

A Nucleotide Substitution in the tRNA^{Lys} Primer Binding Site Dramatically Increases Replication of Recombinant Simian Immunodeficiency Virus Containing a Human Immunodeficiency Virus Type 1 Reverse Transcriptase

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A recombinant simian immunodeficiency virus (SIV) derived from strain 239 (SIVmac239) with reverse transcriptase (RT) sequences from human immunodeficiency virus type 1 (HIV-1) strain HXB2 was severely impaired for replication. Detectable p27^{Gag} levels were not observed until day 65 and peak p27^{Gag} levels were not reached until day 75 after transfection of CEMx174 cells with the recombinant DNA. Sequences from the latter time point did not contain amino acid substitutions in HIV-1 RT; however, a single nucleotide substitution (thymine to cytosine) was found at position eight of the SIV primer binding site. We engineered an RT/SHIV genome with the thymine-to-cytosine substitution, called RT/SHIV/TC, and observed dramatically faster replication kinetics than were observed with the parental RT/SHIV from which this variant was derived. RT/SHIV/TC provides an improved system for study of the impact of drug resistance mutations in HIV-1 RT in a relevant animal model.

For the replication of retroviruses, virion-associated RNA is reverse transcribed into double-stranded DNA prior to integration into the host cell genome (11). The virion-encoded enzyme reverse transcriptase (RT) mediates this reverse transcription, and cellular tRNA serves as the primer for this reaction (5, 25, 26, 28, 30). Polymerization is initiated through the binding of the 18 3'-terminal nucleotides of the tRNA to an 18-nucleotide sequence in the 5' long terminal repeat (LTR), termed the primer binding site (PBS) (24, 29). The sequence of the PBS, and hence of the tRNA used for reverse transcription, is specific for different retroviruses (21, 34). tRNA^{Lys} is the primer typically used for human (HIV-1 and HIV-2) and simian (SIV) immunodeficiency viruses. While mutational analyses of HIV-1 PBS sequences have demonstrated that other tRNA primers can be utilized, these PBS sequences are less efficient than tRNA^{Lys} in initiating reverse transcription; mutated PBS sequences typically revert to wild-type HIV-1 PBS sequences in the context of replicating virus (12, 19, 20, 33, 36). These mutants can be stabilized through the introduction of sequences that complement the anticodon loop (A-loop) of these alternative tRNAs (19, 32).

The essential role of reverse transcription in the HIV-1 life cycle and its importance as a drug target have led to intense studies of HIV-1 RT sequences (11). Conversely, studies of RT in the context of SIV and experimentally infected monkeys have been limited. This is likely due at least in part to the limited homology between SIV and HIV-1 RT (approximately

60% at the amino acid level) and to the fact that SIV RT is relatively insensitive to nonnucleoside reverse transcriptase inhibitors (14). To help alleviate these limitations, an RT/SHIV genome was constructed in which SIV RT sequences were replaced with HIV RT sequences (6). The kinetics of RT/SHIV replication following recombinant DNA transfection was not included in the original or subsequent publications describing this recombinant (6, 7, 22, 31). However, it was demonstrated that stocks of RT/SHIV replicated comparably to wild-type SIV in experimentally infected rhesus macaques (31) and that replication of RT/SHIV was significantly decreased by the administration of either class of RT inhibitor (6, 7).

We transfected RT/SHIV recombinant DNA into the SIV-permissive cell line CEMx174 by using DEAE-dextran (23). The cells, which were grown in RPMI 1640 (Gibco-BRL, Grand Island, N.Y.) that was supplemented with 10% fetal calf serum (Gibco-BRL), were inspected periodically for cytopathic effects (CPE) in the initial weeks posttransfection as an indicator of viral replication. CPE were not detected (data not shown). Cell-free supernatants were also obtained in the initial weeks posttransfection and assayed for the production of p27^{Gag} using an SIV core antigen kit (Coulter, Hialeah, Fla.). We did not observe detectable levels of viral protein in this assay (Fig. 1A). The severe delay in RT/SHIV replication following transfection contrasts with the rapid replication following infection of cultured cells reported previously (6, 7, 22, 31).

We continued to monitor the transfected cells and began to observe CPE on day 62 posttransfection, which became more prominent in subsequent days (data not shown). On day 62, we began to harvest the cell-free supernatant on a daily basis to assay for p27^{Gag} production. Detectable levels of viral protein

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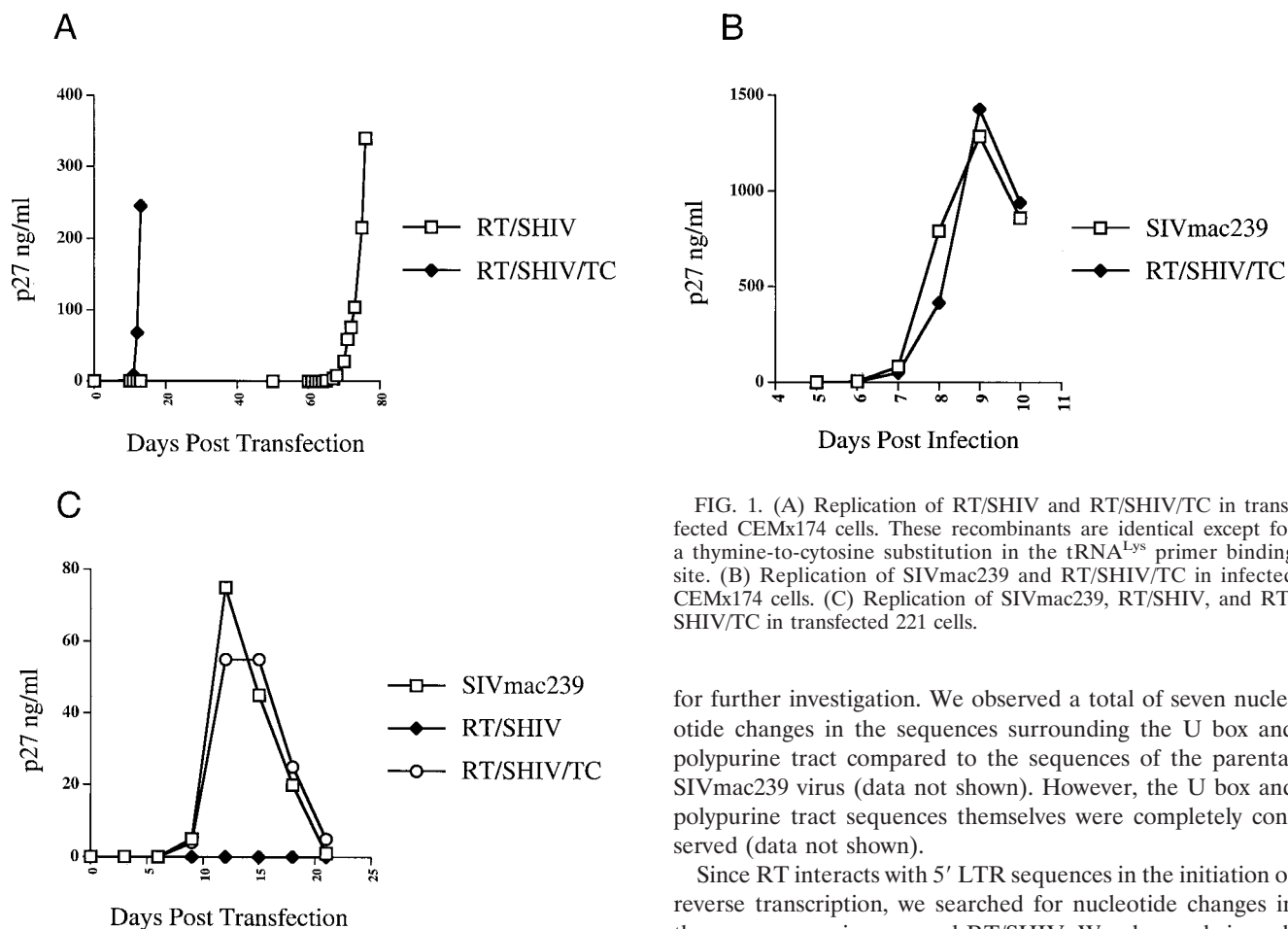


FIG. 1. (A) Replication of RT/SHIV and RT/SHIV/TC in transfected CEMx174 cells. These recombinants are identical except for a thymine-to-cytosine substitution in the tRNA^{Lys} primer binding site. (B) Replication of SIVmac239 and RT/SHIV/TC in infected CEMx174 cells. (C) Replication of SIVmac239, RT/SHIV, and RT/SHIV/TC in transfected 221 cells.

were observed on day 65, which increased dramatically in subsequent days (Fig. 1A).

The pattern of replication observed in the transfection experiment suggested to us that RT/SHIV underwent sequence changes that significantly increased its replication. We thus explored the possibility that the HIV-1 RT sequences changed during RT/SHIV passage in CEMx174 cells. Cellular DNA was isolated from these cells on day 75 posttransfection using a previously described saturated NaCl precipitation technique (3). The HIV-1 RT sequences were amplified by PCR, sequenced, and aligned to the RT sequences of HXB2, those contained in the RT/SHIV cloned DNA. The sequence of the amplified product revealed two nucleotide changes in RT (data not shown). Neither of these changes affected the amino acid sequence of RT, which was completely conserved in the sequences isolated on day 75 posttransfection (data not shown). Thus, we concluded that a change in the HIV-1 RT sequences was not a likely determinant of the observed increase in RT/SHIV replication in the transfected cells.

Highly conserved sequences immediately upstream of the 5' extent of the 3' LTR have been shown to contribute to the efficiency of reverse transcription and viral replication (18, 27). These sequences have been termed the polypurine tract and the U box. To determine if these sequences contributed to delay RT/SHIV replication, we again used the day 75 sample

for further investigation. We observed a total of seven nucleotide changes in the sequences surrounding the U box and polypurine tract compared to the sequences of the parental SIVmac239 virus (data not shown). However, the U box and polypurine tract sequences themselves were completely conserved (data not shown).

Since RT interacts with 5' LTR sequences in the initiation of reverse transcription, we searched for nucleotide changes in these sequences in passaged RT/SHIV. We observed six such changes in the day 75 sample (Table 1). Interestingly, one change was observed in the PBS (T to C). This change was striking because it was previously observed in SIVmac239 and SHIVnef sequences isolated from four experimentally infected rhesus monkeys (1). We aligned the LTR sequences isolated from one animal (Mm 258-95) at 40 weeks postinoculation with the sequences isolated from the cell-passaged RT/SHIV. The 5' LTR sequences from Mm 258-95 contained 13 nucleotide changes from the parental SIVmac239 sequences, although only the T-to-C change in the PBS was common with changes contained in RT/SHIV at 75 days posttransfection (Table 1).

We investigated if the change in PBS sequences in monkey-passaged SIV was also observed in culture-passaged SIV. Cellular DNA was isolated from CEMx174 cells transfected with SIVmac239 DNA at the time of peak virus production (day 12; data not shown). The analysis of SIV sequences did not reveal any changes in the 5' LTR (data not shown). SIV harvested from the transfected cells was diluted to contain 1 μ g of p27^{Gag} and used to infect 10^7 CEMx174 cells. Analysis of SIV sequences isolated from these cells at the time of peak virus production (day 10) again did not reveal changes in 5' LTR sequences (Table 1). These observations indicate that a thymine at position eight of the PBS was well tolerated in the context of SIVmac239 and utilized for efficient SIVmac239 replication in culture.

TABLE 1. Nucleotides contained in SIV LTR sequences^a

Position	Parental	SIV infection	Mm258-95	RT/SHIV transfection
15	T	T	T	A
89	T	T	G	T
97	C	C	T	C
185	G	G	A	G
186	A	A	G	A
224	G	G	A	G
302	T	T	A	T
351	G	G	A	G
555	T	T	C	T
558	G	G	T	G
648	C	C	C	A
655	A	A	A	C
831	T	T	C	C
871	G	G	A	G
897	A	A	G	A
905	T	T	G	T
964	T	T	T	A

^a Parental, sequences in parental SIVmac239 and RT/SHIV; SIV infection, SIVmac239 sequences passaged twice in culture; Mm258-95, SIVmac239 sequences passaged in a rhesus macaque; RT/SHIV transfection, RT/SHIV sequences passaged once in culture. Position 831 is in the primer binding site.

A thymine located at position eight in the PBS is rarely found in primate lentiviral sequences (1, 13). Instead, a cytosine is typically observed at this position. The thymine at this position renders the PBS exactly complementary to the tRNA₅^{Lys} 3'-terminal nucleotides (Fig. 2) whereas the cytosine at this position renders the PBS exactly complementary to tRNA₃^{Lys} 3'-terminal nucleotides. Since it has been documented that tRNA₃^{Lys} is the preferred primer utilized by primate lentiviruses for the initiation of reverse transcription (12, 20, 33), we hypothesized that the thymine in the SIV PBS sequences was an important determinant of the restricted RT/SHIV replication.

In order to test this hypothesis, we engineered a recombinant to introduce a cytosine (C) in place of a thymine (T) at position eight of the SIV PBS RT/SHIV using an overlap extension technique (17). This recombinant (RT/SHIV/TC) differed from the parental sequences (RT/SHIV) only at this position. The RT/SHIV/TC DNA was transfected into CEMx174 cells. In contrast to the transfection with the parental DNA, RT/SHIV/TC replicated rapidly in these cells. We observed high levels of virus in the culture supernatant by day 13 posttransfection (Fig. 1A). These data indicate that the T-to-C change was critical for efficient replication of RT/SHIV/TC in culture.

The rate of replication observed in the RT/SHIV/TC transfection was very similar to that observed for wild-type SIVmac239 transfections (data not shown). To directly compare the replication kinetics of SIVmac239 and RT/SHIV/TC, stocks of both viruses diluted to contain 1 µg of p27^{Gag} were used to infect 10⁷ CEMx174 cells. Assay of viral protein in the cell-free supernatant revealed that RT/SHIV/TC replicated similarly to SIVmac239 (Fig. 1B). To determine if the impaired replication of RT/SHIV in CEMx174 cells (Fig. 1A) was due to the human origin of these cells, RT/SHIV, RT/SHIV/TC, and SIVmac239 replication efficiency was investigated in the rhesus monkey T-cell line 221 (2). In 221 cells transfected with RT/SHIV/TC and SIVmac239 DNA, high levels of virus were

detected in the culture supernatant on day 12 posttransfection (Fig. 1C). Conversely, cells transfected with RT/SHIV DNA did not produce detectable levels of virus at the times assayed (Fig. 1C), indicating that the impairment in RT/SHIV replication was not CEMx174 cell dependent.

We have shown that the requirement for cytosine at position eight in the PBS is significantly higher for RT/SHIV than SIVmac239 in culture. This change renders the PBS exactly complementary to the tRNA₃^{Lys} 3'-terminal 18 nucleotides and likely reflects striking preference of HXB2 RT for this primer for the initiation of reverse transcription. Mutant HIV-1 PBS sequences that complement other tRNA sequences are unstable in culture and revert to PBS sequences that complement tRNA₃^{Lys} sequences (12, 19, 20, 33, 36). These mutants can be stabilized through the introduction of sequences that complement the A-loop of these alternative tRNAs (Ile, Pro, His, and Trp) (19, 32). Although we provide no direct evidence, our data suggest that SIVmac239 RT sequences utilize tRNA₅^{Lys} for efficient viral replication in culture (Fig. 1A), which leads us to believe that SIVmac239 RT interactions with tRNA sequences are distinct from HXB2 RT interactions.

The essential role of reverse transcription in the HIV-1 life cycle has led to the intense study of RT sequences (11). Numerous mutations have been engineered which affect particular RT functions in vitro or in culture (4, 8–11, 15, 16, 35). Supplementary information regarding the effect on viral fitness of these altered RT sequences could be realized through experimental infection of rhesus monkeys with RT/SHIV/TC.

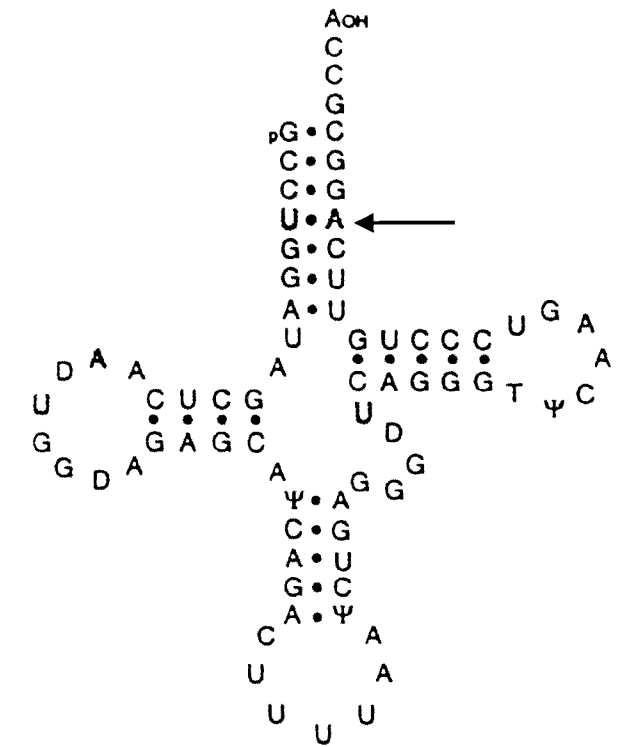


FIG. 2. Secondary structure of tRNA₅^{Lys}. The arrow indicates the nucleotide which complemented the thymine contained in the parental RT/SHIV primer binding site, which changed to a cytosine in the tissue culture-passaged RT/SHIV.

The availability of RT/SHIV/TC, which exhibits markedly increased replication in comparison to parental RT/SHIV (Fig. 1A), may expedite such studies. Cloned RT/SHIV/TC sequences will allow mutations in HIV-1 RT sequences to be introduced into a defined genetic background, where parental sequences replicate comparably to wild-type SIVmac239 (Fig. 1B). RT/SHIV/TC could thus facilitate an increased understanding of HIV-1 reverse transcription mechanisms and the impact of escape mutations through the utilization of the rhesus monkey AIDS model.

The first two authors contributed equally to this work.

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