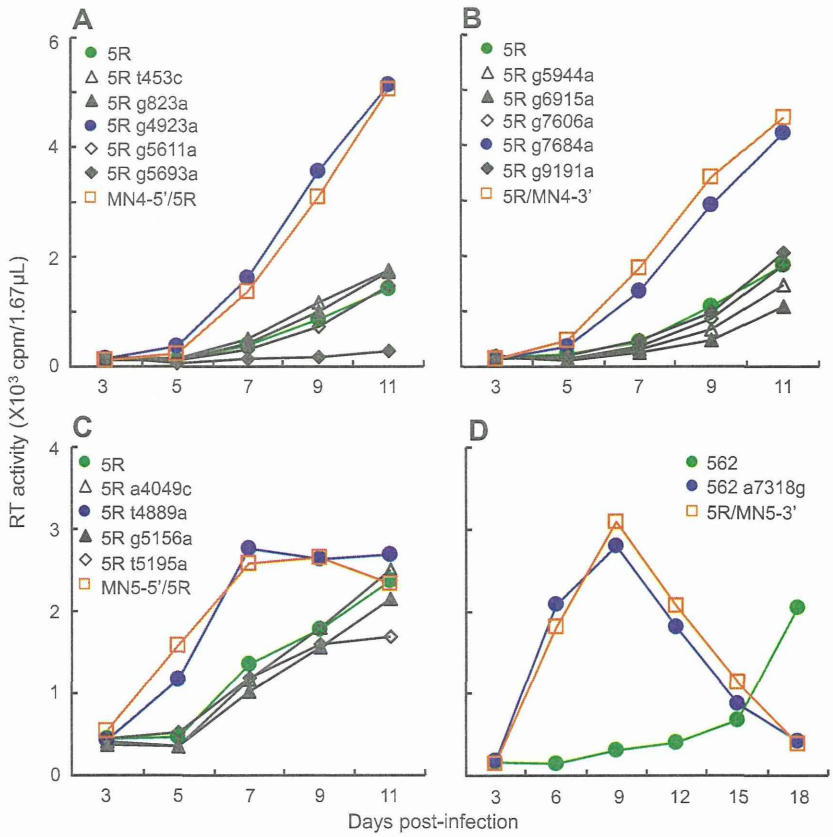
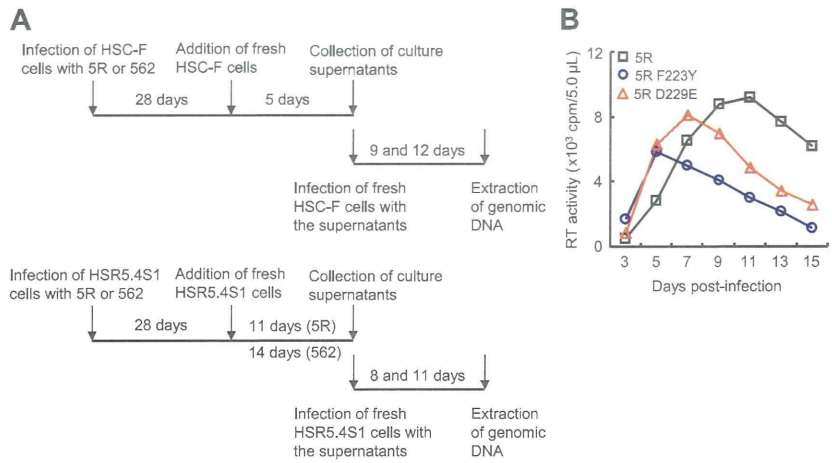


Fig. 3. Effect of individual mutations found in MN4 and MN5 on viral replication in HSC-F cells. Virus samples were prepared from 293T cells transfected with the indicated proviral clones, and inoculated into HSC-F cells (10^6) with an equal amount of viruses (A; 1.6×10^7 RT units, B; 8.5×10^6 RT units, C; 3.9×10^7 RT units, and D; 1.2×10^7 RT units). Proviral clones, MN4-5′/5R, 5R/MN4-3′, MN5-5′/5R, and 5R/MN5-3′, carry 5′ or 3′ half genomes of MN4 or MN5 in the context of 5R genome (Fig. 2). These clones served as positive controls. Virus replication was monitored by RT activity released into the culture supernatants.

(E427K in gp120) (Fig. 3B). 5R carrying t4889a (N222K in IN) showed a similar growth property with MN5-5′/5R (Fig. 3C). Two mutations (non-synonymous and synonymous) were found in MN5-3′ (Fig. 2), but only the non-synonymous mutation (a7318g, S304G in gp120) was tested. The viral clone 562 a7318g showed similar growth kinetics to those of 5R/MN5-3′ (Fig. 3D). In total, the growth-enhancing mutations were found only in *pol*-IN (V234I in 5R and N222K in 562) and *env*-gp120 (E427K in 5R and S304G in 562) regions despite the presence of mutations in the other regions.

3.2. Adaptive mutations in Pol-IN and 562 Env-gp120 are critical for augmentation of pHIV-1mt replication

Growth potential of both MN4 and MN5 obtained from adaptation of two distinct viruses was augmented by mutations in Pol-IN and Env-gp120 (Figs. 2 and 3). To clarify the requirements of adaptive mutations in these regions for enhancement of viral replication, we repeated the virus adaptation experiment (Fig. 4). Because it has been suggested that cellular environment is different between cell lines and affects outcome of virus adaptation [25], we used two distinct viruses (5R and 562) and two different macaque lymphocyte cell lines (CyM HSC-F and RhM HSR5.4S1) in this experiment. During long-term cultures of HSC-F and HSR5.4S1 cells infected with 5R or 562, adapted viruses emerged as judged by the growth property of viruses produced in the culture supernatants as previously described [17]. A number of proviral clones derived from adapted viruses were constructed similarly as above, and test viruses were prepared from transfected 293T cells. Viruses were then inoculated into HSC-F or HSR5.4S1 cells depending on which cells were used for adaptation. We selected proviral clones that show clearly enhanced viral replication in macaque cells, and sequenced their entire genomes. As shown in Fig. 4, of viral clones that bear 5′ half of genomes from adapted 5R, we selected four clones from HSC-F cells and one clone from HSR5.4S1 cells. All of them carried a genetic mutation in Pol-IN (D229E or F223Y) that enhances viral growth potential (Fig. 4B, note the peak day of virus production). Four adaptive mutations identified in Pol-IN (N222K, F223Y, D229E and V234I) were located in a narrow region of IN C-terminal domain (IN-CTD). On the other hand, none of viral clones carrying 3′ half of genomes from adapted 5R exhibited augmentation of viral



C

Virus/Macaque cells used for long-term culture	Half genome derived from adapted viruses	Number of clones examined for growth kinetics	Number of clones with enhanced viral growth	Adaptive mutations in IN-CTD and Env-gp120
5R/HSC-F	5′	39	4	IN-CTD D229E (4)
	3′	43	0	NA
5R/HSR5.4S1	5′	24	1	IN-CTD F223Y (1)
	3′	62	0	NA
562/HSC-F	5′	41	1	IN-CTD N222K (1)
	3′	16	13	V3 S304G (8) V1 G150R (2) C1 L124F (1) V1 N132K (1) V3 I307V (1)
562/HSR5.4S1	5′	27	0	NA
	3′	32	20	V3 S304G (9) V2 F174L (7) V1 L124F (1) V2 P181T (1) V3 G308E (1) V3 G310R (1)

Fig. 4. Various adaptive (growth-enhancing) mutations found in the second adaptation experiment. (A) Generation of molecular clones from adapted 5R and 562 viruses. Schedule of virus adaptation and the harvest of adapted viruses are shown. Cellular genomic DNA preparations for molecular cloning of viruses are indicated. For details, see Materials and methods. (B) Growth kinetics of Pol-IN mutants in HSC-F cells. Virus samples (3.5×10^6 RT units) were prepared from 293T cells transfected with the indicated proviral clones, and inoculated into CyM HSC-F (10^6). Virus replication was monitored by RT activity released into the culture supernatants. (C) Frequency of adaptive mutations in IN-CTD and Env-gp120. Number in parentheses refers to the number of clones carrying the indicated mutation. In total, all viral clones with enhanced growth potential were found to have adaptive mutations in IN-CTD or Env-gp120. NA, not applicable.

replication. This may imply that 5R has been already better adapted to HSC-F cells, since 5R has acquired mutations in *env* and LTR by virus adaptation within the cells [14]. In viral clones bearing 5′ half genome from adapted 562, one selected clone carried the same N222K mutation in IN-CTD as MN5. Enhanced growth potentials were noted for many clones that carry 3′ half genome from adapted 562. Around half of the selected clones carried S304G in Env-gp120 V3 loop, and the rest of clones carried adaptive (growth-enhancing) mutations in the C1, V1, V2, or V3 region of Env-gp120. Similar results with those in HSC-F cells were obtained in HSR5.4S1 cells (data not shown). The results described above indicate that adaptive mutations in IN-CTD and 562 Env-gp120 are critical for enhancement of viral growth potential in macaque cells.

3.3. Introduction of either single or double adaptive IN-CTD mutations (N222K and V234I) enhances viral growth both in macaque and human cells

Replication of two distinct pHIV-1mt was augmented by acquiring N222K or V234I in IN-CTD during virus adaptation in macaque cells (Figs. 2 and 3). To examine whether these adaptive mutations have an additive effect on viral replication, we constructed 5R carrying single (designated 5R N222K and 5R V234I) or double (designated 5R NKVI) mutations. We were also interested in determining the effect of the mutations on viral growth in human cells. Viruses prepared from transfected 293T cells were inoculated into HSC-F and human MT4/CCR5 cells. As shown in Fig. 5A, 5R NKVI exhibited similar growth kinetics in macaque HSC-

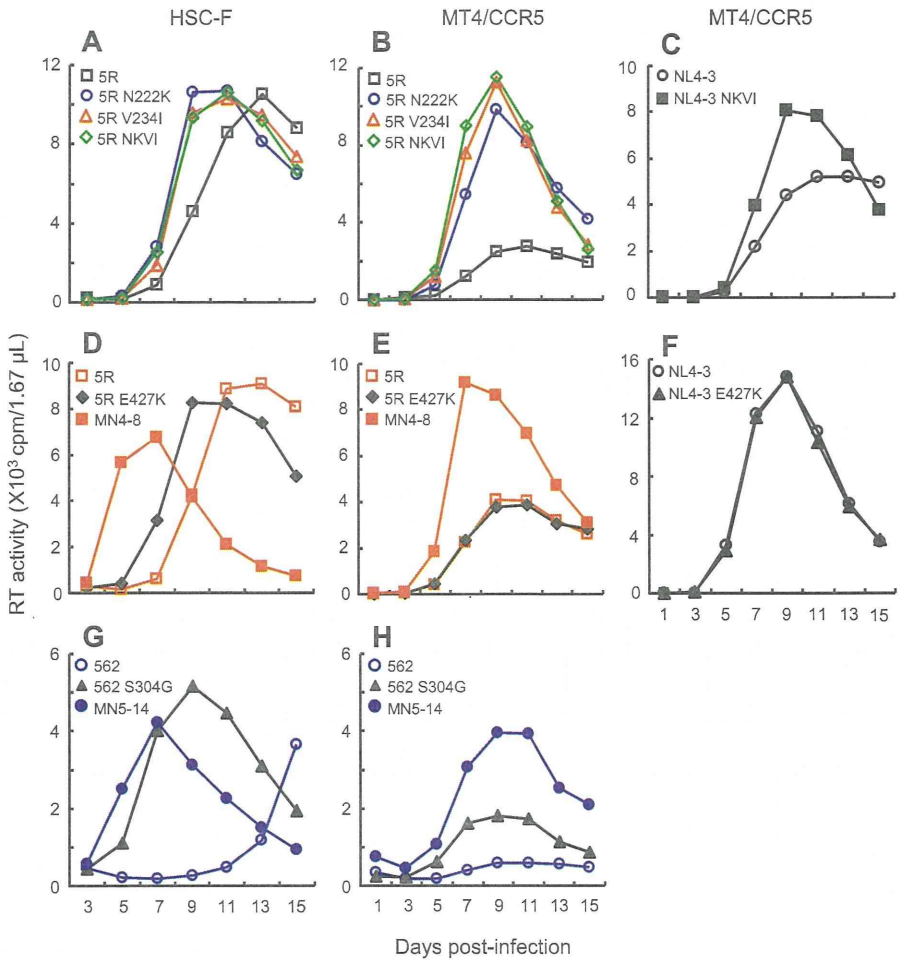


Fig. 5. Effect of adaptive mutations on viral replication. Growth kinetics in the indicated cell lines of various mutant viruses are presented. Virus samples were prepared from 293T cells transfected with the indicated proviral clones, and inoculated into CyM HSC-F (10^6) or spinoculated into human MT4/CCR5 (10^6) cells. Input viral amounts used were 1.0×10^7 RT units, 1.0×10^6 RT units, 1.1×10^5 RT units, 1.2×10^7 RT units, 1.0×10^6 RT units, 1.3×10^5 RT units, 1.2×10^7 RT units and 2.8×10^7 RT units for panels A, B, C, D, E, F, G and H, respectively. Virus replication was monitored by RT activity released into the culture supernatants. (A and B) Growth kinetics of 5R carrying single or double adaptive mutations in IN-CTD. 5R NKVI contains both N222K and V234I mutations. 5R served as a control. (C and F) Growth kinetics of NL4-3 viruses bearing IN NKVI (N222K and V234I) or Env E427K mutation. NL4-3 served as control. (D, E, G and H) Growth kinetics of various viruses carrying adaptive mutations. 5R and 562 served as controls.

F cells to those of 5R N222K and 5R V234I, and all these viruses grew better than 5R. Essentially the same results were obtained in human MT4/CCR5 cells (Fig. 5B). To determine whether the viral growth enhancement by adaptive mutations in IN-CTD is specific to pHIV-1mt, the mutational effect in the context of a standard HIV-1 clone NL4-3 was examined in MT4/CCR5 cells. As is clear in Fig. 5C, the adaptive mutations in IN-CTD also enhanced NL4-3 replication. Unexpectedly, we found that the four adaptive mutations in IN-CTD (Figs. 2 and 4) augment virion production independently of the IN authentic function (manuscript in preparation). In sum, these results show that adaptive mutations in IN-CTD augment growth potential of HIV-1, and that this effect is neither host-cell species-specific nor viral clone-specific.

3.4. E427K in 5R Env-gp120 promotes viral growth specifically in macaque cells, whereas enhancement of viral growth by S304G in 562 Env-gp120 is observed both in macaque and human cells

We constructed proviral clones that have both adaptive mutations in Pol-IN and Env-gp120 in anticipation of additive positive effects. Two mutations in Pol-IN (N222K and V234I) were introduced into clones 5R E427K and 562 S304G, which carry an adaptive mutation in Env-gp120, and designated MN4-8 and MN5-14, respectively (Fig. 2). Viruses prepared from transfected 293T cells were inoculated into HSC-F and MT4/CCR5 cells, and monitored for their growth kinetics (Fig. 5). 5R E427K exhibited rapid growth kinetics relative to 5R in HSC-F cells, but similar in MT4/CCR5 cells (Fig. 5D

and E). To confirm this result, E427K was introduced into NL4-3 Env, which is the origin of 5R Env. NL4-3 carrying E427K showed similar growth kinetics to those of NL4-3 in MT4/CCR5 cells (Fig. 5F). Quite in contrast, the replication of 562 S304G was accelerated compared to that of 562 both in human and macaque cells (Fig. 5G and H). These results indicate that the effect of E427K mutation is host cell species-specific, whereas S304G shows the species-independent positive effect. Notably, E427K and S304G mutations were found to increase viral binding efficiency to CD4 and affinity to CCR5, respectively (manuscript in preparation). Growth potential of MN4-8 and MN5-14 was further enhanced both in macaque and human cells relative to 5R E427K and 562 S304G, respectively (Fig. 5), showing that combination of adaptive mutations in IN-CTD and Env-gp120 has an additive effect on viral replication (note the peak day of virus production).

3.5. Enhancement of viral growth by adaptive Env-gp120 mutations is dependent on the env sequence context

E427K in 5R and S304G in 562 were located in a distinct region within Env-gp120, C4 region and V3 loop, respectively. Alignment of Env-gp120 amino acid sequence of 5R and 562 indicated that the corresponding sites of E427K and S304G are E419K for 562 and S304G for 5R, respectively (Fig. 6A). Since functional relationship between C4 region and V3 loop for viral infection has been reported [26,27], we examined the effect of combination of adaptive mutations in these regions on viral growth. Proviral clones were generated by introducing S304G into 5R and 5R E427K (designated 5R S304G and 5R SGEK, respectively), and E419K into 562 and 562 S304G (designated 562 E419K and 562 SGEK, respectively). Viruses prepared from transfected 293T cells were inoculated into HSC-F cells, and monitored for their growth property. As shown in Fig. 6B and C, while enhancement of viral replication was again observed for 5R E427K and 562 S304G relative to 5R and 562, respectively, the growth of 5R S304G and 562 E419K was undetectable. Clones 5R SGEK and 562 SGEK somewhat restored their infectivity. These results show that the positive effect of adaptive mutations in Env-gp120 is sequence-specific. This is similar to the result in a previous report that the impact of one amino acid change within V3 loop on sensitivity to entry inhibitors is dependent on *env* context [28].

4. Discussion

In this study, we generated MN4 and MN5 clones with enhanced growth potential by virus adaptation in macaque cells. MN4 and MN5 have a number of nucleotide substitutions throughout their genomes (Fig. 2). Interestingly, only the mutations in IN-CTD and Env-gp120 were responsible for augmentation of viral replication (Figs. 2 and 3). In the second round of virus adaptation experiment using two distinct viruses and two different kinds of macaque cells, the mutations in IN-CTD and in 562 Env-gp120 were frequently found in the genomes of viral clones that exhibit rapid growth kinetics (Fig. 4). These results indicate that adaptive mutations in

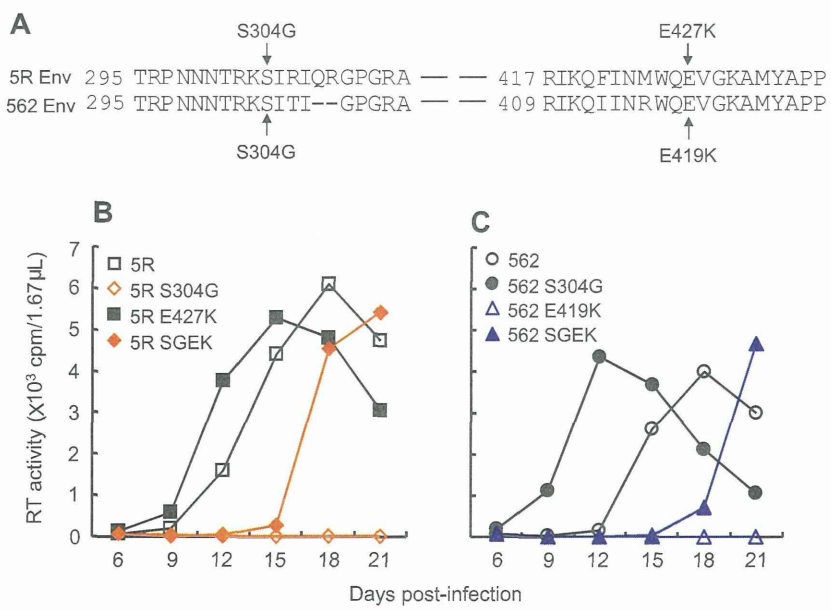


Fig. 6. Effect of combination of the adaptive mutations in Env-gp120 on viral replication. (A) Amino acid alignment of the regions close to adaptive mutations in 5R and 562. The corresponding sites to adaptive mutations in 5R and 562 are indicated. (B and C) Growth kinetics in HSC-F cells of 5R and 562 viruses carrying Env E419K/E427K and/or S304G mutation. Virus samples were prepared from 293T cells transfected with the indicated proviral clones, and inoculated into HSC-F cells (10^6) with an equal amount of viruses (1.0×10^7 RT units). 5R SGEK and 562 SGEK carry both S304G and E419K/E427K mutations. 5R and 562 served as controls. Virus replication was monitored by RT activity released into the culture supernatants.

a narrow region of IN-CTD and in Env-gp120 are critical for growth-adaptation of pHIV-1mt in macaque cells. These results also suggest that pHIV-1mt may evolve in a certain direction to enhance their replication under uniform and strong restrictive environment in macaque cells. Notably, of the adaptive mutations examined, almost all were effective in both macaque and human cells with respect to promoting virus replication (Fig. 5).

In virus adaptation experiments, we obtained numerous proviral clones carrying the mutations in IN-CTD or S304G in 562, which exhibit enhanced growth potential (Figs. 2 and 4). Because genetic changes found in the other regions were different, the mutations came from different virus clones. Reason for the observed high frequency of adaptive IN-CTD and S304G mutations is presently unclear. Viruses may be apt to acquire the mutations in IN-CTD and Env-gp120 regions during adaptation by unknown mechanism. Alternatively, the rapid replication of mutant clones expanded the mutations in a mass of viruses, and resulted in their frequent selection. On the other hand, mutations in IN-CTD and S304G in 562 gp120 enhanced viral growth both in macaque and human cells (Fig. 5). These results indicate that the mutations are not relevant to evasion from the restriction factors responsible or critical for species-tropism. In fact, genetic alterations that influence viral replication were not found in *vif*, *gag*-CA, and *vpu*, which target APOBEC3G/F, CypA/TRIM5 proteins, and tetherin, respectively (Figs. 2 and 4). In this regard, it is interesting to note that replication efficiency of pHIV-1mt (5R and 562) is quite low in macaque cells [14,15]. Strong restriction of replication imposed by macaque cells may serve as a selective pressure on viruses during adaptation. Under this pressure, pHIV-1mt would have evolved to enhance growth ability itself, probably at a basal level, by acquiring the IN-CTD and gp120 mutations rather than to overcome the species barrier. HIV-1mt derivatives described in this report are still poorly infectious for CyM peripheral blood mononuclear cells (PBMCs) relative to the standard SIVmac and SHIV (chimera between SIVmac and HIV-1) clones. However, the enhancing effect of IN-CTD and Env-gp120 mutations was also observed in $CD8^+$ cell-depleted CyM PBMCs [29], confirming the results obtained in the cell lines. To further enhance growth potentials of HIV-1mt clones, it was found to be necessary to efficiently evade the species barrier [29,30]. Detailed functional and structural analyses of the mutations are required to elucidate the whole picture of the adaptive evolution described here. Studies in this direction are in progress in our laboratory.

In conclusion, we have demonstrated here that our adaptation system using virus-infected cells is a powerful tool to study virus replication and its underlying mechanism. We have readily and successfully identified the mutations, from mixtures of biologically insignificant and significant ones, that play a key role in viral replication, as also shown for SIVcpz (SIV isolated from chimpanzees) in human lymphoid tissue [31]. Since how viruses change their phenotypes is dependent on a power balance between viruses and cells, the combination of various primate lentiviruses (HIVs versus SIVs) and cells (human versus macaque) can produce different results upon adaptation. Information of adaptive mutations obtained from these experiments would be invaluable to learn and understand mechanisms for virus replication and evolution.

Acknowledgments

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ARTICLES
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Oligomerization transforms human APOBEC3G from an efficient enzyme to a slowly dissociating nucleic acid-binding protein

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The human APOBEC3 proteins are a family of DNA-editing enzymes that play an important role in the innate immune response against retroviruses and retrotransposons. APOBEC3G is a member of this family that inhibits HIV-1 replication in the absence of the viral infectivity factor Vif. Inhibition of HIV replication occurs by both deamination of viral single-stranded DNA and a deamination-independent mechanism. Efficient deamination requires rapid binding to and dissociation from ssDNA. However, a relatively slow dissociation rate is required for the proposed deaminase-independent roadblock mechanism in which APOBEC3G binds the viral template strand and blocks reverse transcriptase-catalysed DNA elongation. Here, we show that APOBEC3G initially binds ssDNA with rapid on–off rates and subsequently converts to a slowly dissociating mode. In contrast, an oligomerization-deficient APOBEC3G mutant did not exhibit a slow off rate. We propose that catalytically active monomers or dimers slowly oligomerize on the viral genome and inhibit reverse transcription.

APOBEC3 proteins are DNA-editing enzymes that are part of the innate human immune response to viral pathogens, including retroviruses and retrotransposons[1–4]. Of all the A3 proteins, A3G[5] is the most potent inhibitor of HIV-1 replication[1,4,6], reducing viral infectivity by several orders of magnitude in the absence of the HIV-1 viral infectivity factor Vif[7–9]. In fact, the function of Vif is to specifically counteract the antiviral activity of A3G[1,9].

Although A3G is the most studied of all the APOBEC proteins, the molecular mechanism for A3G-mediated HIV-1 restriction is still not fully understood. A3G is a deoxycytidine deaminase, which converts deoxycytidine bases in single-stranded DNA (ssDNA) to deoxyuridine[5,10–14]. A3G deamination of minus-strand viral DNA formed during reverse transcription results in G to A hypermutation in the plus-strand[11,12,15], which effectively impairs viral replication. However, there are several lines of evidence that suggest that a deaminase-independent mechanism is also involved[1,6,16]. First, A3G catalytic mutants retain antiviral activity[17–20]. Second, A3G inhibits hepatitis B virus replication without G to A hypermutation[21]. Third, A3A inhibits LINE-1 and Alu retrotransposition[22–28] and parvovirus replication[24,29] independent of deaminase activity. Further evidence for a non-editing mechanism is based on the reduction of minus-strand viral DNA levels in HIV-1 particles during endogenous reverse transcription[30], inhibition of reverse transcriptase (RT)-catalysed viral DNA elongation *in vitro* by catalytic A3G mutants[31,32], inhibition of strand transfer reactions *in vitro* and in cell-based assays[32–34], and A3G-induced inhibition of reverse transcription in viruses from human $CD4^+$ T cells[35]. A roadblock model—in which A3G molecules bind the template strand at one or a few locations and physically block viral DNA synthesis—has therefore been proposed as a molecular mechanism for deaminase-independent inhibition[32].

Because only 7 ($\pm$4) A3G molecules are incorporated into each *vif*-deficient virion[36], RT inhibition by an A3G roadblock requires a slow A3G off rate from single-stranded nucleic acids. In contrast, these few A3G molecules must have fast on–off rates to deaminate up to 1,000 sites in several minutes[14] using a rapid search mechanism on viral ssDNA[37,38]. To resolve this apparent paradox, we hypothesize that A3G exhibits fast binding kinetics as a monomer or dimer in order to function as an efficient enzyme, and slow kinetics on oligomerization in order to block RT from elongating viral DNA. To test this idea, we used optical tweezers to monitor A3G binding kinetics on a single DNA molecule.

Results

Single-molecule measurements of A3G binding to ssDNA. For these studies, a single double-stranded λ-DNA molecule was tethered to two polystyrene beads, with one bead held in an optical trap and another on a micropipette tip. As the fixed bead was gradually moved away from the optically trapped bead, the force on the DNA molecule was measured at each extension, yielding a force–extension curve (Fig. 1, solid black line). The solution surrounding the single DNA molecule can be exchanged to measure the effects of DNA-binding ligands on the properties of DNA.

In the absence of binding ligands, force-induced melting occurs at a constant force of 61.0 $\pm$ 0.5 pN, generating ssDNA, either by

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