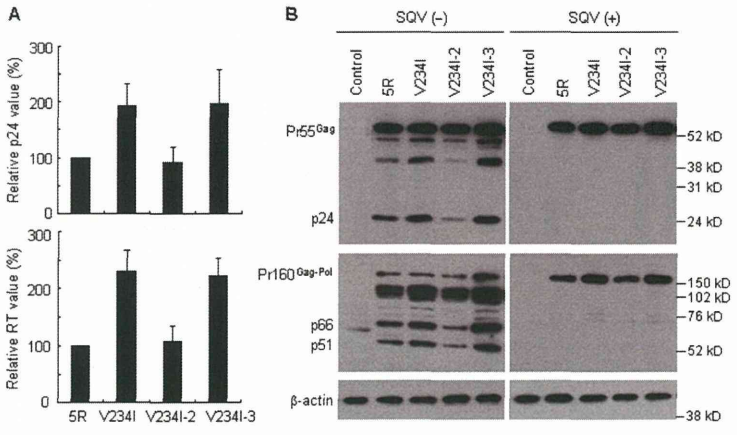


C

	SQV (-)			
	Actin	p24	p66	p51
Control	0.98 ± 0.10	NA	NA	NA
5R	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
V234I	1.18 ± 0.12	2.65 ± 0.42	1.58 ± 0.17	2.55 ± 0.24
V234I-2	1.25 ± 0.13	0.30 ± 0.20	0.32 ± 0.14	0.25 ± 0.15
V234I-3	1.10 ± 0.07	3.07 ± 0.26	3.08 ± 0.71	3.91 ± 0.37

	SQV (+)		
	Actin	$Pr55^{Gag}$	$Pr160^{Gag-Pol}$
Control	0.85 ± 0.11	NA	NA
5R	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
V234I	0.94 ± 0.19	1.22 ± 0.13	1.73 ± 0.10
V234I-2	1.03 ± 0.28	0.73 ± 0.18	0.61 ± 0.11
V234I-3	0.90 ± 0.16	1.34 ± 0.19	1.84 ± 0.17

FIG 6 Effect of codon alterations at the V234I site on intracellular viral protein expression. (A) Expression levels of Gag-p24 and Pol-RT as determined by ELISA. 293T cells were transfected with the indicated proviral clones, and on day 2 posttransfection, lysates were prepared from transfected cells for the analysis of Gag-p24 and Pol-RT. The amounts of Gag-p24 and Pol-RT relative to those expressed by 5R are presented. Mean values ± SD from three independent experiments are shown. (B) Expression pattern of viral proteins as analyzed by Western blotting. 293T cells were transfected with the indicated proviral clones in the absence (−) or presence (+) of 2 μM SQV. Cell lysates were prepared from transfected cells on day 1 posttransfection, and analyzed by Western blotting using anti-p24 (upper panels), anti-RT (middle panels), and anti-β-actin (lower panels) antibodies. The migration positions of mass standards are indicated on the right. Representative data from two independent transfection experiments are shown. Control, pUC19. (C) Quantitative analysis of the Western blot. Signal intensities of viral proteins were quantitated, and the intensities relative to those with 5R proteins are shown. Mean values ± SD obtained from the two independent transfection experiments in panel B are indicated. NA, not applicable.

nucleotide synonymous mutations) in the 3′ region of the HIV-1 *pol* gene (nt 4889 to 4923 for 5R and nt 4895 to 4929 for NL4-3) can alter viral replication potential.

Natural synonymous changes influence the expression levels of intracellular viral proteins (Gag, Gag-Pol, Vpu, and Env) but not those of Nef and Rev. Gag/Gag-Pol expression levels in V234I codon variants varied correlatively with their virion production/replication abilities (Fig. 5 and 6). Env, in addition to Gag/Gag-Pol, is of course required for infectious virion formation. HIV-1-encoded proteins are translated from distinct viral mRNA species, and the regulation of gene expression is different for early transcripts (completely spliced form) and late transcripts (singly spliced and unspliced forms). To investigate further the virological effect of single-nucleotide natural variations, we determined the expression levels of products from various mRNA species: Gag and Gag-Pol from the unspliced form, Vpu and Env from the singly spliced form, and Nef and Rev from the completely spliced form. Proviral clones (5R, Y226tac, D229gat, P233ccc, V234I, and V234gtg) were transfected into 293T cells, and cell lysates prepared on day 1 posttransfection were analyzed by Western blotting. Although V234I was not a synonymous change in the *Pol*4889-4923 region, this clone was included in this analysis because V234I is a single-nucleotide natural variant of V234 in 5R, and its virion production/replication ability was increased or decreased by single-nucleotide substitutions at this position (Table 2). As shown in Fig. 8A, we confirmed that virion production levels in transfected 293T cells correlated with viral replication potential (Fig. 2, 7, and 8A) (upregulated, Y226tac, D229gat, and V234I; downregulated, P233ccc and V234gtg). We then examined the intracellular expression level of each viral protein (Fig. 8B).

TABLE 2 Amino acid/codon frequency in the 3′ region of the *pol* gene (nt 4889 to 4923 for 5R and nt 4895 to 4929 for NL4-3) of HIV-1/SIVcpz[a]

Amino acid position in IN	Amino acid frequency[b]			Codon frequency[b]			
	Amino acid	No.	%	Codon	No.	%	Growth[c]
222	N[d]	179	91.3	AAT[d]	175	89.3	++
				AAC	4	2.0	++/+++
	K[e]	14	7.1	AAA[e]	14	7.1	+++
	Q	2	1.0	CAA	2	1.0	ND[f]
	D	1	0.5	GAT	1	0.5	ND
223	F[d]	196	100.0	TTT[d]	183	93.4	++
				TTC	13	6.6	++/+++
	Y[e]	0	0.0	TAT[e]	0	0.0	+++
225	V[d]	195	99.5	GTT[d]	175	89.3	++
				GTC	20	10.2	++
	L	1	0.5	CTT	1	0.5	ND
226	Y[d]	194	99.0	TAT[d]	193	98.5	++
				TAC	1	0.5	+++
	F	1	0.5	TTT	1	0.5	ND
	H	1	0.5	CAT	1	0.5	ND
229	D[d]	196	100.0	GAC[d]	194	99.0	++
				GAT	2	1.0	+++
	E[e]	0	0.0	GAA[e]	0	0.0	+++
233	P[d]	194	99.0	CCA[d]	135	68.9	++
				CCT	35	17.9	+
				CCC	23	11.7	+
				CCG	1	0.5	+
	S	1	0.5	TCA	1	0.5	ND
	T	1	0.5	ACC	1	0.5	ND
234	V[d]	26	13.3	GTT[d]	25	12.8	++
				GTG	1	0.5	+
	I[e]	128	65.3	ATT[e]	127	64.8	+++
				ATC	1	0.5	+
	L	40	20.4	CTT	39	19.9	ND
				CTG	1	0.5	ND
	S	1	0.5	AGC	1	0.5	ND
	T	1	0.5	ACT	1	0.5	ND

[a] For 196 sequences of HIV-1/SIVcpz complete genomes from the HIV Sequence Compendium, 2011 (Los Alamos National Laboratory; http://www.hiv.lanl.gov).
[b] Relative to 196 sequences of HIV-1/SIVcpz strains.
[c] +++, replication peaked earlier or virus production levels on the peak day were higher than those of the parental clones; ++, replication kinetics were similar to those of the parental clones; +, replication peaked later or virus production levels on the peak day were lower than those of the parental clones. Data were obtained in M8166 or MT4/CCR5 cells.
[d] Amino acid encoded or codon usage in parental clones.
[e] Amino acid encoded or codon usage in adapted (growth-enhanced) viruses.
[f] ND, not done.

The intracellular expression levels of Gag, Gag-Pol, Vpu, and Env varied among the clones tested and correlated with viral replication potential: clones with enhanced growth efficiencies expressed higher levels of these proteins, and vice versa (Fig. 2, 7, and 8B and C). In contrast, the expression levels of Nef and Rev by all proviral clones tested were similar to that of 5R, and they were constant for all variants with different virion production/replication potentials (Fig. 2, 7, and 8). Furthermore, Tat activity was determined by cotransfection assays with 5R, its single-nucleotide variants, and the 5RLTR-Luc reporter construct. No significant difference in the abilities of these proviral clones to *trans*-activate luciferase gene expression was observed, which indicated that 5R and its variants had similar Tat activity (Fig. 8D). These results suggest that single-nucleotide changes in the *Pol*4889-4923 region can alter virion production/replication potential by modulating the expression levels of late (but not early) viral proteins.

Naturally occurring single-nucleotide variations change the expression patterns of HIV-1 mRNA species. Naturally occurring single-nucleotide mutations that alter the expression levels of viral late proteins were Y226tac, D229gat, V234I, P233ccc, and V234gtg (Fig. 8). We noted that these mutations clustered in the region proximal to the splice acceptor A1 (SA1) site (designated SA1prox in Fig. 9A). To determine the mechanistic basis for the altered phenotype, we analyzed the effect of single-nucleotide changes on the profiles of viral mRNA expression. 293T cells were transfected with parental clones (5R and NL4-3) or their mutants,

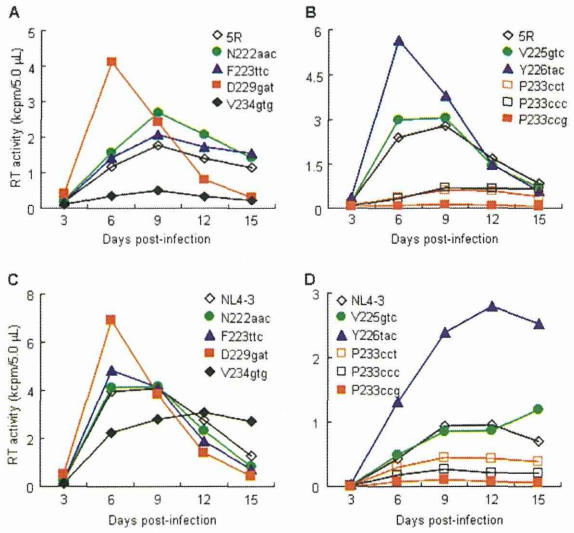


FIG 7 Effect of single-nucleotide synonymous changes in the *Pol*4889-4923 region on viral replication potential. Viruses were prepared from transfected 293T cells, and equal amounts were inoculated into MT4/CCR5 cells. Virus replication was monitored by RT activity released into the culture supernatants. Representative data from two independent experiments are shown.

and DNase I-treated poly(A)$^+$ RNAs were prepared from total cell RNAs at 10 to 20 h posttransfection. Northern blot analysis of these samples using a probe (U probe) to detect all HIV-1 mRNA species (Fig. 9B) was then performed (Fig. 9C). Because no significant difference in the RNA expression patterns for the samples of each clone prepared at 10 and 20 h posttransfection was noted (data not shown), we comparatively analyzed viral mRNAs in cells at the latter time point. As shown in Fig. 9C, the expression levels of viral late proteins did not directly correlate with the steady-state levels of their respective transcripts (9 kb and 4 kb) (Fig. 8B and 9C, left panel). The total amounts of viral mRNAs, especially the 1.8-kb mRNA species, were lower for the clones with increased viral late protein expression (Y226tac, D229gat, and V234I) than for 5R and the other mutants (P233ccc and V234gtg). However, single-nucleotide mutations always gave unique viral mRNA expression profiles. Several bands (*1 to *4 in Fig. 9C, left panel) other than the three major viral RNA species (1.8 kb, 4 kb, and 9 kb) were more or equally intense for P233ccc and V234gtg, with the poor expression of late proteins, and less intense for Y226tac, D229gat, and V234I, with the high expression of late proteins, than for 5R. Similar results, albeit to a lesser extent, were obtained for NL4-3 and its mutants with higher or lower abilities to replicate in cells (Fig. 9C, right panel). As such, viruses with a relatively high replication ability in each group (5R and NL4-3) appeared to be tuned up to have three major mRNA species. Northern blot analysis using HeLa cells was performed to exclude the possibility that the viral mRNA expression pattern described above was 293T cell specific. Results similar to those in 293T cells were obtained for 5R, NL4-3, and their mutants (D229gat and V234gtg) (data not shown). Taken together, our results here show that single-nucleotide changes in the SA1prox affect the expression patterns of viral mRNA species and suggest that this transition may lead to the enhancement or reduction in viral late protein expression/replication efficiency.

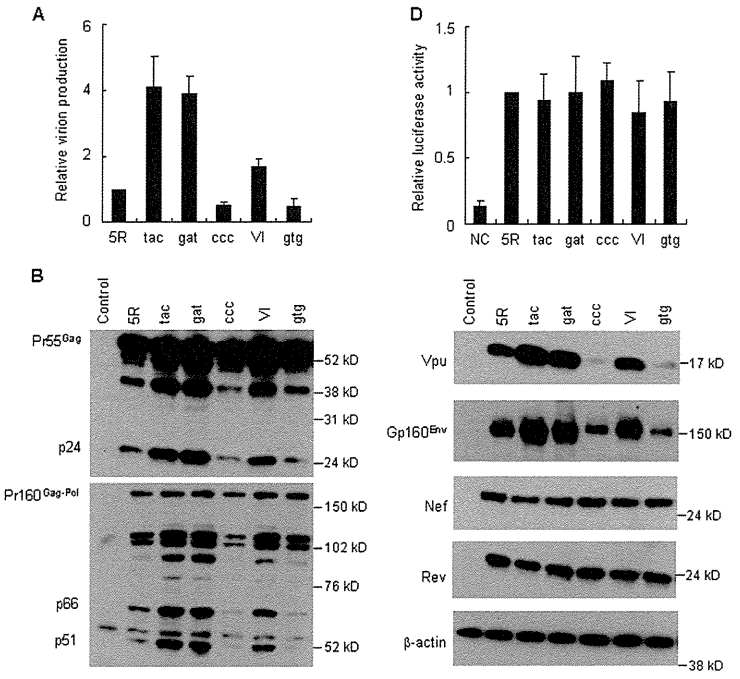
To identify the nature of transcripts detected as extra bands (*1 to *4) (Fig. 9C), we performed Northern blot analysis using RRE, vif, and vpr probes (Fig. 9B). As expected, 9-kb transcripts but not 1.8-kb transcripts were detected by the RRE, vif, and vpr probes (Fig. 9D). While all transcripts longer than 4 kb were detected by the RRE probe, the vif and vpr probes recognized the species *1 to *3 and *1 to *4, respectively. The *2 band was more intense with the vpr probe than with the vif probe. Thus, the *1 species contained the *vif* transcript (Fig. 9B), and the *2 band consisted mainly of the *vpr* transcript (Fig. 9B). Transcripts *3 and *4 contained the Vif/Vpr-coding region and Vpr-coding region, respectively, without the RRE region.

Northern blot analysis revealed that the lower expression of late proteins by 5R, P233ccc, and V234gtg was linked to the abundance of transcripts, especially the *1 and *3 species. We hypothesized that these abundant transcripts may disturb the expression of late proteins. To test this possibility, we performed interference assays by the cotransfection of D229gat and 5R/V234gtg (Fig. 10). If the extra transcripts *1 and *3 interfere with translation from transcripts corresponding to late proteins, the Gag-p24 expression levels of D229gat would be proportionally decreased upon cotransfection with increasing amounts of 5R or V234gtg. As shown in Fig. 10 (top and middle panels), p24 expression levels were increased in cells upon single transfection of D229gat, 5R, or V234gtg with an increasing DNA amount, and marked differences were observed between each clone (D229gat > 5R > V234gtg). When a constant amount of D229gat and an increasing amount of 5R or V234gtg were cotransfected, p24 expression levels reflected just the addition of the amount produced by each clone. Virion production from cells correlated well with the intracellular p24 expression levels (data not shown). Cotransfection assays using D229gat and the *gag/gag-pol* frameshift mutant of V234gtg (gtg-Spe), which is incapable of producing p24, were performed to confirm this result. As is clearly observed in Fig. 10 (bottom panel), the increase in the amount of the gtg-Spe clone did not affect p24 expression levels produced from D229gat. Taken together, these results suggest that a large amount of transcripts (especially the *1 and *3 species) in 5R and V234gtg clones does not interfere with Gag-p24 expression and also that the single-nucleotide changes in the SA1prox act on the expression of late proteins in *cis*.

DISCUSSION

In this study, we demonstrated that four adaptive mutations in the 3′ region of the *pol* gene encoding IN upregulated the viral replication potential by increasing virion production levels without any effects on the early replication phase (Fig. 2). Moreover, the identification of V234I codon variants that have different abilities to produce virions and replicate in cells suggested regulation by single-nucleotide changes (Fig. 5). A comparative investigation of nucleotide sequences in the 3′ region of the *pol* gene (nt 4889 to 4923 for 5R and nt 4895 to 4929 for NL4-3) has revealed that these variants naturally coexist in a viral population (Table 2). We show here that naturally occurring synonymous changes (Y226tac, D229gat, P233cct/ccc/ccg, and V234gtg) can alter the viral replication potentials of HIV-1 5R and NL4-3 (Fig. 7 and Table 2).

The naturally occurring single-nucleotide variations that alter viral replication potential clustered in the SA1prox (Fig. 9A).



C

	Actin	p24	p66	p51
Control	0.86 ± 0.16	NA	NA	NA
5R	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
tac	1.03 ± 0.04	3.80 ± 0.66	4.36 ± 1.86	6.78 ± 2.09
gat	0.99 ± 0.03	4.23 ± 0.63	4.16 ± 1.06	6.48 ± 0.89
ccc	1.09 ± 0.09	0.19 ± 0.05	0.14 ± 0.05	0.08 ± 0.05
VI	1.03 ± 0.11	2.25 ± 0.62	2.82 ± 0.75	3.08 ± 1.07
gtg	0.94 ± 0.04	0.28 ± 0.13	0.12 ± 0.02	0.05 ± 0.04

	Vpu	Env	Nef	Rev
Control	NA	NA	NA	NA
5R	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
tac	2.61 ± 0.01	2.06 ± 0.58	0.94 ± 0.18	0.90 ± 0.10
gat	2.19 ± 0.03	1.96 ± 0.33	1.01 ± 0.08	0.96 ± 0.06
ccc	0.10 ± 0.13	0.35 ± 0.03	1.12 ± 0.20	1.12 ± 0.13
VI	1.49 ± 0.14	1.55 ± 0.34	1.03 ± 0.06	1.01 ± 0.10
gtg	0.10 ± 0.09	0.35 ± 0.10	0.99 ± 0.14	1.04 ± 0.16

FIG 8 Effect of single-nucleotide changes on virion production and intracellular expression levels of viral proteins. (A) Virion production in transfected cells. 293T cells were transfected with the indicated proviral clones, and on day 1 posttransfection, virion production was monitored by the amount of Gag-p24 in the culture supernatants. The amount of p24 relative to that produced by 5R is presented. Mean values ± SD from three independent experiments are shown. (B) Expression of various viral proteins in transfected cells. 293T cells were transfected with the indicated proviral clones, and on day 1 posttransfection, cell lysates were prepared for Western blotting using anti-Gag-p24, anti-RT, anti-Vpu, anti-Env-gp160, anti-Nef, anti-Rev, and anti-β-actin antibodies. The migration positions of mass standards are indicated on the right. Representative data from two independent transfection experiments are shown. Control, pUC19; tac, Y226tac; gat, D229gat; ccc, P233ccc; VI, V234I; gtg, V234gtg. (C) Quantitative analysis of the Western blot. Signal intensities of viral proteins were quantitated, and the intensities relative to those for 5R proteins are shown. Mean values ± SD obtained from the two independent transfection experiments in panel B are indicated. NA, not applicable. (D) Analysis of Tat activity. 293T cells were cotransfected with the indicated proviral clones and an LTR-driven luciferase reporter clone, and on day 1 posttransfection, cell lysates were prepared for luciferase assays. Luciferase activity relative to that exhibited by 5R is presented. Mean values ± SD from three independent experiments are shown. NC, negative control (basal luciferase activity of the LTR-driven luciferase reporter clone in the absence of proviral clones).

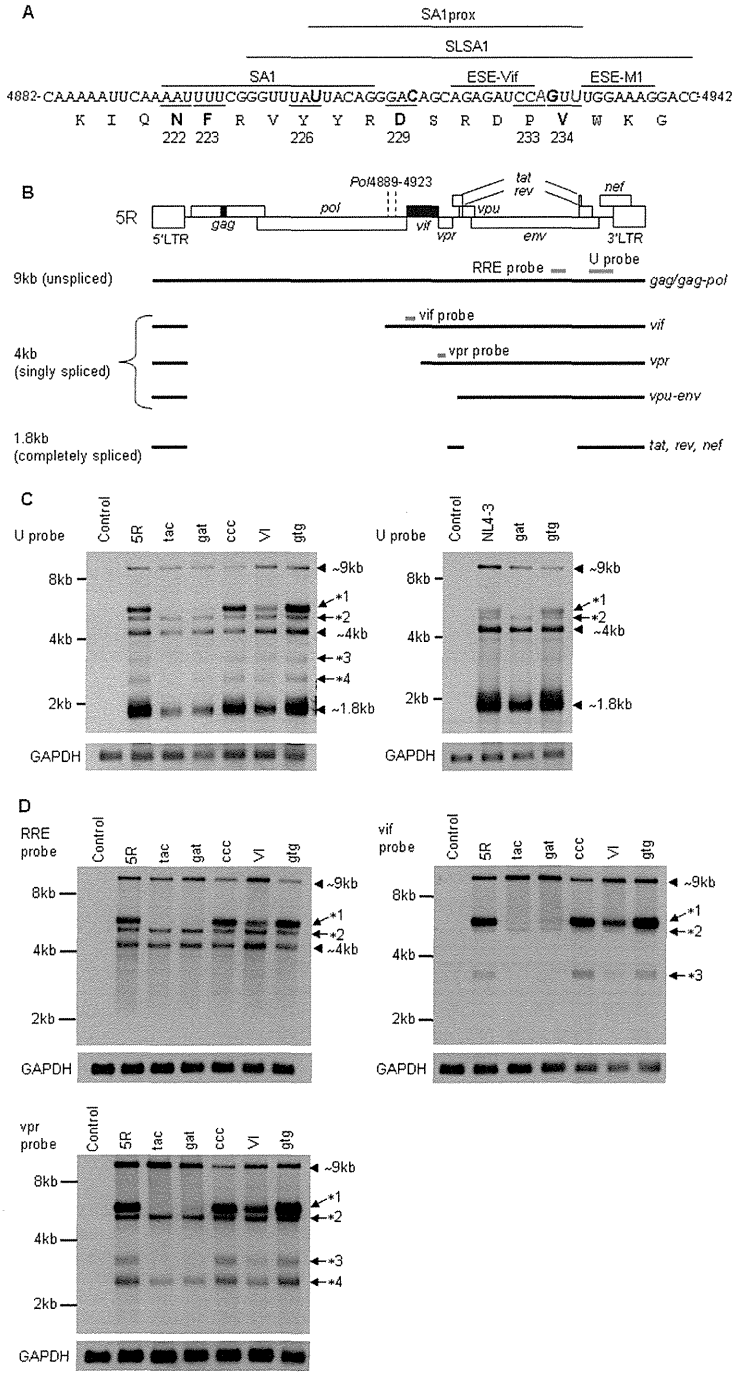


FIG 9 Effect of single-nucleotide changes on viral mRNA expression. (A) Nucleotide and amino acid sequences in the 3′ region of the *pol* gene. Nucleotides 4882 to 4942 correspond to the NL4-3 sequence (35) (GenBank accession no. AF324493). Black and gray bold letters in the nucleotide sequence show the sites at which the single-nucleotide substitution promoted or decreased viral replication efficiency, respectively. Bold letters in the amino acid sequence represent adaptive mutation sites. The SA1prox (this study), SA1 site (3, 4), ESE-Vif (27), ESE-M1 (28), and SLSA1 region (45) are as indicated. Numbers and underlines show the positions of amino acids in IN and their codons, respectively. (B) Schematic presentation of the 5R genome organization and various HIV-1 mRNA species. The genome structure of 5R is shown as in Fig. 1. Gray bars represent the regions used as probes for Northern blot analyses. The *Pol*4889-4923 region is also indicated.

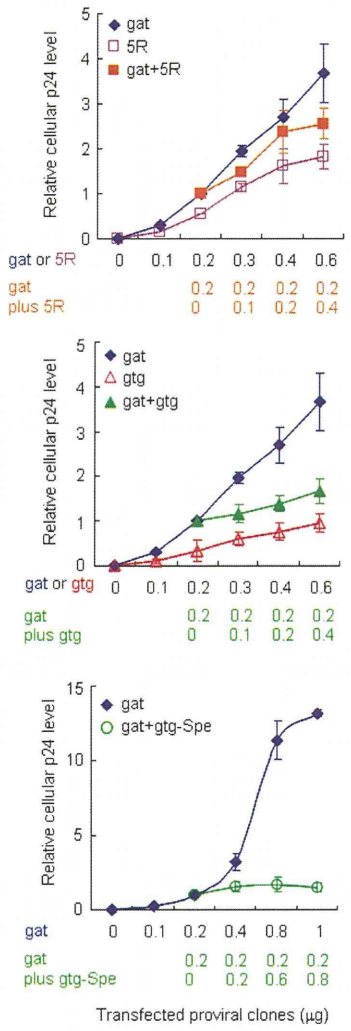


FIG 10 Expression level of Gag-p24 in singly or doubly transfected cells. 293T cells were transfected with proviral clones as indicated, and on day 1 posttransfection, Gag-p24 expression level in cell lysates was determined. The amount of p24 relative to that produced upon transfection with 0.2 μg of the D229gat (gat) clone is presented. Mean values ± SD from three independent experiments are shown. gat, D229gat; gtg, V234gtg; gtg-Spe, a frameshift mutant of V234gtg.

These mutations were identified by both viral adaptation experiments (32) and comparative analysis of numerous HIV-1/SIVcpz genomes (Table 2). These findings suggest that the nucleotide sequence in the SA1prox may be involved in the viral adaptation/evolution process. Recent RNA structure analysis has shown that the nucleotide sequence proximal to SA1 within the HIV-1 genome forms a stem-loop structure (45, 46). Moreover, Pollom et al. reported that this stem-loop structure, designated "SLSA1," was conserved between HIV-1 NL4-3 and SIVmac239, suggesting the virological importance of the SLSA1 structure (45). Interestingly, all single-nucleotide mutations analyzed in Fig. 8 and 9 were mapped onto the SLSA1 sequence (at positions 4901 to 4942 in Fig. 9A). The emergence of novel mutations within this region may be limited due to its effect on IN functions, SA1 functions, and the SLSA1 structure. This may explain the low frequency of single-nucleotide mutations that alter viral replication efficiencies among HIV-1 genomes (Table 2). Nevertheless, the presence of such single-nucleotide variations in SLSA1 represents the plasticity of viruses with the ability to adapt themselves under various constraints. Our results on the replication-altering mutations within SLSA1 may also be useful for analyzing changes in the RNA sequence/structure and their effects on viral replication. Because the SLSA1 structure is conserved between HIV-1 NL4-3 and SIVmac239, it is of interest to determine whether naturally occurring single-nucleotide synonymous mutations in this region affect the replication efficiency of SIVmac239 and its closely related primate lentiviruses.

The change in virion production/replication ability was reflected in the expression levels of viral late proteins (Gag, Gag-Pol, Vpu, and Env) but not in those of the early proteins (Nef and Rev) (Fig. 7 and 8). However, a direct positive correlation between the steady-state levels of viral mRNAs and their corresponding late proteins was not observed (Fig. 8 and 9). More viral RNAs and a large number of viral RNA species were synthesized in cells producing low expressors of viral late proteins (5R, P233ccc, and V234gtg) than in those producing high expressors (Y226tac, D229gat, and V234I). This may imply that the expression level of mRNAs directed by high expressors is necessary and sufficient for the efficient expression of viral proteins and optimal viral replication. We initially assumed that various kinds of viral transcripts by low expressors may hinder the efficient translation of viral late proteins. However, interference assays between variants (Fig. 10) showed that this prediction may not be accurate. Moreover, no significant difference in the Tat activity of 5R and its variants was observed (Fig. 8D). Therefore, it is reasonable to assume that single-nucleotide changes in the SA1prox act in *cis* and may influence splicing, mRNA stability, mRNA transport, and/or translation efficiency from mRNAs.

The abundance of the *vif* transcript (species *1 in Fig. 9C and D) observed for 5R and low expressors (P233ccc and V234gtg) suggests enhanced splicing at the SA1 site. On one hand, a combination of two synonymous mutations in SLSA1 that changes its RNA structure was reported to affect splicing at the SA3 site downstream of SA1 but not that at the SA1 site, showing long-range RNA interactions and cross talk between splicing sites (45). This may explain variations in the expression levels of transcripts (the

RRE, Rev-responsive element; U, universal. (C and D) Steady-state expression levels of HIV-1 mRNA species. Total RNA was prepared from 293T cells transfected with the indicated proviral clones, and poly(A)$^+$ RNA was selected. After the DNase I treatment, samples were subjected to Northern blot analysis using the indicated probe. GAPDH was used as an internal standard. Three major species of viral mRNA (~9 kb, ~4 kb, and ~1.8 kb) are shown by arrowheads. The other extra bands *1 to *4 are indicated by arrows. RNA size markers (8 kb, 4 kb, and 2 kb) are on the left. Representative data from four independent experiments (C, left panel [5R and its mutants]) and from two independent experiments (C, right panel [NL4-3 and its mutants] and D) are shown. Control, pUC19; tac, Y226tac; gat, D229gat; ccc, P233ccc; VI, V234I; gtg, V234gtg.

*3, *4, and 1.8-kb species) among 5R/low expressors and high expressors. Since these transcripts do not contain the RRE region, it is clear that they are generated via splicing downstream of SA1. The abundance of these transcripts (*3, *4, and 1.8-kb species) for 5R and low expressors may also imply that the overall splicing efficiency of these clones is higher than that of high expressors. Efficient splicing at SA sites may compete with Rev function, and equilibrium between the strength of splicing acceptors and Rev function for the nuclear export of Rev-dependent mRNAs is important for virus replication (28). Thus, increased splicing for 5R and low expressors may obstruct the function of Rev, which results in a decrease in the Rev-dependent expression of late proteins from RRE-containing transcripts. On the other hand, while the amounts of 1.8-kb mRNAs of 5R and low expressors were larger than those of high expressors, the expression levels of viral early proteins were similar among 5R and its variants. A high concentration of Rev was previously shown to inhibit the translation from various RNAs (47). It is possible that the expression of viral early proteins may be regulated at an optimal level for viral replication. Alternatively, the translation efficiency of ~40 mRNA isoforms synthesized by alternative splicing events may vary due to differences in their noncoding sequences and/or structures. Viral mRNA species within 1.8-kb and 4-kb RNAs were shown to be altered by mutations that change splicing efficiency at SA1 or the structure of SLSA1 (27, 45, 48). Viral mRNA isoforms with a low translation efficiency, even if present in abundance, may not express a high level of their corresponding proteins.

vif mRNA expression is strongly influenced by splicing efficiency at the SA1 site. The regulation of splicing at SA1 is complicated and is determined by various elements, including three different exonic splicing enhancers (ESE-Vif and ESE-M1 [Fig. 9A] and ESE-M2 [nt 4956 to 4962 in NL4-3]), a suboptimal D2 splicing site (nt 4960 to 4970 in NL4-3), a GGGG silencer (nt 4968 to 4971 in NL4-3), and a G run (G_{12}-1, nt 5034 to 5038 in NL4-3), which are located within the region from SA1 to just upstream of the *vif* start codon (nt 5041 in NL4-3) (27, 28, 48). The proviral clone 5R was constructed by introducing SIVmac239 *vif* into the downstream region of the *pol* open reading frame in the NL4-3 genome (Fig. 1 and 9B) (34). As a result, while the *pol* and *vif* genes of NL4-3 partially overlap, those of 5R do not. Since splicing efficiency is dependent on the sequence around the splice sites and their distance from the regulatory elements, the insertion of SIVmac239 *vif* into NL4-3 may have changed the splicing event at SA1. Indeed, 5R produced abundant amounts of the *vif* transcript (the *1 species in Fig. 9). The increase in *vif* mRNA was previously shown to decrease virion production, and the proportion between unspliced and spliced mRNAs has been suggested to be important for virion production (27). In agreement with this finding, we found that the virion production level from 293T cells transfected with 5R was lower than that from cells transfected with NL4-3 (data not shown). The decrease in *vif* transcript (*1 species) expression for high expressors may have caused the increase in virion production.

The splicing balance of viral mRNAs has been suggested to have biologically significant effects on viral replication (4, 9–11). Accumulating evidence has shown that HIV-1 gene expression processes, composed of transcription, poly(A) tailing, splicing, mRNA export, and subsequent translation, are mutually affected and coupled, even though these processes are biochemically distinguished (1, 2, 49). In addition, various elements within the HIV-1 genome and a number of virus/host factors have been shown to be involved in HIV-1 gene expression (3, 4, 9–11, 25–30, 50–55). The virological importance of the nucleotide sequence in the SA1prox is evident from the increase or decrease in viral replication caused by naturally occurring single-nucleotide changes. Further studies are needed to elucidate the molecular mechanism underlying the modulation of overall HIV-1 gene expression generated by single-nucleotide changes in the SA1prox.

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We declare that no competing interests exist.

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Lack of Association between Intact/Deletion Polymorphisms of the *APOBEC3B* Gene and HIV-1 Risk

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Abstract

Objective: The human APOBEC3 family of proteins potently restricts HIV-1 replication *APOBEC3B*, one of the family genes, is frequently deleted in human populations. Two previous studies reached inconsistent conclusions regarding the effects of *APOBEC3B* loss on HIV-1 acquisition and pathogenesis. Therefore, it was necessary to verify the effects of APOBEC3B on HIV-1 infection *in vivo*.

Methods: Intact (I) and deletion (D) polymorphisms of *APOBEC3B* were analyzed using PCR. The syphilis, HBV and HCV infection rates, as well as $CD4^+$ T cell counts and viral loads were compared among three *APOBEC3B* genotype groups (I/I, D/I, and D/D). HIV-1 replication kinetics was assayed *in vitro* using primary cells derived from PBMCs.

Results: A total of 248 HIV-1-infected Japanese men who have sex with men (MSM) patients and 207 uninfected Japanese MSM were enrolled in this study. The genotype analysis revealed no significant differences between the *APOBEC3B* genotype ratios of the infected and the uninfected cohorts ($p = 0.66$). In addition, HIV-1 disease progression parameters were not associated with the *APOBEC3B* genotype. Furthermore, the PBMCs from D/D and I/I subjects exhibited comparable HIV-1 susceptibility.

Conclusion: Our analysis of a population-based matched cohort suggests that the antiviral mechanism of APOBEC3B plays only a negligible role in eliminating HIV-1 *in vivo*.

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Introduction

Human APOBEC3 proteins are cellular cytidine deaminases that play crucial roles in the inhibition of retroviral replication, including that of HIV-1 [1–3]. The molecular mechanisms underlying APOBEC3-mediated HIV-1 restriction are primarily dependent on the editing [1,2] and/or non-editing activities [4,5] of these enzymes. The family of genes encoding the seven APOBEC3 proteins (APOBEC3A, B, C, DE, F, G, and H) is positioned in a tandem array on human chromosome 22 [6]. HIV-1 produces an accessory protein, Vif, that invalidates the antiviral functions of the APOBEC3 proteins by mediating the ubiquitination-proteasomal degradation of APOBEC3 in virus-producing cells [7]. APOBEC3C, DE, F, G, and H (haplotype II) are vulnerable to HIV-1 Vif-mediated degradation, whereas APOBEC3A and B are resistant [8–12].

Among the members of the APOBEC3 family, APOBEC3G has been consistently shown to possess powerful anti-HIV-1 activity in cell-based systems [1,2], and this protein may affect the pathogenesis of HIV-1 infection *in vivo* [13–19]. However, there is little consensus regarding the degree to which the other APOBEC3 family members, especially APOBEC3B, are able to restrict HIV-1 replication *in vitro* and *in vivo*. The anti-HIV-1 activity of APOBEC3B is undetectable when this gene is stably expressed in a human T cell line [20] and is detected only weakly after the transient transfection of HEK 293T or HeLa cells [20–22]. Because these findings have varied according to the experimental conditions employed, there is a fundamental