Available online at www.sciencedirect.com
ScienceDirect
Virology 366 (2007) 330–339
VIROLOGY
www.elsevier.com/locate/yviro

Forced selection of tRNA^{Glu} reveals the importance of two adenosine-rich RNA loops within the U5-PBS for SIV$_{smmPBj}$ replication

Maureen C. Kelly, Barry R. Kosloff, Casey D. Morrow *

Department of Cell Biology, University of Alabama at Birmingham, 802 Kaul Building, 720 20th Street South, Birmingham, AL 35294, USA

Received 23 February 2007; returned to author for revision 15 March 2007; accepted 25 April 2007
Available online 1 June 2007

Abstract

Simian immunodeficiency virus (SIV) and human immunodeficiency virus (HIV-1) preferentially select and use tRNA^{Lys,3} as the primer for initiation of reverse transcription. Previous studies have shown that HIV-1 can be forced to use tRNA^{Glu} if mutations are made within the primer-binding site (PBS) and a region upstream, A-loop, to be complementary to the 3′-terminal 18 nucleotides and anticodon loop of tRNA^{Glu}. To examine the primer preference of SIV, mutations were made within the PBS of SIV$_{smmPBj}$ to be complementary to tRNA^{Glu}. Analysis of the production of infectious virus revealed that SIV$_{smmPBj}$ with the PBS complementary to tRNA^{Glu} retained approximately 80% infectivity of the wild type. However, modification of the U5 of SIV$_{smmPBj}$ to alter nucleotides to be complementary to the anticodon of tRNA^{Glu}, in combination with the PBS complementary to tRNA^{Glu}, drastically reduced the production of infectious SIV$_{smmPBj}$ to less than 1% that of wild type. The replication of SIV$_{smmPBj}$ with the PBS complementary to tRNA^{Glu} was similar to that of the wild type virus, while the replication of SIV$_{smmPBj}$ with PBS and A-loop complementary to tRNA^{Glu} was delayed compared to that of wild type virus. Analysis of the PBS regions revealed that the virus with the PBS complementary to tRNA^{Glu} reverted quickly, within 4 days, to be complementary to tRNA^{Lys,3}, while the virus with PBS and A-loop complementary to tRNA^{Glu} retained the PBS for a longer time during *in vitro* culture although following extended replication both the A-loop and PBS of SIV$_{smmPBj}$ reverted to be complementary to tRNA^{Lys,3}. RNA modeling of SIV$_{smmPBj}$ U5-PBS by *m-fold* revealed two potential A-loop regions. Mutations in either A-loop drastically effected replication in human PBMC. Analysis of the A-loops following *in vitro* replication revealed that both reverted to the wild type sequence. The results of these studies demonstrate that SIV$_{smmPBj}$, like HIV-1, preferentially utilizes tRNA^{Lys,3} as a primer for reverse transcription for high level replication, but unlike HIV-1 selection may involve the use of two adenosine-rich loops.

Keywords: Primer-binding site; HIV-1; SIV; Reverse transcription; tRNA

Introduction

A hallmark in the lifecycle of retroviruses is the process by which the viral RNA is converted to a DNA intermediate prior to integration. This process, termed reverse transcription is catalyzed by a virally encoded enzyme reverse transcriptase. In order for the reverse transcriptase to copy the viral RNA, a cellular tRNA is captured and used as the primer for the initiation of reverse transcription (Baltimore, 1970; Temin and Mizutani, 1970). The 3′-terminal nucleotides of the tRNA primer are complementary to a region on the viral genome designated as the primer-binding site (PBS) (Panet and Berliner, 1978; Peters and Dahlberg, 1979; Temin, 1981).

The tRNA selected by different retroviruses for use as a primer for reverse transcription can vary between viral species (Mak and Kleiman, 1997; Marquet et al., 1995). Lentiviruses, such as HIV and SIV, preferentially select tRNA^{Lys,3} as the primer for initiation of reverse transcription (Mak and Kleiman, 1997; Marquet et al., 1995; Muesing et al., 1985; Ratner et al., 1985). HIV-1 can be forced to use many different tRNAs as primers for initiation of reverse transcription by alteration of the PBS to be complementary to the 3′-terminal 18 nucleotides of different tRNAs (Das et al., 1995; Li et al., 1994; Wakefield et al., 1995). However, the preference for the selection and use of tRNA^{Lys,3} as a primer for reverse transcription is manifested by the fact that all of the viruses revert to utilize tRNA^{Lys,3}

* Corresponding author. Fax: +1 205 934 1580.
E-mail address: caseym@uab.edu (C.D. Morrow).

0042-6822/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2007.04.025

following a short-term *in vitro* culture. A previous study found a region in U5 upstream of the PBS, designated as the A-loop, contained adenosines displayed on a loop structure of a RNA stem–loop that is postulated to interact with the anticodon region of $tRNA^{Lys,3}$ (Goldschmidt et al., 2002; Isel et al., 1995). Previous studies from our laboratory have found that viruses with the A-loop and PBS complementary to the anticodon and 3′ nucleotides of $tRNA^{His}$, $tRNA^{Met}$, $tRNA^{Lys1,2}$ and $tRNA^{Thr}$ could stably utilize these tRNAs as primers for initiation of reverse transcription (Ni and Morrow, 2007; Wakefield et al., 1996; Zhang et al., 1998). We have also found that viruses with the A-loop and PBS complementary to $tRNA^{Glu}$ could stably utilize $tRNA^{Glu}$ as a primer for reverse transcription (Dupuy et al., 2003). The integrity of the RNA stem–loop encompassing the nucleotides complementary to the anticodon of $tRNA^{Glu}$ were demonstrated to be important for the virus to maintain the PBS complementary to $tRNA^{Glu}$ following *in vitro* replication.

SIV_{smmPBj} also contains a PBS that is complementary to $tRNA^{Lys,3}$ (Dewhurst et al., 1990). Presumably, SIV_{smmPBj} also preferentially selects $tRNA^{Lys,3}$ similar to what has been seen for HIV-1, although this has not been formally tested. To further explore the mechanism of lentivirus primer selection, we have compared the stability of HIV-1 and SIV_{smmPBj} genomes in which the PBS has been altered to be complementary to $tRNA^{Glu}$ following growth in human peripheral blood mononuclear cells (PBMC). SIV_{smmPBj} has been shown to infect both macaque or human PBMC (Fultz et al., 1989). The results of our studies demonstrate that the PBS of SIV_{smmPBj} and HIV-1 that were altered to be complementary to $tRNA^{Glu}$ differs with respect to stability of the PBS and virus growth in PBMC. Analysis of the PBS and A-loop regions from SIV_{smmPBj} following replication revealed the presence of at least two adenosine-rich loops that could possibly influence primer selection by SIV_{smmPBj}.

Results

Construction and characterization of SIV_{smmPBj} viral genomes with mutated PBS

In a previous study, we described the construction and characterization of HIV-1 in which the A-loop and PBS have been mutated to be complementary to the anticodon and 3′-terminal nucleotides of $tRNA^{Glu}$ (Dupuy et al., 2003). The A-loop region of HIV-1 consists of four adenosine residues that previous studies have modeled to be on the crown of an RNA stem–loop structure. In this study, we found that mutation of these adenosines to nucleotides that are complementary to the anticodon of $tRNA^{Glu}$, along with mutations in the PBS complementary to $tRNA^{Glu}$, resulted in a virus that stably utilized this tRNA for *in vitro* replication. SIV_{smmPBj} contains a PBS that is complementary to $tRNA^{Lys,3}$ (Dewhurst et al., 1990). Upstream of this PBS there are nucleotides identical to the A-loop region of HIV-1 (a sequence GAAAAT) (Fig. 1A). For the initial set of experiments, we generated SIV-Glu in which the PBS alone has been altered to the 3′-terminal 18 nucleotides of $tRNA^{Glu}$, and a second mutant, SIV-GluAC in which the putative A-loop region was also modified to be complementary to the anticodon of $tRNA^{Glu}$.

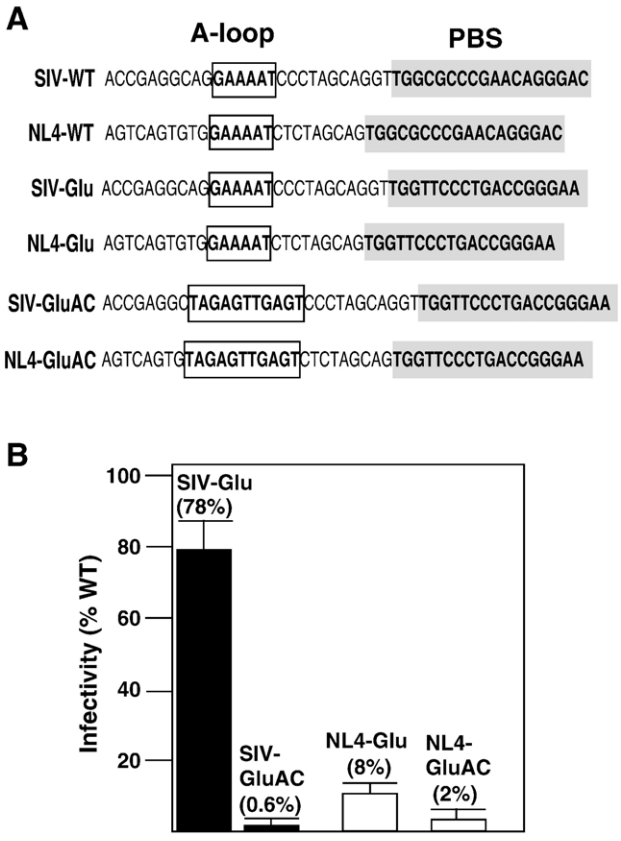

Fig. 1. HIV-1 and SIV_{smmPBj} U5 and PBS. (A) Nucleotide sequence of HIV-1 and SIV_{smmPBj} A-loop and PBS. The nucleotides sequence of the wild type SIV (SIV-WT) and HIV-1 (NL4-WT) A-loop and PBS regions are depicted. The PBS for both is complementary to the 3′-terminal 18 nucleotides of $tRNA^{Lys,3}$. A second region consisting of GAAAAT is found in both SIV-WT and NL4-WT. Proviral genomes were generated in which the PBS was made complementary to the 3′-terminal nucleotides of $tRNA^{Glu}$ (SIV-Glu or NL4-Glu). Proviral genomes were constructed in which the A-loop region of SIV-Glu and NL4-Glu was mutated to be complementary to the anticodon region of $tRNA^{Glu}$. This resulted in the alteration of the GAAAAT to TAGAGTTGAGT; proviral clones were named SIV-GluAC and NL4-GluAC. (B) Infectivity of viruses obtained following transfection into 293T cells. The proviral genomes were transfected into 293T cells and activity of the viruses were determined. Analysis of the p27 (SIV) or p24 (HIV-1) produced from the transfected cultures revealed no differences between the wild type and proviral genomes containing the A-loop or PBS mutations (data not shown). The amount of infectious virus was determined using the JC53-BL assay. The infectivity produced from transfection of wild type (SIV-WT or NL4-WT) was determined and the amounts produced by the PBS and A-loop mutant proviral genomes were compared to this value. The data presented represent infectivity compared to the wild type (SIV-WT or NL4-WT). The data presented are representative of three independent transfections.

Our first set of experiments characterized the infectivity of viruses obtained following transfection of the wild type or mutated proviral genomes into 293T cells. The modification of the PBS and A-loop did not effect the production of total virus as measured by p24 antigen (HIV-1) or p27 (SIV_{smmPBj}) of the transfection supernatant (data not shown). Previous studies from our laboratory have shown that HIV-1 genomes in which the PBS has been altered to be complementary to alternative

tRNAs results in viruses with approximately 10–20% infectivity of that of the wild type virus. Consistent with those previous studies, we found that NL4-Glu or NL4-GluAC had infectivities approximately 10% that of the wild type virus as determined in JC53-BL cells. Analysis of the infectivity of SIV_{smmPBj} with the PBS complementary to $tRNA^{Glu}$ (SIV-Glu) though gave a completely different result. In this case, the infectivity obtained for this virus following transfection was approximately 80% that of the wild type virus (Fig. 1B). However, inclusion of both A-loop and PBS complementary to $tRNA^{Glu}$ (SIV-GluAC) resulted in a virus that had considerably reduced infectivity approximately 1% of that of the wild type virus.

We next analyzed the replication of these viruses in which the PBS alone or U5 and PBS were altered to be complementary to $tRNA^{Glu}$. Viral replication was determined by measuring the luciferase activity produced from JC53-BL cells infected with supernatants from human PBMC at different times of infection. As expected, wild type HIV-NL4 replicated efficiently in human PBMC demonstrating a rapid rise in the production of infectious virus that peaked by 14 days (Fig. 2A). The alteration of the PBS to be complementary to $tRNA^{Glu}$ (NL4-Glu) had a drastic effect on the replication of HIV-1. Consistent with the previous study, we found that the virus with the PBS complementary to $tRNA^{Glu}$ grew slowly early in culture but at later times demonstrated a rapid increase in production of infectious virus that coincided with the reversion of the PBS to be complementary to $tRNA^{Lys,3}$. The virus with the U5 and PBS complementary to $tRNA^{Glu}$ NL4-GluAC also grew slowly during the culture period, peaking at approximately 18 days. Analysis of the PBS of this virus revealed that it maintained complementarity to $tRNA^{Glu}$ (data not shown).

The replication of SIV_{smmPBj} in PBMC was considerably different than HIV-1. First, the wild type virus, as expected, grew in PBMC reaching a peak at approximately 14 days post initiation of culture (Fig. 2B). However, the production of infectious virus by SIV_{smmPBj} was approximately 20-fold less than that for HIV-1 grown in human PBMC. SIV-Glu also grew well in human PBMC reaching a peak at approximately the same time as the wild type virus. The rapid growth of this virus in human PBMC is consistent with the infectivity, which demonstrated that this virus retained approximately 80% of the infectivity of the wild type virus. In contrast, SIV-GluAC demonstrated a delay in replication, reaching a peak level similar to that for the wild type virus after 21 days of the infection. Thus, in contrast to HIV-1, SIV_{smmPBj} does not grow as robustly in human cells and mutations within the PBS and U5 do not seem to effect virus replication as significantly as that for HIV-1.

We next analyzed the sequence of the U5-PBS region of the SIV_{smmPBj} following growth in human PBMC. For these studies, we utilized PCR to amplify the region containing the U5-PBS from integrated provirus. In most cases, the PCR product was TA cloned and individual clones were sequenced to determine the identity of the PBS. Analysis of SIV-Glu revealed that by Day 4 post-infection, all of the PBS sequences recovered were complementary to $tRNA^{Lys,3}$, indicating that a reversion to the wild type PBS had occurred (Table 1). A few additional

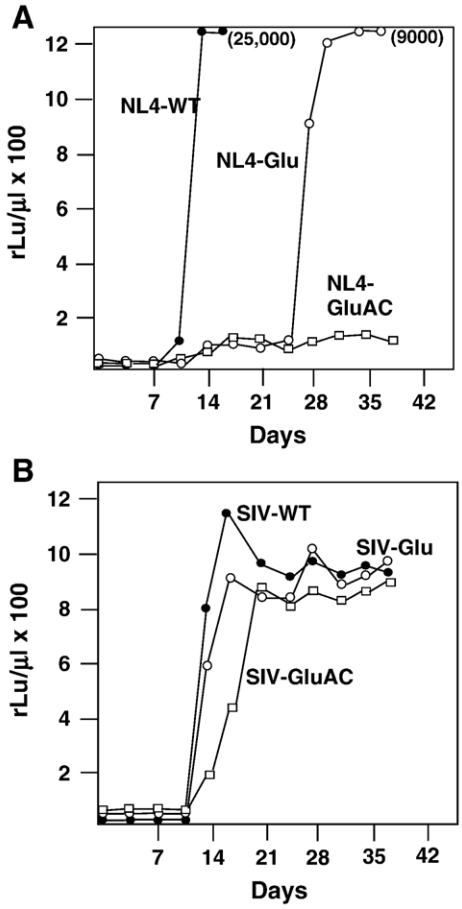

Fig. 2. Replication of HIV-1 and SIV_{smmPBj} with U5-PBS complementary to $tRNA^{Glu}$. (A) Replication of wild type HIV-1 and A-loop-PBS mutants. Infections were initiated in human PBMC using equal amounts of infectious virus. Supernatants were assayed at the designated intervals for infectious virus using the JC53-BL assay. The identity of the samples are as indicated. Note that for NL4-WT and NL4-Glu a break occurred in the production of infectious virus such that the wild type by Day 14 had peaked at 25,000 rLu/µL whereas NL4-Glu peaked at Day 31 at 9000 rLu/µL. The amount of infectious virus produced from NL4-GluAC increased throughout the culture, but peaked at approximately 100 rLu/µL. The data presented are representative of three independent experiments. (B) Replication of SIV-WT and A-loop-PBS mutants. Infections were initiated in human PBMC using equal amounts of infectious virus. At 3 to 4 day intervals, supernatants were collected from the infected cultures and analyzed for production of infectious virus using the JC53-BL assay. The identity of the samples are as indicated. The data presented is representative of three independent experiments.

nucleotide changes were also noted upstream of the PBS. Thus, the rapid rise of virus similar to the wild type virus at Day 11 was probably due to the fact that SIV-Glu had already reverted to utilize $tRNA^{Lys,3}$. We next analyzed the U5-PBS sequence of SIV-GluAC following *in vitro* replication. At early time points, Days 4 and 7 the virus maintained a PBS complementary to $tRNA^{Glu}$ indicating that the alteration in the A-loop sequence of SIV_{smmPBj} had stabilized the use of $tRNA^{Glu}$ as the primer for replication. However, by Day 14 and Day 21, PBS sequences complementary to $tRNA^{Lys,3}$ were observed. Strikingly though, we noted the emergence of additional mutations in the mutated A-loop sequence which had inserted adenosine residues for the majority of the mutated nucleotides. This would have the

Table 1
DNA sequences of U5-PBS region of proviruses derived from SIV-Glu and SIV-GluAC grown in human PBMC

Sequences (5′-3′)[a]			Sample[b]	Days[c]
GGAAACCGAGGCAGGAAAATCCCTAGCAGGT	TGG**TT**CCC**TG**AC**C**GGG**A**	TTGAAGGAGAGTGAG	Glu input	
	Lys,3[d]			
**********G*T**********	***CG***GA**A****C	***************	5/5	4
AAACCGAGGC**TA**GA**GTTGAG**TCCCTAGCAGGT	TGG**TT**CCC**TG**AC**C**GGG**A**	TTGAAGGAGAGTGAG	GluAC input	
	Glu[e]			
******************************	**********	***************	PCR	4,7
	Lys,3			
**********AG**AAA---**********	***CG***GA**A****C	***************	2/8	14
	Glu			
******************************	**********	***************	6/8	
	Glu			
******************************	**********	***************	5/10	21
******************************	**********A**	******A*A**A**A	1/10	
**********G***	**********	***************	1/10	
	Lys,3			
*****AAA**AG**AAA---**********	***CG***GA**A****C	***************	2/10	
**********AG**AAA---**********	***CG***GA**A****C	**********C***	1/10	

[a] Spaces separate the PBS from flanking sequences. Boldface characters indicate nucleotide changes made from the wild type sequence. Asterisks represent conserved nucleotides with the input sequence.
[b] Glu and GluAC input refers to either SIV-Glu or GluAC viruses used to initiate culture. PCR products were sequenced directly when indicated. Sequences of cloned PCR products are shown as frequencies of TA clones analyzed (i.e. number observed over total number analyzed).
[c] Number of days post inoculation of culture.
[d] PBS complementary to the 3′-terminal 18 nucleotides of $tRNA^{Lys,3}$.
[e] PBS complementary to the 3′-terminal 18 nucleotides of $tRNA^{Glu}$.

consequence of re-forming the adenosine-rich loop that was postulated to interact with the anticodon of $tRNA^{Lys,3}$. These changes that evolved in SIV_{smmPBj} occurred during the first 21 days of culture in human PBMC, even though the amount of virus produced as determined by the JC53-BL assay is less than that seen for the wild type virus.

Effect of mutations on two adenosine-rich RNA stem–loops in the U5 of SIV_{smmPBj}

To further understand the significance of the nucleotide changes within the U5 region in SIV-GluAC that lead to a stabilization of the PBS complementary to $tRNA^{Glu}$ for a short time, we analyzed the RNA structure of the U5-PBS using *m-fold*. A previous study reported that the *m-fold* of the U5-PBS of HIV-2, which is identical to this region in SIV_{smmPBj} revealed a RNA stem–loop structure containing two adenosine-rich regions (Berkhout, 1997) (Fig. 3). In our SIV-GluAC construct, we mutated the second A-loop region to be complementary to the anticodon region of $tRNA^{Glu}$ resulting in a new RNA stem–loop that displayed the nucleotides complementary to the anticodon region of $tRNA^{Glu}$ on an exposed loop (shaded area in A-loop 2, Fig. 3). What was striking about the *m-folds* of SIV_{smmPBj} was the existence of a second A-loop at the tip of a RNA stem. The question arose as to whether or not this loop structure could also impact the selection of the tRNA primer by SIV_{smmPBj} and virus replication. To address this question, we made two additional constructs. In the first, the adenosine residues of the A-loop structure were mutated to cytidines, resulting in a virus that we designated as SIV-CCA1 (Fig. 4A). In the second virus construct the adenosines of the second A-loop were also mutated to cytidines, designated as SIV-CCA2. Refolding of the new U5-PBS of these RNAs predicted an RNA stem–loop structure in which only the loop region was altered by the substitution of cytosines for adenosines.

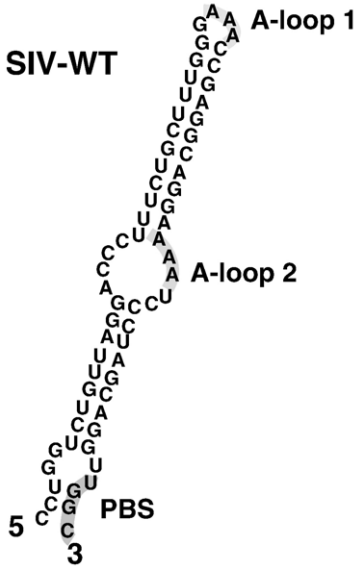

Fig. 3. Potential A-loop regions of SIV_{smmPBj}. A *m-fold* of the potential A-loop regions of SIV_{smmPBj} is presented. Depicted are two potential A-loop regions designated as A-loop 1 and A-loop 2. Note that A-loop 2 was altered in the construction of SIV-GluAC. The first four nucleotides of the PBS complementary to $tRNA^{Lys,3}$ are also depicted.

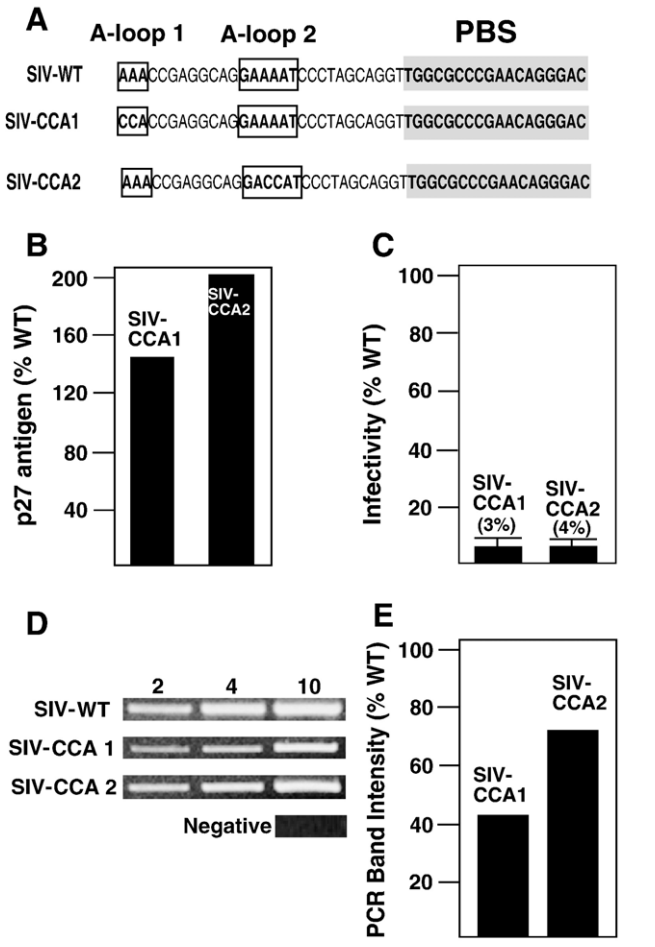

Fig. 4. SIV_{smmPBj} genomes with mutations in the A-loop 1 or A-loop 2. (A) The wild type SIV_{smmPBj} U5-PBS is presented with the two A-loop regions (boxed); the PBS complementary to $tRNA^{Lys,3}$ is shaded. The first mutant SIV-CCA1, contains a mutation in A-loop 1 with two adenosines mutated to cytosines. A second proviral genome, SIV-CCA2 contains mutation of two adenosines to cytosines in A-loop 2. *m-folds* of the RNA from SIV-CCA1 and SIV-CCA2 revealed similar RNA structures as the wild type with the mutated nucleotide substituted in the loop regions (data not shown). (B) Effect of the A-loop mutations on virus production. The proviral genomes were transfected in the 293T cells and the amount of virus determined. There were no different effects on the amount of virus produced as determined by the p27 antigen ELISA capture assay. (C) Effect of the A-loop mutations on infectivity. The amount of infectious virus produced from transfection was determined by the JC53-BL assay and was compared to that obtained from transfection of SIV-WT (set at 100%). Both A-loop mutations resulted in a reduction in the amount of infectious virus to 3 and 4% that of the wild type virus. Data are representative of three independent transfections. (D) Effect of A-loop mutations on the completion of reverse transcription. Equal amounts of the transfection supernatant were used to infect human PBMC; 24-h later, PCR was used to determine the amount of viral DNA present. The agarose gel is shown and the lanes are labeled with the amount of high molecular weight DNA used in the PCR reactions (i.e., 2, 4, and 10 ng). (E) The amount of product produced from PCR was determined from the band intensity on a 1% agarose gel divided by that obtained from SIV-WT (set at 100%); 2 ng samples are presented in figure. Values presented are for three independent PCR reactions; values varied 2% from each PCR reaction.

We first examined the impact of these mutations on the production of infectious virus following transfection of the proviral genomes. The mutations had no effect on the production of virus as measured by p27 in the supernatant from transfected cells (Fig. 4B). However, we found that both SIV-CCA1 and SIV-CCA2 produced infectious virus at a level that was approximately 3–4% that of the wild type as measured by the JC53-BL assay (Fig. 4C). Thus, consistent with SIV-GluAC, we found that mutations within the U5 drastically reduced the production of infectious virus. We next analyzed the replication of these viruses in PBMC. In the first experiments, we infected PHA stimulated PBMC and then harvested high molecular weight DNA after 24 h. PCR was used to analyze for the presence of SIV_{smmPBj} DNA using primers specific for U5-PBS (Fig. 4D). Compared to wild type, both the SIV-CCA1 and SIV-CCA2 had less SIV_{smmPBj} DNA, consistent with the infectivity results (Fig. 4C). We next analyzed the replication in long-term PBMC culture. SIV-CCA1 replicated similar to the wild type virus peaking at Day 11 post initiation of culture (Fig. 5). In contrast, the virus SIV-CCA2 replicated much slower than the wild type virus, demonstrating a gradual rise in production of infectious virus throughout the culture period (39 days). Thus, the mutations within the different A-loop regions had different effects on the replication of these viruses.

To determine if the mutations were stable, we analyzed the sequence of the U5 region of each virus at different times post initiation of culture. Results of these studies demonstrated that SIV-CCA1 rapidly reverted to the wild type sequence by approximately 4 days after initiation of the culture (Table 2). In fact, analysis of the DNA sequence of PCR product obtained after a 24-h infection (Fig. 4C) revealed the presence of the wild type sequence from the SIV-CCA1 culture. In contrast, the virus SIV-CCA2 remained stable for the majority of the culture period up to 32 days post initiation of culture. By Day 39 the virus replication was approaching a level similar to that of the wild type and we noted the reversion of the two cytidine to adenosines, resulting in the wild type U5 sequence. Collectively, the results of these studies demonstrate that SIV_{smmPBj} contains two A-loop regions that have different effects on the replication of the virus and in contrast to HIV-1, has a strong

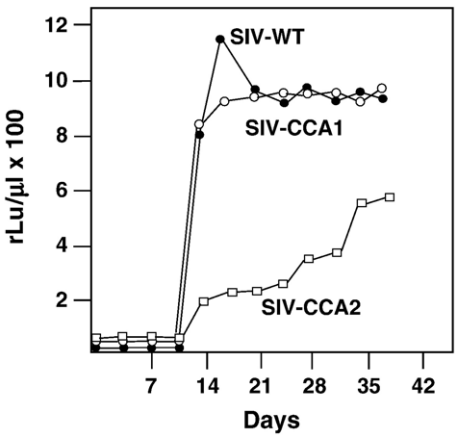

Fig. 5. Replication of SIV_{smmPBj} genomes with mutations in A-loop 1 or A-loop 2. Infections were initiated with equal amounts of infectious virus in human PBMC. The amount of virus produced was monitored by JC53-BL assay. At the end of the experiment, the U5-PBS was amplified from the integrated provirus of infected cells and DNA sequenced to determine the presence of the mutations. The data presented are representative of three independent experiments.

Table 2
DNA sequences of U5-PBS region of proviruses derived from SIV-CCA1 and SIV-CCA2 grown in human PBMC

Sequences (5′–3′)[a]			Sample[b]	Days[c]
TGCTTTGGGA**CCA**CGAGGCAGGAAAATCCCTAGCAGGT	TGGCGCCCGAACAGGGAC	TTG	CCA1 input	4
	Lys,3[d]			
**********AAC*************************	**********	***	5/5	
TGCTTTGGGAAACCGAGGCAGGA**CC**ATCCCTAGCAGGT	TGGCGCCCGAACAGGGAC	TTG	CCA2 input	
	Lys,3			
*************************************	**********	***	PCR	4,7,11,18
	Lys,3			
**********************A***************	**********	***	4/5	32
**********************A***************	**********	***	1/5	
	Lys,3			
**********************AA**************	**********	***	5/5	39

[a] Spaces separate the PBS from flanking sequences. Boldface characters indicate nucleotide changes made from the wild type sequence. Asterisks represent conserved nucleotides with the input sequence.
[b] CCA1 and CCA2 input refers to either SIV-CCA1 or CCA2 viruses used to initiate culture. PCR products were sequenced directly when indicated. Sequences of cloned PCR products are shown as frequencies of TA clones analyzed (i.e. number observed over total number analyzed).
[c] Number of days post inoculation of culture.
[d] PBS complementary to the 3′-terminal 18 nucleotides of $tRNA^{Lys,3}$.

preference for the wild type sequence leading to reversion of mutations to restore these A-loop regions.

Discussion

In the current study, we have investigated the primer selection preference of SIV_{smmPBj} using mutants in which the PBS was altered to be complementary to $tRNA^{Glu}$. A virus with the PBS complementary to $tRNA^{Glu}$ was approximately 80% as infectious as the wild type virus. However, after limited replication in human PBMC, the virus reverted to utilize $tRNA^{Lys,3}$ as the primer. In HIV-1, a region 5′ to the PBS in U5 that is complementary to $tRNA^{Lys,3}$ (A-loop) is important in primer selection. A SIV_{smmPBj} construct in which the PBS and a similar A-loop region were mutated to be complementary to $tRNA^{Glu}$ was less than 1% as infectious as the wild type virus. Although this virus did replicate in human PBMC, analysis of the U5-PBS of this virus following replication revealed that both the U5 and PBS had reverted to be complementary to $tRNA^{Lys,3}$ in some clones. To further explore the importance of the U5 region in primer selection for SIV_{smmPBj}, we made additional proviruses which contained mutations in two adenosine-rich RNA stem–loop regions that could potentially interact with the anticodon of $tRNA^{Lys,3}$. Both viruses exhibited profound reduction in infectivity, replicated more slowly than the wild type virus and eventually reverted to be complementary to the anticodon of $tRNA^{Lys,3}$.

Lentiviruses such as HIV-1 and SIV have evolved to preferentially select $tRNA^{Lys,3}$ as a primer for reverse transcription (Mak and Kleiman, 1997; Marquet et al., 1995). Previous studies with HIV-1 have found that alteration of the PBS to be complementary to alternative tRNAs results in the virus reverting to utilize $tRNA^{Lys,3}$ following short-term *in vitro* replication (Das et al., 1995; Li et al., 1994; Wakefield et al., 1995). In some cases, the virus can be forced to utilize alternative tRNAs by additional mutations upstream in a region that is complementary with the anticodon of the $tRNA^{Lys,3}$ (A-loop) (Dupuy et al., 2003; Kang and Morrow, 1999; Kang et al., 1999; Ni and Morrow, 2007; Wakefield et al., 1996). Previous studies using enzymatic and chemical analysis have shown the A-loop region interacting with the anticodon of $tRNA^{Lys,3}$ in the initiation complex (Isel et al., 1995). The intent of the current study was to determine if SIV_{smmPBj} would also exhibit a preferential selection of $tRNA^{Lys,3}$ after mutation of the U5-PBS. For our studies, we chose to mutate the PBS complementary to $tRNA^{Glu}$, since previous studies from our laboratory have shown that HIV-1 can be forced to use $tRNA^{Glu}$ with mutations both in the U5 and PBS (Dupuy et al., 2003). SIV_{smmPBj} was chosen for our studies because previous work demonstrated this virus can replicate in human PBMC (Fultz et al., 1989). Thus, we would be able to compare more directly the primer preference of HIV-1 and SIV_{smmPBj} grown in the same cells. Analysis of primer preference following replication in the same cells should also eliminate concerns over differences in availability of tRNAs for primer capture.

The first difference between HIV-1 and SIV_{smmPBj} was evident upon the analysis of the effect of altering the PBS in SIV_{smmPBj} to be complementary to $tRNA^{Glu}$. This virus, SIV-Glu retained approximately 80% of the infectivity of the wild type virus while a similar mutation in HIV-1 results in a virus with only 10% of the infectivity of the wild type virus. Analysis of the replication of SIV-Glu in human PBMC revealed that the replication kinetics was very similar to the wild type virus. Analysis of the PBS of integrated proviruses from cultures only 4 days post initiation of infection revealed the presence of PBS complementary to $tRNA^{Lys,3}$, indicating that reversion had occurred rapidly during replication of this virus. In contrast, HIV-1 with a PBS complementary to $tRNA^{Glu}$ replicated more slowly than the wild type virus and only reverted after an extended time in culture (Dupuy et al., 2003). The replication of HIV-1 in PBMC was more vigorous than SIV_{smmPBj} as determined by production of infectious virus (JC53-BL assay). The rapid reversion of SIV_{smmPBj} even with the lower production of infectious virus suggests that the

primer selection process might actually be more stringent for SIV_{smmPBj}. Why there would be a difference between SIV_{smmPBj} and HIV-1 with respect to primer selection is unclear. Previous studies from our laboratory have linked primer selection with viral translation (Kelly and Morrow, 2003; Kelly et al., 2003; Palmer et al., 2007). It is possible that inherent differences between SIV_{smmPBj} and HIV-1 viral translation, such as codon preference within Gag and Gag-pol could impact on primer selection (Palmer et al., 2007). Additional studies with more PBS mutants will be needed to resolve the reason for the differences between SIV_{smmPBj} and HIV-1.

A second difference between SIV_{smmPBj} and HIV-1 with respect to primer preference comes from the analysis of SIV_{smmPBj} in which both the PBS and a region upstream of the PBS (in the U5) were mutated to be complementary to $tRNA^{Glu}$ (SIV-GluAC). In contrast to SIV-Glu, this virus had greatly reduced infectivity compared to the wild type virus (approximately 1%) and the replication of SIV-GluAC in PBMC was delayed compared to that of the wild type virus, eventually reaching wild type replication levels after extended culture. Analysis of the U5-PBS at different times during the culture period revealed that in some clones both the PBS and A-loop region had reverted to wild type sequence. In contrast, the A-loop regions of HIV-1 A-loop-PBS mutants did not revert back to wild type (Dupuy et al., 2003; Kang and Morrow, 1999; Kang et al., 1999; Wakefield et al., 1996; Zhang et al., 1998). Previous studies have described the *m-fold* of the HIV-2 U5-PBS RNA (Berkhout, 1997) (there is a sequence identity of the region of HIV-2 and SIV_{smmPBj}). Two possible A-loop regions were noted in the *m-fold*. One of the A-loop regions corresponded to the region mutated in the construction of the SIV-GluAC (A-loop 2), while another A-loop region was found upstream at the tip of a long RNA stem–loop (A-loop 1). To ascertain the importance of these A-loop regions, we mutated them individually and analyzed the effect on virus replication. Although mutation of either A-loop did not effect the production of total virus as measured by p27 (Fig. 4B), the mutation of either A-loop had a great impact on the infectivity of the virus derived from 293T cells as measured by PCR of infected PBMC or the JC53-BL assay. Further support for the importance of the adenosines in virus replication, comes from the analysis of the stability of the mutations following replication in PBMC. The mutation in A-loop 1 rapidly reverted to the wild type sequence following short-term *in vitro* culture (first detected after 24 h of infection). The reversion correlated with the rapid increase in production of infectious virus in these cultures, while the virus with mutations in A-loop 2 had delayed replication resulting in slower growth as compared to the wild type virus. However, during replication a reversion also occurred which restored the second A-loop. The kinetics of the reversion of virus with mutations in the second A-loop region were similar to that observed for SIV-GluAC, which also reverted the mutations in the A-loop back to the wild type sequence. Based on our studies with HIV-1, we speculate that the A-loop regions in SIV_{smmPBj} are involved in primer selection. The dynamics of the interaction between the A-loops and $tRNA^{Lys,3}$ though are unclear. Since previous studies have suggested the A-loop of HIV-1 interacts with the anticodon of $tRNA^{Lys,3}$ in a manner similar to codon:anticodon interaction (Puglisi and Puglisi, 1998), it is possible that A-loop 1 of SIV_{smmPBj} could function primarily to attract $tRNA^{Lys,3}$. The sequence of the A-loop 2 region is identical to that of the HIV-1 A-loop. Previous studies have found that the A-loop of HIV-1 forms an intricate interaction with the anti-codon of $tRNA^{Lys,3}$ in the initiation complex required for reverse transcription (Goldschmidt et al., 2002; Isel et al., 1995). Although it has been suggested that this region could also be involved with integration (Masuda et al., 1998; Yoshinaga and Fujiwara, 1995), the key nucleotides for integration did not overlap with our mutants. Thus, it is possible that SIV_{smmPBj} has evolved to separate the individual functions of the HIV-1 A-loop. Further studies though will be needed to resolve the roles of the individual A-loops of SIV_{smmPBj} in reverse transcription.

Materials and methods

Construction of HIV-NL4.3 and $SIV_{smmPBj14}$ mutant proviral genomes

Construction of pHXB2(Glu) and pHXB2(Glu Loop 1) with U5-PBS changes complimentary to $tRNA^{Glu}$ was previously described (Dupuy et al., 2003). The proviral clones pHXB2 (Glu) and pHXB2(Glu Loop 1) were digested with the restriction enzymes *Hpa*I and *Bss*HII (New England Biolabs, Beverly, MA) to release a 868 base pair fragment containing the 5′ long terminal repeat, PBS, and leader region of *gag*. This 868 base pair fragment was isolated via gel extraction using the Qiagen QIAquick Gel Extraction kit (Qiagen, Valencia, CA) and cloned between the *Hpa*I and *BssH*II sites of the HIV-NL4.3 proviral plasmid, producing the molecular clones pNL4-Glu and pNL4-GluAC.

The infectious molecular clone 1.9 of SIV_{smm} isolate PBj14 (SIV_{smmPBj}) was obtained from Patricia Fultz, Department of Microbiology, University of Alabama at Birmingham. The 5′ long terminal repeat, PBS, and leader region of *gag* of this clone were inserted into the multiple cloning site, *Xho*I-to-*Eco*47III (New England Biolabs and Promega, Madison, WI, respectively), of the pSE420 vector (Invitrogen, Carlsbad, CA). The resulting vector, named pSE420-PBj, served as a template for mutagenesis using the QuikChange II Site-Directed *in vitro* mutagenesis kit (Stratagene, La Jolla, CA). The PBS was mutated to a sequence complementary to the 3′-terminal 18 nucleotides of $tRNA^{Glu}$ forming the mutant pSE420-PBj(Glu). The pSE420-PBj(Glu) vector was then used as the template to make additional mutations upstream of the PBS sequence in a region that appeared to resemble the A-loop of HIV-1, forming the mutant vector pSE420-PBj(GluAC). The pSE420-PBj vector was used to make mutations to disrupt two adenosine-rich loops forming the mutant vectors pSE420-PBj(CCA1) and pSE420-PBj(CCA2). The mutagenic primers used to make these mutations were: GluF(5′CCCTAGCAGGTTGGTTCCCTGACCGGGAATTGAAGGAGAGTGAGAGCTCC3′), GluR (5′ GGAGCTCTCACTCTCCTTCAATTCCCGGTCAGGGAACCAACCTGCTAGGG3′), GluACF (5′ CCTTTCTGCTTTGG-

GAAACCGAGGCTAGAGTTGAGTCCCTAGCAGGTTG-GTTCCCTGAGGG 3′), GluACR(5′CCCGGTCAGGGAAC-CAACCTGCTAGGGACTCAACTCTAGCCTCGGTTTCCC-AAAGCAGAAAGG3′), CCA1F (5′G GGAAACCGAGGCA-GGACCATCCCTAGCAGGTTGGCGC CCG3′), CCA1R (5′ CGGGCGCCAACCTGCTAGGGATGGTCCTGCCT-CGGTTTCCC3′), CCA2F(5′ GGACCCTTTCTGCTTTGGG-ACCACGAGGCAGGAAAATCCCTAGCAGG3′), CCA2R (5′CCTG CTAGGGATTTTCCTGCCTCGTGGTCCCAAAG-CAGAAAGGGTCC3′). All mutagenesis was performed according to the manufacturer's instructions, and the resulting mutant sequences were verified by DNA sequencing. The 1356 base pair *Xho*I-to-*Eco*47III fragments of pSE420-PBj(Glu), pSE420-PBj(GluAC), pSE420-PBj(CCA1) and pSE420-PBj (CCA2) containing the U5 and PBS regions were subcloned between the *Xho*I and *Eco*47III sites in the molecular clone 1.9 of $SIV_{smmPBj14}$ to form the complete proviral clones pSIV-Glu, pSIV-GluAC, pSIV-CCA1 and pSIV-CCA2. All proviral clones were screened by restriction digestion, and the sequences were verified by DNA sequencing to ensure successful mutagenesis and subsequent ligation into the proviral plasmid, either pNL4 or pSIV.

Tissue culture

All cultures were maintained at 37 °C with 5% CO_2. Both JC53-BL, obtained from the National Institutes of Health AIDS Research and Reference Reagent Program, and 293T cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Rockville, MD) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT) and 1% penicillin–streptomycin–L-glutamine mix (penstrep-glu; Cellgro® by Mediatech, Inc., Herdon, VA). Human peripheral blood mononuclear cells (PBMC) were isolated from HIV-1-seronegative whole blood (from two donors) by Ficoll-Hypaque (Amersham Pharmacia Biotech, Piscataway, NJ) density gradient centrifugation. PBMC were aliquoted and stored in liquid nitrogen at −140 °C. To begin an infection, PBMC were thawed and stimulated with 3 μg/mL of phytohemagglutinin (Sigma, St. Louis, Mo.) for 3 days in PBMC media, which contains Roswell Park Memorial Institute 1640 medium (RPMI; Cellgro) supplemented with 15% FBS, 1% penstrep-glu, and 30 U/mL recombinant human interleukin-2 (rIL-2; Roche Molecular Biochemicals, Indianapolis, IN). After stimulation, the medium was removed and replaced with fresh PBMC medium. PBMC were incubated for another 2 days before being infected with virus.

Virus production and analysis of viral infectivity

Transfections were performed in 293T cells that were approximately 70% confluent in a six-well cell culture plate using the appropriate pNL4 or pSIV proviral DNA plasmid and FuGENE 6 transfection reagent (Roche Molecular Biochemicals). Briefly, 3 μg of FuGENE 6 transfection reagent and 2 μg of proviral DNA plasmid were added to 100 μL DMEM (without FBS) and incubated at room temperature for 45 min. Each transfection cocktail was added, drop-wise, to one of the wells containing the 293T cells. The plates were incubated at 37 °C with 5% CO_2. After 24 h, the media were removed and replaced with fresh DMEM supplemented with 10% FBS and 1% penstrep-glu. At 72 h post-transfection, the virus supernatants were removed, microcentrifuged to remove cells, and then stored at −80 °C. To determine the amount of virus produced from the transfection, the supernatants were assayed for either p24 for HIV-1 or p27 for SIV_{smmPBj} using the Beckman Coulter p24 antigen ELISA kit (Beckman Coulter, Miami, FL) and the Retro Tek SIV p27 antigen ELISA kit (Zepto Metrix, Buffalo, NY). The number of infectious units (IU) contained in the supernatants was determined by the JC53-BL β-Gal assay. JC53-BL indicator cells are a derivative of HeLa cells that not only express high levels of CD4, CCR5, and CXCR4, but also contain a reporter cassette of β-galactosidase that is expressed from an HIV-1 promoter. Expression of the reporter genes is dependent on production of the viral protein *Tat*. Therefore, the cells are indicators of viral infectivity that is contained within a given volume. The JC53-BL β-Gal assays were preformed as described previously (Derdeyn et al., 2000). To initiate PBMC infection, virus transfection supernatant containing 250 IUs was used to infect 2×10^7 PBMC. The long-term PBMC cultures were passaged 1:2 alternating every 3 and 4 days. Supernatants were collected from PBMC infected cultures at every passage. Every 14 days, 5×10^6 newly thawed and stimulated PBMC, as described above, were added to replenish cells that had died. To determine the replication kinetics of the viruses in culture, the culture supernatants were used to infect JC53-BL cells and a JC53-BL luciferase assay was performed. In addition to the β-galactosidase, JC53-BL cells also contain a reporter cassette of luciferase that is controlled by the same HIV-1 promoter. For each assay, 6×10^3 JC53-BL cells were plated 1 day prior to infection. Equal amounts of the culture supernatants in the presence of DEAE-dextran (3 μg) in a final volume of 60 μL were added to the monolayer of JC53-BL cells. Plates were incubated at 37 °C with 5% CO_2 and then 140 μL of DMEM with 10% FBS and 1% penstrep-glu was added to the wells. Plates were incubated for another 48 h at 37 °C with 5% CO_2. To determine the luciferase activity, cells were lysed using M-PER mammalian protein extraction reagent (Pierce, Rockford, IL) and 20 μL of each lysate was transferred to a microplate. Reporter lysis buffer (Promega) was added to each sample in the microplate, and the light intensity was measured using a LUMIstar luminometer (BMG Labtech, Durham, NC). The luminescence emitted from uninfected cells was used as background levels and was subtracted from all other samples. The luciferase values for two dilutions per sample were averaged. Relative light units (rLu) per μL were calculated by dividing the luciferase values by their corresponding dilution values.

DNA sequence analysis of proviral U5 and PBS regions

Cell pellets were collected 24-h post-infection of PBMC for single-round culture and from the long-term PBMC cultures at

every passage. High molecular weight (HMW) DNA was obtained from these cell pellets using the Wizard Genomic DNA isolation kit (Promega) according to the manufacturer's protocol. A 506 base pair fragment containing the U5 and PBS regions of the integrated provirus was amplified from the HMW template DNA by PCR with the primers PBjF (5′TTTCCACAAAGGGGATGTTACG3′) and PBjR (5′CGCCTTTATAGGAGCACTCCG3′) under the following PCR conditions: Step 1: 95 °C for 5 min, Step 2: 95 °C for 1 min, Step 3: 62 °C for 1 min, Step 4: 72 °C for 1 min, Step 5: Goto Step 2, 33 more times, Step 6: 72 °C for 15 min, and Step 7: Keep at 4 °C. The PCR reactions for the 24-h PBMC cultures of SIV-WT, SIV-CCA1, and CCA2 contained 2 ng, 4 ng, and 10 ng of HMW template DNA. The negative control PCR reaction contained 10 ng of HMW from uninfected PBMC. The PCR products were run on a 1% agarose gel containing ethidium bromide. The intensity of the bands was then quantified using the image analysis software ImageJ 13.7v (Abraham et al., 2004). The PCR products from the long-term PBMC cultures were sequenced directly or clones of the PCR product were ligated into the pGEM-T Easy vector (Promega) according to the manufacturer's protocol and then sequenced using the primer PBjF.

Acknowledgments

We thank members of the Morrow laboratory for the helpful discussions, especially Uros Djekic, David McPherson, and Sylvia McPherson for help with the construction of mutant viruses. We thank Patricia Fultz for the SIV_{smmPBj} proviral plasmid. Adrienne Ellis is thanked for preparation of the manuscript. MCK is supported by training grant AI007439. DNA Sequencing was carried out at the UAB Center for AIDS Research Sequencing Core (AI27767). CDM acknowledges help from MAR. The research was supported by a grant from the NIH to CDM (AI34749).

References

Abraham, K.E., McMillen, D., Brewer, K.L., 2004. The effects of endogenous interleukin-10 on gray matter damage and the development of pain behaviors following ecitotoxic spinal cord injury in the mouse. NeuroScience 124 (4), 945–952.

Baltimore, D., 1970. RNA-dependent DNA polymerase in virions of RNA tumour viruses. Nature 226 (5252), 1209–1211.

Berkhout, B., 1997. The primer binding site on the RNA genome of human and simian immunodeficiency viruses is flanked by an upstream hairpin structure. Nuc. Acids Res. 25, 4013–4017.

Das, A.T., Klaver, B., Berkhout, B., 1995. Reduced replication of human immunodeficiency virus type 1 mutants that use reverse transcription primers other than the natural tRNALys,3. J. Virol. 69 (5), 3090–3097.

Derdeyn, C.A., Decker, J.M., Sfakianos, J.N., Wu, X., O'Brien, W.A., Ratner, L., Kappes, J.C., Shaw, G.M., Hunter, E., 2000. Sensitivity of human immunodeficiency virus type 1 to the fusion inhibitor T-20 modulated by coreceptor specificity defined by the V3 loop of gp120. J. Virol. 74, 8358–8367.

Dewhurst, S., Embretson, J.E., Anderson, D.C., Mullins, J.I., Fultz, P.N., 1990. Sequence analysis and acute pathogenicity of molecularly cloned SIVSMM-PBj14. Nature 345, 636–640.

Dupuy, L.C., Kelly, N.J., Elgavish, T.E., Harvey, S.C., Morrow, C.D., 2003. Probing the importance of tRNA anticodon: human immunodeficiency virus type 1 (HIV-1) RNA genome complementarity with an HIV-1 that selects tRNAGlu for replication. J. Virol. 77 (16), 8756–8764.

Fultz, P.N., McClure, H.M., Anderson, D.C., Switzer, W.M., 1989. Identification and biologic characterization of an acutely lethal variant of simian immunodeficiency virus from sooty mangabeys (SIV/SMM). AIDS Res. Hum. Retroviruses 5, 397–409.

Goldschmidt, V., Rigourd, M., Ehresmann, C., Le Grice, S.F.J., Ehresmann, B., Marquet, R., 2002. Direct and indirect contributions of RNA secondary structure elements to the initiation of HIV-1 reverse transcription. J. Biol. Chem. 277 (45), 43233–43242.

Isel, C., Ehresmann, C., Keith, G., Ehresmann, B., Marquet, R., 1995. Initiation of reverse transcription of HIV-1: secondary structure of the HIV-1 RNA/tRNA(3Lys) (template/primer). J. Mol. Biol. 247, 236–250.

Kang, S.-M., Morrow, C.D., 1999. Genetic analysis of a unique human immunodeficiency virus type 1 (HIV-1) with a primer binding site complementary to tRNAMet supports a role for U5-PBS stem–loop RNA structures in initiation of HIV-1 reverse transcription. J. Virol. 73, 1818–1827.

Kang, S.-M., Zhang, Z., Morrow, C.D., 1999. Identification of a human immunodeficiency virus type 1 that stably uses tRNALys1,2 rather than tRNALys,3 for initiation of reverse transcription. Virology 257, 95–105.

Kelly, N.J., Morrow, C.D., 2003. Yeast tRNAPhe expressed in human cells can be selected by HIV-1 for use as a reverse transcription primer. Virology 313, 354–363.

Kelly, N.J., Palmer, M.T., Morrow, C.D., 2003. Selection of retroviral reverse transcription primer is coordinated with tRNA biogenesis. J. Virol. 77, 8695–8701.

Li, X., Mak, J., Arts, E.J., Gu, Z., Kleiman, L., Wainberg, M.A., Parniak, M.A., 1994. Effects of alterations of primer-binding site sequences on human immunodeficiency virus type 1 replication. J. Virol. 68 (10), 6198–6206.

Mak, J., Kleiman, L., 1997. Primer tRNAs for reverse transcription. J. Virol. 71 (11), 8087–8095.

Marquet, R., Isel, C., Ehresmann, C., Ehresmann, B., 1995. tRNAs as primer of reverse transcriptases. Biochimie 77, 113–124.

Masuda, T., Kuroda, M.J., Harada, S., 1998. Specific and independent recognition of U3 and U5 att sites by human immunodeficiency virus type 1 integrase in vivo. J. Virol. 72 (10), 8396–8402.

Muesing, M.A., Smith, D.H., Cabradilla, C.D., Benton, C.V., Lasky, L.A., Capon, D.J., 1985. Nucleic acid structure and expression of the human AIDS/lymphadenopathy retrovirus. Nature 313, 450–458.

Ni, N., Morrow, C.D., 2007. Impact of forced selection of tRNAs on HIV-1 replication and genome stability highlight preferences for selection of certain tRNAs. Virus Res. 124 (1–2), 29–37.

Palmer, M.T., Kirkman, R., Kosloff, B.R., Eipers, P.G., Morrow, C.D., 2007. tRNA isoacceptor preference prior to retrovirus Gag-pol junction links primer selection and viral translation. J. Virol. 81 (9), 4397–4404.

Panet, A., Berliner, H., 1978. Binding of tRNA to reverse transcriptase of RNA tumor viruses. J. Virol. 26, 214–220.

Peters, G., Dahlberg, J.E., 1979. RNA-directed DNA synthesis in Moloney murine leukemia virus: interaction between the primer tRNA and the genome RNA. J. Virol. 31, 398–407.

Puglisi, E.V., Puglisi, J.D., 1998. HIV-1 A-rich RNA loop mimics the tRNA anticodon structure. Nat. Struct. Biol. 5 (12), 1033–1036.

Ratner, L., Haseltine, W., Patarca, R., Livak, K.J., Starcich, B., Josephs, S. F., Doran, E.R., Rafalski, J.A., Whitehorn, E.A., Baumeister, K., Ivanoff, L., Petteway Jr., S.R., Pearson, M.L., Lautenberger, J.A., Papas, T.S., Ghrayeb, J., Chang, N.T., Gallo, R.C., Wong-Staal, F., 1985. Complete nucleotide sequence of the AIDS virus, HTLV-III. Nature 313, 277–284.

Temin, H.M., 1981. Structure, variation and synthesis of retrovirus long terminal repeat. Cell 27, 1–3.

Temin, H.M., Mizutani, S., 1970. RNA-dependent DNA polymerase in virions of Rous sarcoma virus. Nature 226, 1211–1213.

Wakefield, J.K., Wolf, A.G., Morrow, C.D., 1995. Human immunodeficiency virus type 1 can use different tRNAs as primers for reverse transcription but

selectively maintains a primer binding site complementary to $tRNA^{Lys,3}$. J. Virol. 69, 6021–6029.

Wakefield, J.K., Kang, S.-M., Morrow, C.D., 1996. Construction of a type 1 human immunodeficiency virus that maintains a primer binding site complementary to $tRNA^{His}$. J. Virol. 70, 966–975.

Yoshinaga, T., Fujiwara, T., 1995. Different roles of bases within the integration signal sequence of human immunodeficiency virus type 1 in vitro. J. Virol. 69 (5), 3233–3236.

Zhang, Z., Kang, S.-M., Li, Y., Morrow, C.D., 1998. Genetic analysis of the U5-PBS of a novel HIV-1 reveals multiple interactions between the tRNA and RNA genome required for initiation of reverse transcription. RNA 4, 394–406.