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Ⅴ.　研究論文抜粋

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Natural Single-Nucleotide Polymorphisms in the 3′ Region of the HIV-1 *pol* Gene Modulate Viral Replication Ability

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ABSTRACT

We previously showed that prototype macaque-tropic human immunodeficiency virus type 1 (HIV-1) acquired nonsynonymous growth-enhancing mutations within a narrow genomic region during the adaptation process in macaque cells. These adaptive mutations were clustered in the 3′ region of the *pol* gene, encoding a small portion of the C-terminal domain of integrase (IN). Mutations in HIV-1 IN have been reported to have pleiotropic effects on both the early and late phases in viral replication. *cis*-acting functions in the IN-coding sequence for viral gene expression have also been reported. We here demonstrated that the adaptive mutations promoted viral growth by increasing virion production with no positive effects on the early replication phase. Synonymous codon alterations in one of the adaptive mutations influenced virion production levels, which suggested nucleotide-dependent regulation. Indeed, when the single-nucleotide natural polymorphisms observed in the 3′ regions of 196 HIV-1/simian immunodeficiency virus (SIVcpz) *pol* genes (nucleotides [nt] 4895 to 4929 for HIV-1 NL4-3) were introduced into macaque- and human-tropic HIV-1 clones, more than half exhibited altered replication potentials. Moreover, single-nucleotide mutations caused parallel increases or decreases in the expression levels of viral late proteins and viral replication potentials. We also showed that the overall expression profiles of viral mRNAs were markedly changed by single-nucleotide mutations. These results demonstrate that the 3′ region of the HIV-1 *pol* gene (nt 4895 to 4929) can alter viral replication potential by modulating the expression pattern of viral mRNAs in a nucleotide-dependent manner.

IMPORTANCE

Viruses have the plasticity to adapt themselves under various constraints. HIV-1 can mutate and evolve in growth-restrictive cells by acquiring adaptive changes in its genome. We have previously identified some growth-enhancing mutations in a narrow region of the IN-coding sequence, in which a number of *cis*-acting elements are located. We now focus on the virological significance of this *pol* gene region and the mechanistic basis underlying its effects on viral replication. We have found several naturally occurring synonymous mutations within this region that alter viral replication potentials. The effects caused by these natural single-nucleotide polymorphisms are linked to the definite expression patterns of viral mRNAs. We show here that the nucleotide sequence of the *pol* gene (nucleotides 4895 to 4929 for HIV-1 NL4-3) plays an important role in HIV-1 replication by modulating viral gene expression.

The gene expression process of human immunodeficiency virus type 1 (HIV-1) (transcription, capping, polyadenylation, splicing, nuclear export, and translation) is highly coordinated and regulated by interactions between host/viral proteins and *cis*-acting elements located within the viral genome (1, 2). During this process, more than 40 mRNA species with nine viral genes are generated by alternative splicing (3, 4). These mRNA species are divided into three major groups: ~9-kb mRNAs (unspliced form) encoding Gag and Gag-Pol proteins, ~4-kb mRNAs (singly spliced form) encoding Vif, Vpr, Vpu, and Env proteins, and ~1.8-kb mRNAs (completely spliced form) encoding Tat, Rev, and Nef proteins. In the early phase of HIV-1 gene expression, ~1.8-kb mRNA species are transported to the cytoplasm and translated to synthesize Tat, Rev, and Nef proteins. Tat, along with some host factors, *trans*-activates HIV-1 transcription (5, 6). Rev facilitates the nuclear export of ~4-kb and ~9-kb HIV-1 mRNAs, and their encoded proteins are subsequently produced (7, 8). Alterations in the tightly regulated process of HIV-1 gene expression can affect viral replication (3, 4, 9–11).

HIV-1 integrase (IN) is generated from a Gag-Pol precursor and mediates integration, a hallmark of retroviruses. HIV-1 IN is involved not only in integration but also in reverse transcription, viral DNA nuclear import, and virion assembly/production (12–19). The deletion or C-terminal truncation of HIV-1 IN has been shown to reduce virion production in producer cells (20, 21). Although mutations in IN have negative effects on virion production, they also affect the early phase of viral replication (15, 22). Different amino acid substitutions at the same sites often have diverse effects on viral replication potential (13, 15, 23, 24). Furthermore, a number of splicing sites and *cis*-acting elements have been identified in the IN-coding sequence (3, 4, 25–30). There-

Received 9 July 2013 Accepted 20 January 2014
Published ahead of print 29 January 2014
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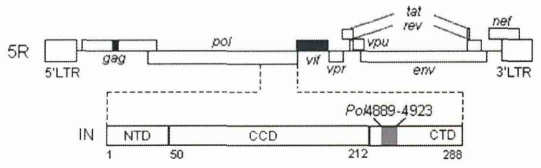
doi:10.1128/JVI.01859-13



Amino acid position in IN	5R		Adapted clones	
	Amino acid	Codon	Amino acid	Codon
222	N	AAT	K	AAA
223	F	TTT	Y	TAT
229	D	GAC	E	GAA
234	V	GTT	I	ATT

FIG 1 Growth-enhancing mutations identified in the 3′ region of the *pol* gene. The genome structure of 5R (34) (GenBank accession no. AB266485) is schematically depicted. Black areas show the sequence derived from SIVmac239 (56) (GenBank accession no. M33262). The *pol* region encoding IN and the IN domain structure are indicated. The gray area represents a region in the *pol* gene (nt 4889 to 4923) designated *Pol*4889-4923, in which adaptive mutations are located. NTD, N-terminal domain; CCD, catalytic core domain; CTD, C-terminal domain. Details of the adaptive mutations found in the *Pol*4889-4923 region are shown at the bottom.

fore, mutations in the IN-coding sequence can change the nucleotide sequence important for viral replication as well as the protein-coding sequence associated with IN activity. The replication-defective mutant IN E246K represents a good example. It showed a processing defect in Gag, and its virion production level was markedly reduced (15). The E246K mutation, which is located within splicing site D2 and affects viral RNA splicing, was shown to result in the loss of viral infectivity (31). Thus, *cis*-acting functions in the IN-coding sequence must be considered to delineate its possible roles in viral replication.

In a previous virus adaptation study, we demonstrated that growth-enhanced viruses, which emerged following a long-term culture of cells infected with macaque-tropic HIV-1 NL-DT5R (5R) or NL-DT562 (562), frequently and reproducibly acquired mutations in the 3′ region of the *pol* gene (nucleotides [nt] 4889 to 4923 for 5R [Fig. 1] and nt 4895 to 4929 for the standard HIV-1 NL4-3), which encodes a small portion of the IN C-terminal domain (CTD) (32). Four adaptive mutations (N222K, F223Y, D229E, and V234I) in the region were identified in our repeated virus adaptation experiments and were responsible for viral growth enhancements (32). In this study, we aimed to elucidate how mutations in the 3′ region of the *pol* gene promoted viral replication and what the virological significance of this region was. The four mutations mentioned above were found to augment virus replication potential by increasing infectious virion production without any effects on the early replication phase. The CTD has been reported to be the least conserved sequence of the three domains in IN (15, 17, 19), with the 234th amino acid in IN being polymorphic (33). Codon alterations in V234I from ATT to ATC and ATA influenced virion production, which indicated regulation by a single-nucleotide change. An investigation of the sequences in the 3′ region of the *pol* gene in 196 HIV-1/simian immunodeficiency virus (SIVcpz) genomes (HIV sequence compendium 2011, Los Alamos National Laboratory, NM, USA) revealed that natural variants carried ATT or ATC at amino acid position 234. Based on these findings, single-nucleotide natural variations in the 3′ region of the *pol* gene (nt 4895 to 4929 for NL4-3) were introduced into macaque- and human-tropic HIV-1 clones. We identified several natural variations that alter virion production/replication efficiency. The observed effects of single-nucleotide variations were attributed to an increase or decrease in the expression levels of viral late proteins (Gag, Gag-Pol, Vpu, and Env), whereas early proteins (Nef and Rev) were invariably expressed. Moreover, single-nucleotide variations caused changes in the viral mRNA expression pattern. Taken together, our results showed that the nucleotide sequence of the 3′ region of the HIV-1 *pol* gene (nt 4895 to 4929 for NL4-3) play an important role in viral replication by modulating viral gene expression.

MATERIALS AND METHODS

Plasmid DNAs. The proviral clones pNL-DT5R (34) and pNL4-3 (35) were used as parental clones in the present study. Proviral clones carrying each mutation in the 3′ region of the *pol* gene were constructed with the QuikChange site-directed mutagenesis kit (Agilent Technologies Inc., Santa Clara, CA). Proviral *env*-deficient clones were constructed by deleting the NdeI (nt 6584)-NheI (nt 7435) fragment of pNL-DT5R. Reporter clones were constructed by introducing the luciferase gene into the *nef* gene of *env*-deficient proviral clones as described previously (36). A long terminal repeat (LTR)-driven luciferase reporter clone (5RLTR-Luc) was constructed by replacing the AatII (5′end of the proviral genome)-NcoI (5′end of the luciferase gene in the *nef* gene) fragment of the pNL-DT5R luciferase reporter clone with the LTR region (nt 1 to 789) of pNL-DT5R. A proviral *gag/gag-pol* frameshift clone (gtg-Spe) was constructed by cutting the V234gtg clone with SpeI (nt 1501) within the *gag* capsid and inserting four nucleotides with T4 DNA polymerase.

Cells. Human kidney 293T cells were cultured in minimal essential medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum (hiFBS). Human lymphocyte M8166 cells were cultured in RPMI 1640 supplemented with 10% hiFBS. Human lymphocyte MT4/CCR5 cells (MT4 cells stably expressing CCR5) were maintained in RPMI 1640 containing 10% hiFBS and 200 μg/ml of hygromycin B (Sigma-Aldrich Co., St. Louis, MO).

Analysis of virus growth kinetics. Virus stocks were prepared from 293T cells transfected with proviral clones as previously described (34, 35). Virion-associated reverse transcriptase (RT) activity was measured as previously described (37). M8166 and MT4/CCR5 cells (10^5) were infected with equal amounts of NL-DT5R and its derivative viruses (35 RT units and 5×10^5 RT units for M8166 and MT4/CCR5 cells, respectively), as previously described (38, 39). Equal amounts (10^5 RT units) of HIV-1 NL4-3 and its derivative viruses were inoculated into MT4/CCR5 cells (10^5). Virus replication was monitored by RT activity released into the culture supernatants.

Analysis of single-cycle viral infectivity. Vesicular stomatitis virus G protein (VSV-G)-pseudotyped viruses were prepared from 293T cells cotransfected with an *env*-deficient luciferase reporter clone and pCMV-G (40) at a molar ratio of 1:1. Virus amounts on day 2 posttransfection were measured with an HIV-1 p24 antigen enzyme-linked immunosorbent assay (ELISA) kit (ZeptoMetrix Corporation, Buffalo, NY). M8166 cells (10^5) were infected with equal amounts of pseudotyped viruses (30 pg of p24), and cells were lysed on day 1 postinfection for luciferase assays (Promega Corporation, Madison, WI).

Analysis of viral cDNA synthesis. DNase I-treated pseudoviruses (150 pg of p24) were inoculated into M8166 cells (5×10^5), and total DNA was extracted on day 1 postinfection using the DNeasy blood and tissue kit (Qiagen GmbH, Hilden, Germany). Quantitative analyses of viral cDNA products using real-time quantitative PCR (AB17500; Life Technologies Corporation, Carlsbad, CA) were performed as previously described (14).

Analysis of virion production. M8166 cells (10^6) were cotransfected with equal amounts of *env*-deficient proviral clones (1 μg) and the pGL3

luciferase reporter vector (Promega Corporation) (1 μg) using the Amaxa human T cell Nucleofector kit (Lonza Ltd., Basel, Switzerland) with Nucleofector II (Lonza Ltd.). Culture supernatants were collected on day 2 posttransfection, and virion production was measured using the HIV-1 p24 antigen ELISA kit (ZeptoMetrix Corporation). Cell lysates were prepared with 1× CCLR buffer (Promega Corporation) and subjected to luciferase assays (Promega Corporation). The luciferase activity in cell lysates was used to normalize the transfection efficiency.

Analysis of viral protein expression. 293T cells for Western blot analysis were transfected with equal amounts of proviral clones by using Lipofectamine 2000 (Life Technologies Corporation) in the absence or presence of 2 μM saquinavir (SQV) (Sigma-Aldrich Co.). On day 1 posttransfection, cells were lysed in 1× TNE buffer (10 mM Tris-HCl [pH 8.0], 1% Nonidet P-40, 150 mM NaCl, 1 mM EDTA [pH 8.0], and 1% protease inhibitor cocktail [Sigma-Aldrich Co.]). The total protein amounts in the cell lysates were measured with the DC protein assay (Bio-Rad Laboratories Inc., Hercules, CA), and equal amounts were loaded onto Mini-Protean TGX gels (Bio-Rad Laboratories Inc.) for electrophoresis (0.5 μg for the anti-gp160 antibody, 1 μg for the anti-Vpu antibody, 2 μg for the anti-p24, anti-Nef, or anti-β-actin antibody, 5 μg for the anti-Rev antibody, and 20 μg for the anti-RT antibody). Following blotting onto Immobilon-P transfer membranes (Merck KGaA, Darmstadt, Germany), the membranes were treated with the anti-β-actin clone AC-15 (Sigma-Aldrich Co.), anti-HIV-1 p24 (183-H12-5C) (catalog number 3537; NIH Research and References Reagent Program), anti-HIV-1 RT (MP Biomedicals, Santa Ana, CA), HIV-1 NL4-3 Vpu antiserum (catalog number 969; NIH Research and References Reagent Program), anti-HIV-1 gp160 (ADP409; Immuno Ltd./the MRC AIDS Directed Programme Reagent Project), anti-Rev (ab25871; Abcam PLC, Cambridge, England), or anti-HIV-1 Nef (Advanced Biotechnologies Inc., Columbia, MD) antibody and visualized with the Amersham ECL Plus Western blotting detection system (GE Healthcare UK Ltd., Buckinghamshire, England). A GS-800 calibrated densitometer and Quantity One software (Bio-Rad Laboratories Inc.) were used to quantify signal intensities. To monitor the expression levels of viral proteins, 293T cells were transfected with proviral clones by using Lipofectamine 2000 (Life Technologies Corporation), and on day 2 posttransfection, samples were prepared as described above. The amounts of cell-associated Gag-p24 and Pol-RT were measured using the HIV-1 p24 antigen ELISA kit (ZeptoMetrix Corporation) and RT capture ELISA kit (ImmunoDX, LLC, Woburn, MA), respectively. To monitor Tat activity, 293T cells were cotransfected with proviral clones (0.2 μg) and the 5RLTR-Luc clone (0.05 μg) by using Lipofectamine 2000 (Life Technologies Corporation). Cells were lysed with 1× CCLR buffer (Promega Corporation) on day 1 posttransfection for luciferase assays (Promega Corporation). 293T cells for the interference experiments were transfected with an appropriate amount of proviral clones (5R and its derivatives) by using Lipofectamine 2000 (Life Technologies Corporation). Cells were lysed with 1× TNE buffer on day 1 posttransfection, and the amount of Gag-p24 in cell lysates was measured using the HIV-1 p24 antigen ELISA kit (ZeptoMetrix Corporation).

Northern blot analysis. 293T cells were transfected with equal amounts of proviral clones by using Lipofectamine 2000 (Life Technologies Corporation), and total RNA was extracted at 10 to 20 h posttransfection using the RNeasy Plus Minikit (Qiagen GmbH). Poly(A)$^+$ RNA was isolated with the Oligotex-dT30 Super mRNA purification kit (TaKaRa Bio Inc., Otsu, Japan) and then treated with DNase I (TaKaRa Bio Inc.). Equal amounts of RNA samples were loaded on a glyoxal denatured 1% agarose gel prepared with NorthernMax-Gly 10× Gel Prep/running buffer (Life Technologies Corporation), electrophoresed, and blotted onto a positively charged nylon membrane (Roche Diagnostics GmbH, Mannheim, Germany). The digoxigenin (DIG)-labeled universal probe (U probe) to detect all HIV-1 mRNA species was prepared by using a PCR DIG probe synthesis kit (Roche Diagnostics GmbH.) with pNL4-3 as a template and primers 5′-GAGGATTGTGGAACTTCTGG-3′ and 5′-CTTTGGGAGTGAATTAGCCC-3′. The DIG-labeled Rev-responsive element (RRE) probe, vif probe, and vpr probe were synthesized using templates and primer pairs as follows: RRE probe, pNL4-3, forward primer 5′-CCATTAGGAGTAGCACCCAC-3′, and reverse primer 5′-GTTCCAGAGATTTATTACTCC-3′; vif probe, pNL-DT5R, forward primer 5′-ATGGAGGAGGAAAAGAGGTGG-3′, and reverse primer 5′-CTGCATAAGTACTGAGCCAC-3′; and vpr probe, pNL4-3, forward primer 5′-ATGGAACAAGCCCCAGAAG-3′, and reverse primer 5′-GCAGAATTCTTATTATGGCTTCC-3′. The membrane was hybridized with the DIG-labeled probe in DIG Easy Hyb (Roche Diagnostics GmbH), and visualized with the DIG High Prime DNA labeling and detection starter kit II (Roche Diagnostics GmbH). To monitor and normalize loading amounts of RNAs, membranes were hybridized with the random-prime DIG-labeled GAPDH (glyceraldehyde-3-phosphate dehydrogenase) probe prepared with the DIG High Prime DNA labeling and detection starter kit II (Roche Diagnostics GmbH) and visualized as described above.

RESULTS

Growth-enhancing adaptive mutations increase virion production but have no effects on the early phase of viral replication. We previously obtained adapted viruses with enhanced growth potential from long-term cultures of macaque cells infected with macaque-tropic HIV-1 5R (34) or its R5-tropic version 562 (41). While proviral clones generated from adapted viruses contained a number of mutations in scattered regions of the viral genomes, only mutations in *pol*-IN or *env*-Gp120 were found to contribute to viral growth enhancement (32). In this study, we investigated the mechanistic basis for acceleration of viral replication by the adaptive mutations in the IN-coding sequence. As indicated in Fig. 1, four mutations (N222K, F223Y, D229E, and V234I in the 3′ region of the *pol* gene designated *Pol*4889-4923) were previously shown to enhance viral growth in macaque cells (32). The introduction of both N222K and V234I into human-tropic HIV-1 NL4-3 had a growth-enhancing effect in human cells similar to that observed for 5R (32). We first studied the effect of the adaptive mutations in the *Pol*4889-4923 region on viral replication in M8166 cells. As shown in Fig. 2A, all mutants grew more efficiently than the parental 5R virus. To examine the early replication phase, single-cycle viral infectivity was determined by infection with VSV-G-pseudotyped viruses containing a luciferase gene in the *nef* gene. All mutant clones exhibited infectivity similar to that of 5R (Fig. 2B). Furthermore, the four mutations did not have positive effects on viral cDNA synthesis, as measured by real-time quantitative PCR (Fig. 2C). All four growth-enhancing mutations resulted in an increase in virion production in transfected M8166 cells (Fig. 2D). An enhancement in virion production was consistently observed for pseudotyped and proviral clones in transfected 293T cells (data not shown). These results showed that the acceleration of viral replication by the adaptive mutations in the *Pol*4889-4923 region could be attributed to the increase in infectious virion production in producer cells.

Amino acid substitutions caused by the adaptive mutations may not be responsible for the enhancement in virion production/replication ability. All growth-enhancing mutations in the *Pol*4889-4923 region were nonsynonymous changes (Fig. 1). To determine whether altered amino acids were critical for the enhancement in virion production/replication efficiency, each amino acid at the four sites (N222, F223, D229, and V234) was replaced by various amino acids with different sizes and chemical properties (detailed in Table 1). The effects of amino acid substitutions at these positions on virion production and viral replica-

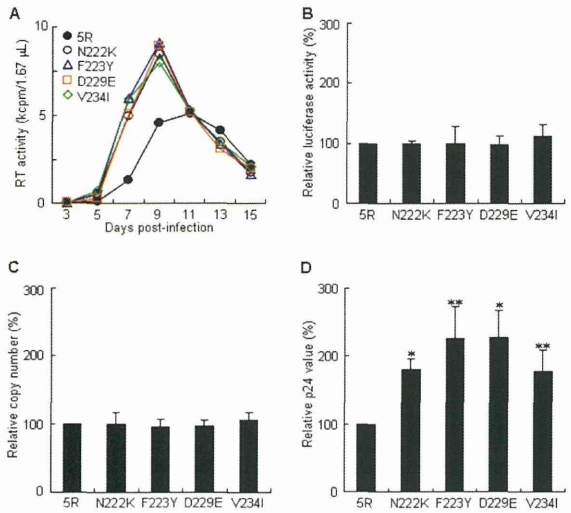


FIG 2 Effect of the adaptive mutations in the *Pol*4889-4923 region on different stages of virus replication in M8166 cells. (A) Replication kinetics. Input viruses were prepared from 293T cells transfected with the indicated proviral clones, and equal amounts were inoculated into M8166 cells. Virus replication was monitored by RT activity released into the culture supernatants. Representative data from at least three independent experiments are shown. (B) Single-cycle viral infectivity. VSV-G-pseudotyped viruses were prepared from 293T cells transfected with the indicated clones, and equal amounts were inoculated into M8166 cells. Cell lysates were prepared on day 1 postinfection and subjected to luciferase assays. Infectivity is presented as luciferase activity relative to that exhibited by 5R. Mean values from three independent experiments are shown with the standard deviations (SD). (C) Viral cDNA synthesis. VSV-G-pseudotyped viruses were prepared from 293T cells transfected with the indicated clones, DNase I treated, and inoculated into M8166 cells. Total DNA was extracted from infected cells on day 1 postinfection and subjected to real-time quantitative PCR analyses with a primer pair specific for the late form (R/gag) of viral cDNA. The DNA copy number relative to that of 5R is presented. Mean values ± SD from three independent experiments are shown. (D) Virion production. M8166 cells were cotransfected with the indicated *env*-deficient proviral clones and a luciferase reporter vector (pGL3) by using a Nucleofector. Virion production on day 2 posttransfection was measured by the amount of Gag-p24 in the culture supernatants. The amount of p24 was normalized by luciferase activity in cell lysates. The amount of p24 relative to that produced by 5R is presented. Mean values ± SD from four independent experiments are shown. Significance relative to 5R as calculated by the Student *t* test is shown (*, $P < 0.01$; **, $P < 0.02$).

tion (Fig. 3) were determined as described above. As shown in Fig. 3A, for N222, the virion production level relative to that of 5R was enhanced by the substitution with Ala (A) and Gly (G) as well as the growth-enhancing adaptive mutation Lys (K) but was decreased by the substitution with Phe (F) and Tyr (Y). The increase and decrease in virion production caused by these amino acid substitutions at N222 positively correlated with viral growth potential (Fig. 3A). For F223 (Fig. 3B), all mutant clones carrying Ala (A), Glu (E), Gly (G), His (H), Lys (K), and Ser (S) in addition to the adaptive mutation Tyr (Y) produced more virions than 5R, whereas only F223Y augmented viral replication efficiency. To study the cause of this apparent discrepancy, we compared the effects of F223Y and F223A/E/H/S on the early replication phase (Fig. 4). While F223Y showed levels of single-cycle viral infectivity and viral cDNA synthesis (late and integrated forms) similar to those for 5R, marked differences were observed in the behaviors of the other mutants. The virus infectivity of F223H was approximately 30% to that of 5R, and the infectivity of F223A/E/S was undetectable (Fig. 4A). A reduction in viral cDNA levels was also observed for F223A/E/H/S (Fig. 4B and C). These results indicate that the F223 residue is essential for the function of IN in the early replication phase. For D229 (Fig. 3C), virion production/replication ability was enhanced only by the adaptive mutation Glu (E), and the other mutants carrying D229A/G/T/W had reduced virion production/replication potential. For V234 (Fig. 3D), the Gly (G) substitution as well as the adaptive mutation Ile (I) augmented virion production/replication capability, but V234A/W decreased these. Because mutants D229K and V234E produced virions at the 5R level upon transfection (Fig. 3C and D), they are most likely to be defective for the early replication phase like the F223 mutants (Fig. 3B and 4). The results in Fig. 3 clarify the clear correlation between virion production and viral growth potential, except for the F223 mutants and the D229K/V234E mutants. However, increases or decreases in virion production were not dependent on altered amino acid sizes or chemical properties (Table 1). For example, virion production levels were enhanced by any altered amino acids tested at F223, and V234G and V234A increased and decreased virion production levels, respectively, despite their similar amino acid properties. Therefore, these results indicate that amino acid residues may not be a determinant for virion production.

Synonymous codon changes in V234I alter virion production/replication ability. Although no clear relationship was observed between the amino acids in the *Pol*4889-4923 region and increase in virion production (Table 1), virion production levels were markedly affected by the substitutions with different amino acids (Fig. 3). Since nucleotide mutations in the IN-coding sequence can induce viral phenotypic changes as described above, we speculated about the possible involvement of codon/nucleotide sequences in virion production enhancements. Thus, proviral clones carrying different codons for growth-enhancing mutations were constructed (N222K-2, F223Y-2, D229E-2, V234I-2, and V234I-3), and their growth potentials were compared to those of 5R and parental clones with each adaptive mutation. As shown in Fig. 5A, the growth of codon-altered viral clones (N222K-2, F223Y-2, and D229E-2) was more efficient than that of 5R, as observed for N222K, F223Y, and D229E. A fluctuation in viral replication potential among the V234I codon variants was noted: the growth abilities of V234I and V234I-3 were higher than that of 5R, but that of for V234I-2 was slightly impeded (Fig. 5A). While the single-cycle early infectivities of 5R and these codon variants were similar (Fig. 5B), their virion production levels varied in parallel with their replication potentials (Fig. 5A and C). Only V234I-2 exhibited a virion production level similar to that of 5R, and the other codon-altered mutants showed an enhanced level of virion production. The results for the V234I codon variants demonstrate the alteration in virion production levels in a nucleotide sequence-dependent manner, resulting in the modulation of viral replication ability.

Virion production levels of V234I codon variants correlate with Gag and Gag-Pol expression levels in producer cells. Synonymous codon changes in Ile (I) at amino acid position 234 in *pol*-IN caused an alteration in infectious virion production levels (Fig. 5C). We assumed that at least Gag and Gag-Pol expression levels, prerequisites for virion formation, correlatively varied with the amount of progeny virions. It has also been shown that cell-

TABLE 1 Characteristics of viral clones carrying various mutations in the 3′ region of the *pol* gene

Amino acid position in IN	Viral clone name	Amino acid	Codon	Size of amino acid	Chemical property(ies) of amino acid	Early replication[a]	Virion production[a]	Growth[b]
222	5R	N	AAT	Medium-small	Neutral, hydrophilic	++	++	++
	N222K[c]	K	AAA	Medium-large	Basic	++	+++	+++
	N222K-2	K	AAG	Medium-large	Basic	++	+++	+++
	N222A	A	GCT	Small	Aliphatic, hydrophobic	ND[d]	+++	+++
	N222G	G	GGT	Small	Aliphatic, hydrophobic	ND	+++	+++
	N222F	F	TTC	Large	Aromatic, hydrophobic	ND	+	−
	N222Y	Y	TAT	Large	Aromatic	ND	++	+
223	5R	F	TTT	Large	Aromatic, hydrophobic	++	++	++
	F223Y[c]	Y	TAT	Large	Aromatic	++	+++	+++
	F223Y-2	Y	TAC	Large	Aromatic	++	+++	+++
	F223H	H	CAT	Large	Basic	+	+++	+
	F223G	G	GGT	Small	Aliphatic, hydrophobic	ND	+++	−
	F223A	A	GCT	Small	Aliphatic, hydrophobic	−	+++	−
	F223S	S	TCT	Small	Neutral, hydrophilic	−	+++	−
	F223E	E	GAA	Medium-large	Acidic	−	+++	−
	F223K	K	AAA	Medium-large	Basic	ND	+++	−
229	5R	D	GAC	Medium-small	Acidic	++	++	++
	D229E[c]	E	GAA	Medium-large	Acidic	++	+++	+++
	D229E-2	E	GAG	Medium-large	Acidic	++	+++	+++
	D229K	K	AAA	Medium-large	Basic	ND	++	+
	D229A	A	GCC	Small	Aliphatic, hydrophobic	ND	+	−
	D229G	G	GGC	Small	Aliphatic, hydrophobic	ND	+	+
	D229T	T	ACC	Medium-small	Neutral, hydrophilic	ND	−	−
	D229W	W	TGG	Large	Aromatic, hydrophobic	ND	−	−
234	5R	V	GTT	Medium-small	Aliphatic, hydrophobic	++	++	++
	V234I[c]	I	ATT	Medium-small	Aliphatic, hydrophobic	++	+++	+++
	V234I-2	I	ATC	Medium-small	Aliphatic, hydrophobic	++	++	+
	V234I-3	I	ATA	Medium-small	Aliphatic, hydrophobic	++	+++	+++
	V234G	G	GGT	Small	Aliphatic, hydrophobic	ND	+++	+++
	V234A	A	GCT	Small	Aliphatic, hydrophobic	ND	+	+
	V234E	E	GAA	Medium-large	Acidic	ND	++	+
	V234W	W	TGG	Large	Aromatic, hydrophobic	ND	+	+

[a] +++, >150% of 5R activity; ++, >70 to 150% of 5R activity; +, 10 to 70% of 5R activity; −, <10% of 5R activity. Data were obtained in M8166 cells.
[b] +++, replication peaked earlier or virus production levels on the peak day were higher than those of 5R; ++, replication kinetics were similar to those of 5R; +, replication peaked later or virus production levels on the peak day were lower than those of 5R; −, replication was not detected during the observation period. Data were obtained in M8166 cells.
[c] Adaptive (growth-enhancing) mutation.
[d] ND, not done.

associated Gag was decreased for *pol*-IN deletion mutant viruses defective in virion production (20). To determine intracellular Gag and Gag-Pol expression levels, the proviral clones of 5R and V234I codon variants were transfected into 293T cells in the presence or absence of the HIV-1 protease inhibitor SQV. First, intracellular Gag-p24 and Pol-RT in the absence of SQV were measured by ELISA. While V234I and V234I-3, with improved virion production potential, expressed higher levels of Gag-p24 and Pol-RT than 5R, V234I-2 and 5R generated similar amounts (Fig. 6A). We then examined the intracellular expression patterns of Gag and Gag-Pol by Western blotting analyses. The expression profiles of $Pr55^{Gag}$/p24 and $Pr160^{Gag\text{-}Pol}$/p66/p51 for 5R and V234I codon variants were similar in the absence of SQV, which strongly suggested the absence of an effect of V234I mutations on viral protein processing [Fig. 6B SQV(−)]. The expression levels of Gag/Gag-Pol-related proteins were higher for V234I and V234I-3 than for 5R [Fig. 6B and C, SQV(−)]. In contrast, V234I-2 appeared to express slightly lower levels of these viral proteins than 5R [Fig. 6B and C, SQV(−)]. This small decrease in Gag/Gag-Pol expression levels may be consistent with the similar difference observed for the viral replication kinetics of 5R and V234I-2 (Fig. 5A). The variations in $Pr55^{Gag}$ and $Pr160^{Gag\text{-}Pol}$ expression levels in the presence of SQV appeared to be smaller than those obtained by Western blotting analyses [Fig. 6B and C, SQV(+)]. This may have been due to the weak recognition of $Pr55^{Gag}$ and $Pr160^{Gag\text{-}Pol}$ by the antibodies used. The results described above show that the alteration in the virion production/replication potential of V234I codon variants is in parallel with the increase or decrease in Gag/Gag-Pol expression levels.

Viral replication capability can be altered by natural variations in the sequences of the 3′ region of the HIV-1 *pol* gene. As observed for V234I codon variants, single-nucleotide changes resulted in alterations in virion production/replication potential through the modulation of Gag/Gag-Pol expression levels, which indicated the importance of the nucleotide sequence in the *Pol*4889-4923 region (Fig. 5 and 6). Recent studies revealed that a

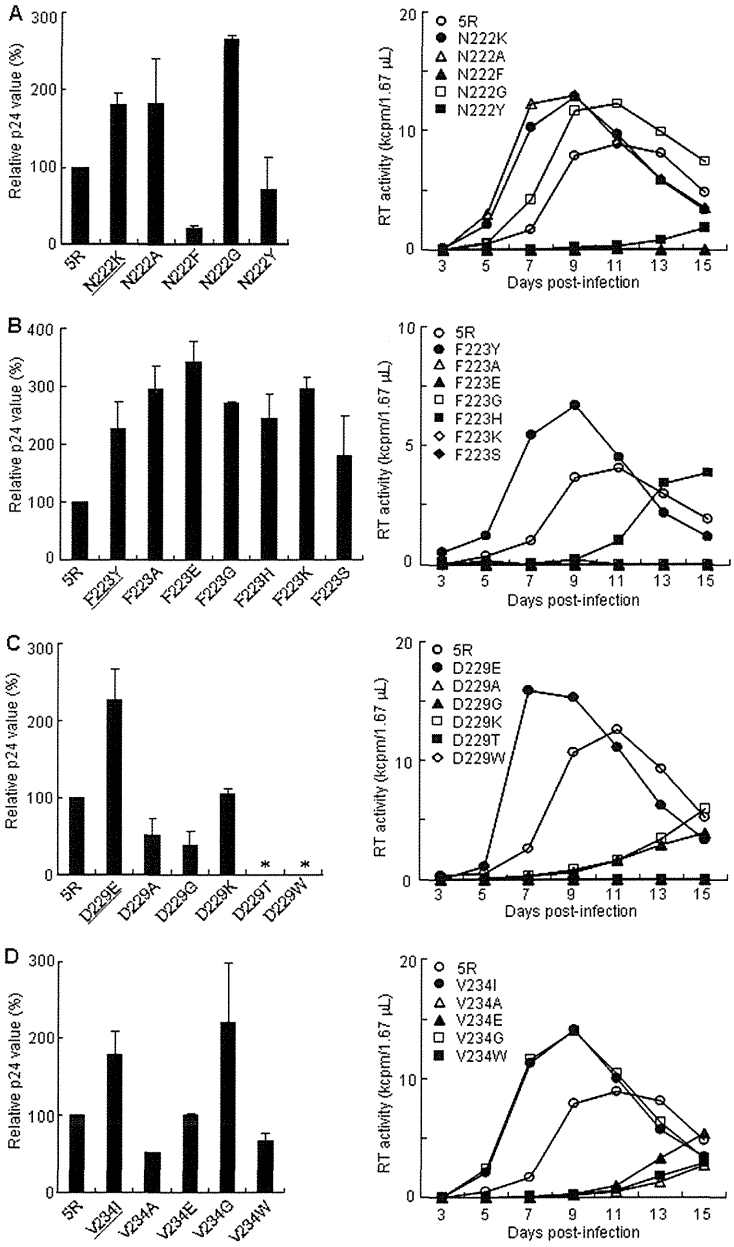


FIG 3 Effect of various amino acid substitutions at the N222 (A), F223 (B), D229 (C), and V234 (D) sites in the *Pol*4889-4923 region on virion production and replication kinetics. Details of the substitution for each mutant are shown in Table 1. Left panels, virion production. M8166 cells were cotransfected with the indicated *env*-deficient proviral clones and a luciferase reporter vector (pGL3) by using a Nucleofector. Virion amounts in the culture supernatants on day 2 posttransfection were measured. The amount of p24 was normalized by luciferase activity in cell lysates. The amount of p24 relative to that produced by 5R is presented. Mean values ± SD from at least two independent experiments are shown. Results for N222K, F223Y, D229E, and V234I shown in Fig. 2D (underlined) are incorporated into each panel for an easy comparison. *, under the detection limit. Right panels, viral replication kinetics. Viruses were prepared from transfected 293T cells, and equal amounts were inoculated into M8166 cells. Virus replication was monitored by RT activity released into the culture supernatants. Data in panels A and D were obtained from the same experiment, and the same result for 5R is shown separately in panels A and D as a control. Representative data from three independent experiments are shown.

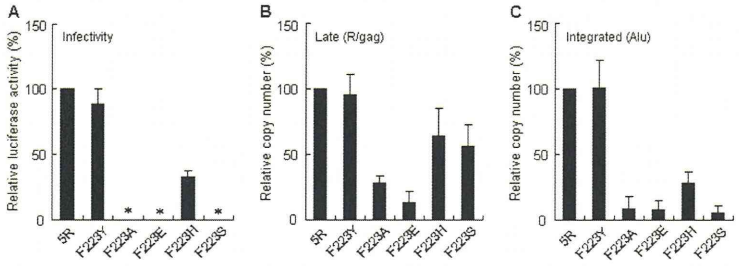


FIG 4 Effect of various amino acid substitutions at the F223 site on the early viral replication phases. (A) Single-cycle viral infectivity. VSV-G-pseudotyped viruses were prepared from transfected 293T cells, and equal amounts were inoculated into M8166 cells. Cells were collected and lysed on day 1 postinfection for luciferase assays. Infectivity is presented as luciferase activity relative to that exhibited by 5R. Mean values ± SD from at least four independent experiments are shown. *, mean values are <0.1%. (B and C) Monitoring viral cDNA synthesis. VSV-G-pseudotyped viruses from transfected 293T cells were treated with DNase I, and equal amounts were inoculated into M8166 cells. Total DNA was extracted from infected cells on day 1 postinfection and subjected to real-time quantitative PCR analyses with primer pairs specific for the late (B) and integrated (C) forms of viral cDNA. The DNA copy number relative to that of 5R is presented. Mean values ± SD from at least four independent experiments are shown.

naturally occurring synonymous polymorphism in some genes can influence the expression levels, structures, and functions of their encoded proteins (42–44). In addition, the IN CTD sequence is more heterogenous than those of the other IN domains (15, 17, 19). To clarify the significance of the nucleotide sequence in the *Pol*4889-4923 region for viral replication, we examined HIV-1 sequences within the region obtained from the HIV Sequence Compendium (Los Alamos National Laboratory, NM, USA). As shown in Table 2, while viruses carrying F223Y or D229E, which we identified as growth-enhancing mutations, were not found, those with N222K or V234I were present. We noted that codon variants with distinct growth abilities, V234I (ATT) and V234I-2 (ATC), coexisted in a viral population with different frequencies (Table 2). This suggested that there may be natural variants of HIV-1 with distinct replication potentials. Moreover, a sequence comparison in the *Pol*4889-4923 region revealed the presence of natural synonymous variations for parental clone 5R-encoded amino acid residues, even though the frequency was lower than that of 5R (Table 2). Thus, we examined whether viral replication can be affected by natural synonymous changes at the sites of adaptive mutations (N222, F223, D229, and V234) as well as other sites (V225, Y226, and P233) within the *Pol*4889-4923 region. The viral growth kinetics of 5R and its natural variants at adaptive mutation sites (N222aac, F223ttc, D229gat, and V234gtg) were determined in human MT4/CCR5 cells (Fig. 7A and Table 2). While N222aac and F223ttc exhibited growth kinetics similar or slightly better than those of 5R, the viral replication potentials of D229gat and V234gtg was markedly higher and lower, respectively, than that of 5R (Fig. 7A and Table 2). We next determined the viral growth kinetics of additional natural synonymous variants (V225gtc, Y226tac, and P233cct/ccc/ccg). Alterations in the viral replication potentials of these clones were evident: the viral replication kinetics of V225gtc were similar to those of 5R, whereas growth ability was higher for Y226tac and lower for P233cct/ccc/ccg than for 5R (Fig. 7B and Table 2). On the other hand, the genome structure of 5R is different from that of natural human-tropic HIV-1 due to the change of the cyclophilin A-binding loop-coding region in *gag* and of an entire *vif* to the corresponding regions of SIVmac239 (34). Thus, we determined whether these natural synonymous variations also affect the viral replication of human-tropic HIV-1 (NL4-3 clone). The viral growth potential of NL4-3 was altered similarly as that of 5R by natural synonymous changes: the replication abilities of D229gat and Y226tac were higher than that of NL4-3, and those of V234gtg and P233cct/ccc/ccg were lower than that of NL4-3 (Fig. 7C and D). We conclude from these results that natural variations (single-

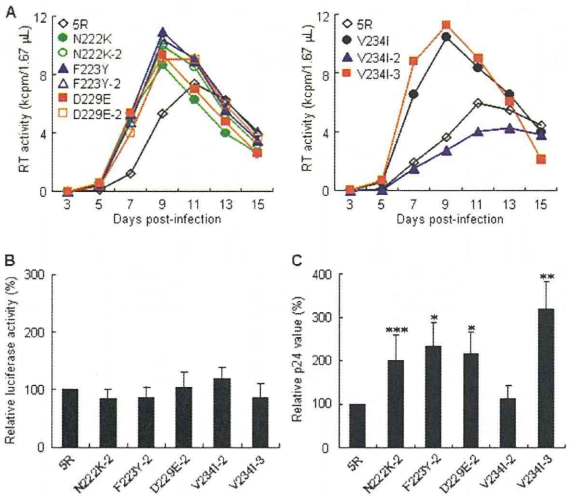


FIG 5 Effect of codon alterations at N222K, F223Y, D229E, and V234I sites on different stages of viral replication. N222K-2, F223Y-2, D229E-2, V234I-2, and V234I-3 indicate mutant clones containing an altered codon. The codon used for each mutant is shown in Table 1. (A) Viral replication kinetics. Input viruses were prepared from transfected 293T cells, and equal amounts were inoculated into M8166 cells. Virus replication was monitored by RT activity released into the culture supernatants. Representative data from at least two independent experiments are shown. (B) Single-cycle viral infectivity. VSV-G-pseudotyped viruses were prepared from transfected 293T cells, and equal amounts were inoculated into M8166 cells. Cell lysates were prepared on day 1 postinfection and subjected to luciferase assays. Infectivity is presented as luciferase activity relative to that exhibited by 5R. Mean values ± SD from at least three independent experiments are shown. (C) Virion production. M8166 cells were cotransfected with the indicated *env*-deficient proviral clones and a luciferase reporter vector (pGL3) by using a Nucleofector. Virion production on day 2 posttransfection was measured by the amount of p24 in the culture supernatants. The amount of p24 was normalized by luciferase activity in cell lysates. The amount of p24 relative to that produced by 5R is presented. Mean values ± SD from at least three independent experiments are shown. Significance relative to 5R as calculated by the Student *t* test is shown (*, $P < 0.01$; **, $P < 0.05$; ***, $P < 0.1$).