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Human immunodeficiency virus uses $tRNA^{Lys,3}$ as primer for reverse transcription in HeLa-$CD4^+$ cells

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Abstract

Significant amounts of different tRNA molecules are present in retroviral particles, but one specific tRNA species functions as primer in reverse transcription. It is generally believed that the HIV-1 virus uses the $tRNA^{Lys,3}$ molecule as primer. This is based on sequence complementarity between the 3′ end of $tRNA^{Lys,3}$ and the primer-binding site (PBS) on HIV-1 genomic RNA. Recent biochemical analyses indicated that $tRNA^{Lys,3}$ is indeed incorporated into viral particles. Interestingly, $tRNA^{Lys,3}$ could not be detected in virions produced by HeLa-$CD4^+$ cells [(1992) Biochem. Biophys. Res. Commun. 185, 1105–1115]. In order to test whether alternative tRNA molecules can function as primer in HIV replication, we performed a series of experiments based on the observation that tRNA primer sequences are inherited by the viral progeny. We cultured HIV-1 for prolonged periods of time in HeLa-$CD4^+$ cells, but did not detect sequence changes in the PBS region. Furthermore, we found PBS-mutants to be replication-incompetent, again suggesting that HIV-1 solely uses $tRNA^{Lys,3}$ as primer. Most importantly, we obtained revertants of one such PBS-mutant, which had restored a wild-type PBS sequence. This $tRNA^{Lys,3}$-mediated repair demonstrates a general requirement for this primer in HIV-1 reverse transcription.

Key words: Reverse transcription; $tRNA^{Lys,3}$; Human immunodeficiency virus type 1; RNA–RNA interaction

1. Introduction

The replicatio cycle of human immunodeficiency virus (HIV) and other retroviruses involves the reverse transcription of virion RNA into DNA, which then becomes integrated into the host cell genome (reviewed in [2]). The reverse transcription reaction is mediated by the virion-associated enzyme reverse transcriptase (RT) and a cellular tRNA molecule is used as primer. The tRNA molecule associated with the genomic RNA by base-pairing of the 3′-terminal 16–19 nucleotides of the primer with a complementary sequence in the viral genome, referred to as the primer-binding site (PBS). Some diversity exists with regard to the tRNA molecule used as primer by the different retroviruses. The vast majority of the mammalian retroviruses use $tRNA^{Pro}$, and avian retroviruses use $tRNA^{Trp}$ (reviewed in [3]). Both human T-cell leukemia virus types I and II utilize $tRNA^{Pro}$, and the HIV-1 and HIV-2 sequences reveal a PBS corresponding to $tRNA^{Lys,3}$ [4] (see also Fig. 1A). This same $tRNA^{Lys,3}$ molecule, which is one of the major $tRNA^{Lys}$ isoacceptors present in mammalian cells [5], is also used by mouse mammary tumor virus, while $tRNA^{Lys,1,2}$ is used by Mason-Pfizer monkey virus, Visna and Spuma retrovirus. The identity of only a few primer species was substantiated by biochemical studies on the amino-acid-acceptor properties, chromatographic behaviour and tRNA sequence (reviewed in [2]).

Recent work by Jiang et al. [1] showed that $tRNA^{Lys,3}$ is indeed present in HIV-1 viral particles produced in T-lymphocytic cell lines. In general, retroviral particles do not exclusively contain genomic RNA and the corresponding tRNA primer, but in addition a large number of other tRNA molecules. Of the many tRNAs, the primer species is in tight association with the viral RNA, while most other tRNAs are referred to as free tRNA. The primer $tRNA^{Lys,3}$ was shown to be the major component of the genome-assosiated tRNA in HIV-1 virions, which resisted dissociation from the HIV-1 RNA at 65°C [1]. Surprisingly, these authors were unable to detect $tRNA^{Lys,3}$ in virions produced in HeLa-$CD4^+$ cells. Instead, one tightly-associated and abundant tRNA species was found that did not have the electrophoretic mobility of $tRNA^{Lys,3}$ and did not hybridize to $tRNA^{Lys,3}$-specific probes. These results may suggest that HIV can replicate in HeLa-$CD4^+$ cells using a primer other than $tRNA^{Lys,3}$.

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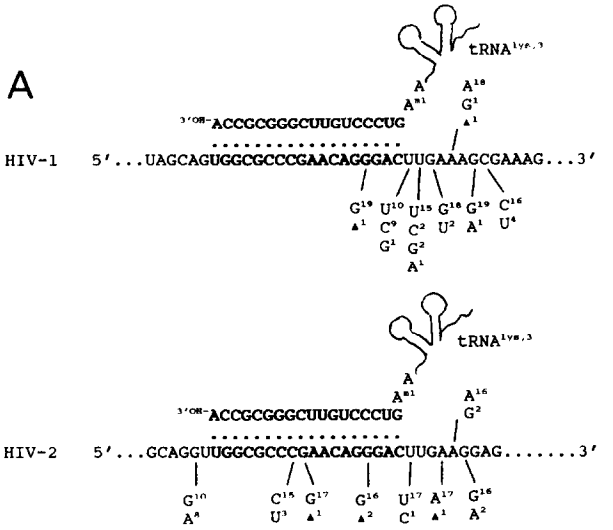
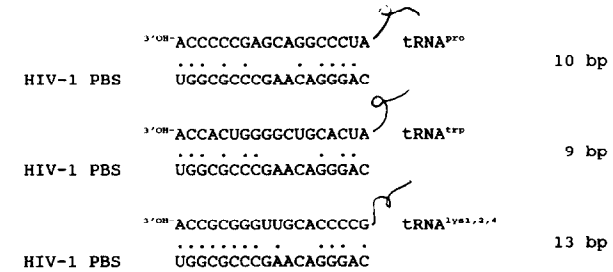
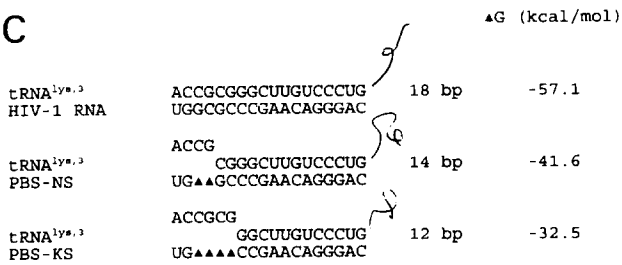
2. Results and discussion

We have tested primer-usage of HIV-1 in HeLa-CD4$^+$ cells by analyzing the PBS sequences of viruses that were cultured for a prolonged period of time in HeLa-CD4$^+$ cells. The rational for this approach stems from the model for retroviral replication [6]. The model predicts that the sequence of the primer-binding site in proviruses is the copy DNA of the 3′ end of the priming tRNA species. This tRNA-copy is formed during strong-stop plus-strand DNA synthesis and most likely proceeds up to the first modified base in the tRNA (A^{m1} in Fig. 1A). Recent data of Rhim et al. [7] strongly support this aspect of the model. Thus, the PBS sequence is regenerated from the priming tRNA itself and usage of an alternative primer should lead to changes in the PBS sequence after one replication cycle. For instance, usage of tRNA^{Pro} will generate HIV-1 progeny with 8 nucleotide substitutions in the PBS and usage of one of the other lysine-accepting tRNAs will change the PBS at 5 positions (Fig. 1B). Such altered PBS sequences will perfectly match the new primer in subsequent replication cycles.







In order to allow viral replication in HeLa cells, we used the HeLa-CD4$^+$ cell line that expresses the HIV-1 receptor on its surface [8]. HeLa-CD4$^+$ cells were infected with a HIV-1 LAI virus stock that was produced in T cells and virus replication was continued for many weeks by frequently passaging the cell-free virus onto fresh HeLa-CD4$^+$ monolayers (see legend to Fig. 2 for experimental details). Since the viral stock was produced in T cells, the first round of reverse transcription in HeLa-CD4$^+$ cells will use a tRNA^{Lys,3} primer derived from T cells. Therefore, we analyzed viral sequences only after several passages in HeLa-CD4$^+$ cells. The proviral DNA sequence obtained at this time point is shown in Fig. 2 (HeLa1). We also analyzed multiple proviral sequences after 32 passages in HeLa-CD4$^+$ cells (HeLa2-6). The results clearly indicate that the PBS^{Lys,3} sequence is not changed in proviral genomes recovered from HeLa-CD4$^+$ cells. In addition, we tested the pool of proviral DNA for the presence of the *Nar*I/*Kas*I restriction site within the PBS sequence (GGCGCC). We could not detect *Nar*I/*Kas*I-resistant HIV sequences, which is consistent with the absolute conservation of the binding site for tRNA^{Lys,3}.

It cannot a priori be excluded that selection of a new primer tRNA in HeLa-CD4$^+$ cells is coupled to the use of another PBS sequence at a different position on the

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Fig. 1. Schematic representation of the PBS-tRNA interaction for the HIV virus. (A) The consensus genomic sequences of HIV-1 and HIV-2 are shown with the 18-nucleotide long PBS region in bold. Nucleotide variation in 20 HIV-1 and 18 HIV-2 isolates (4) is indicated with the number of isolates having the particular sequence in superscript. Nucleotide deetions are shown as ▲. The viral PBS sequence is shown base-paired to the 3′ end of the tRNA^{Lys,3} primer. The tRNA nucleotide flanking the base-paired stretch is the methylated A^{m1} residue. The remaining part of the tRNA molecule is drawn as two stem-loop structures, representing the anticodon- and D-arm, and the single-stranded 5′ end. (B) Putative interactions of the HIV-1 primer-binding site (PBS) with tRNAs other than tRNA^{Lys,3}. This analysis was performed for the primer species frequently used by other retroviruses (tRNA^{Pro}, tRNA^{Trp} and tRNA^{Lys,2,4}). The latter three tRNAs are represented by one sequence because they have an identical 3′-terminus (5). The base-pair potential is indicated by dots and the total number of interactions is listed. (C) PBS-tRNA interaction for the wild-type PBS and mutants PBS-NS and PBS-KS. The 2- or 4-nucleotide deletion was introduced into the full-length HIV-1 plasmid pLAI by digestion with endonucleases *Nar*I or *Kas*I, respectively, followed by digestion of the single-stranded 3′ ends with nuclease S1 and subsequent ligation. This sequence modification was verified by sequence analysis.

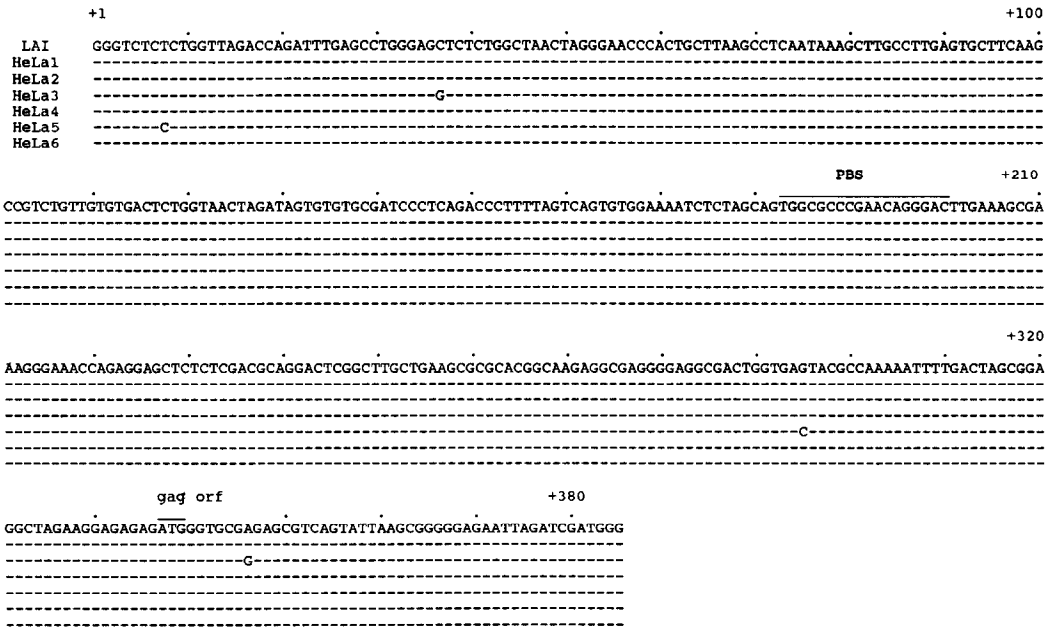

Fig. 2. Nucleotide sequence of HIV-1 proviral DNA obtained from HeLa-CD4$^+$ cells. Subconfluent monolayers of HeLa-CD4$^+$ cells (approximately 0.5×10^6 cells in 2 ml medium per 24-well dish) were infected with a cell-free HIV-1 LAI virus that was obtained from DNA-transfected SupT1 T cells. Viral replication was monitored by the appearance of syncytia and 100 μl of culture supernatant was passed twice a week onto a fresh monolayer of HeLa-CD4$^+$ cells. Proviral sequences were selectively amplified from total cellular DNA by PCR using a 5′ primer (NRE-B) in the U3 region of the LTR and a 3′ primer (Gag-3) in the *gag* gene. The DNA fragment was subcloned into pUC9 plasmid using unique restriction sites present in the primers and analyzed on an Applied Biosystems automatic sequencer. HeLa1 was isolated after 8 passages in HeLa-CD4$^+$ cells, and HeLa2-6 were obtained after 16 weeks (32 passages). The nucleotide sequence of the input LAI isolate is shown for comparison. The position of the transcriptional start site (+1), PBS^{Lys,3} and *gag* start codon is indicated. For the HeLa2-6 clones, we sequenced a total of 3250 nucleotides and found 15 base substitutions (not shown). This would suggest that on average ± 1 mutation is introduced per genome per passage, which is consistent with an error frequency of the RT enzyme of 10^{-4} (10).

viral genome. We therefore analyzed sequences flanking the PBS^{Lys,3} region. The complete HIV-1 leader region from the transcriptional start site at position +1 up to position +385 within the *gag* reading frame was analyzed. No major sequence alterations were observed in the HIV-1 leader region of clones HeLa1-6 compared to the input virus LAI (Fig. 2). In addition, we found that the HeLa-adapted virus was able to initiate an infection in T cells (not shown).

To formally prove the importance of the PBS^{Lys,3} sequence for viral replication in HeLa cells, we constructed two mutant HIV-1 genomes with a 2- or 4-nucleotide deletion in the PBS (Fig. 1C, mutants PBS-NS and PBS-KS, respectively). The reduced basepairing potential of a mutant PBS with the 3′ end of tRNA^{Lys,3} is expected to interfere with initiation of reverse transcription [7,9]. Wild-type and mutant proviral DNAs were individually transfected into HeLa-CD4 cells and viral replication was monitored by visual inspection for syncytium formation and quantitated by Gag p24 antigen levels released into the medium (Fig. 3). Replication of the wild-type virus proceeds at a rapid rate such that after 13 days of culture massive syncytia were observed and high levels of Gag p24 were measured in the supernatant. Although both mutants transiently produced virus, we did not measure subsequent viral replication. No infectious virus was produced in the PBS-KS transfection up to 38 days of culture. In contrast, a rapid increase in Gag p24 levels was detected in the PBS-NS culture at day 16. The supernatant of day 20 was used to infect fresh HeLa-CD4$^+$ cells and the sequence of the PBS of the resulting proviruses was analyzed. It appeared that a reversion to the wild-type PBS sequence had taken place (four clones were analyzed). These results show that PBS-mutated viruses are unable to replicate in HeLa cells. The transient production of normal levels of mutant virus shortly after transfection indicates that the PBS-mutation has no effect on virus production, e.g. protein synthesis or virion assembly. Similar results were obtained in SupT1 T cell transfections (data not shown).

The molecular mechanism of reverse transcription predicts that a mildly mutated PBS can be repaired in one step during tRNA-mediated reverse transcription as long as the primer is able to anneal to the mutated viral genome [7]. This model can explain both the reversion seen for the PBS-NS mutant and the apparent failure to obtain revertants for the more severely mutated PBS-KS variant. Most importantly, the model predicts that the PBS sequence of progeny viruses corresponds to the sequence of the 3′ end of the tRNA primer used. As described above, the viruses resulting from the PBS-NS transfection had regenerated the wild-type PBS se-

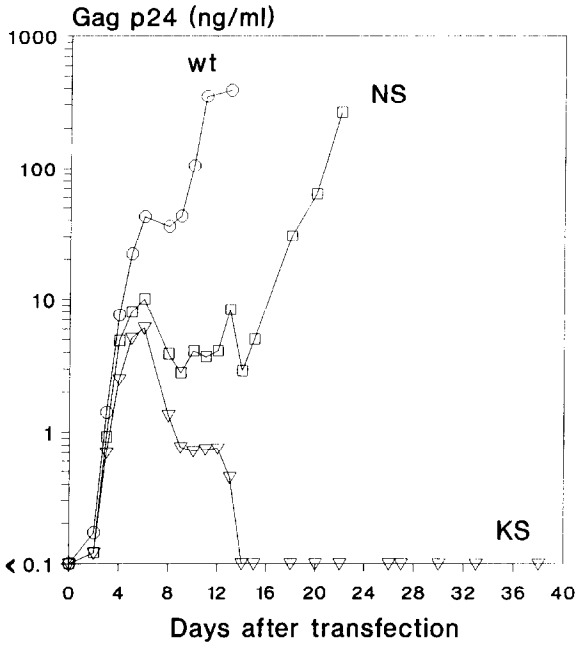

Fig. 3. Virus production upon transfection of wild-type (WT) and PBS-mutated HIV-1 constructs PBS-NS and PBS-NK. HeLa-CD4$^+$ cells were transfected with 10 μg DNA by the DEAE-dextran method. Virus-associated Gag p24 protein was measured in the culture supernatant by elisa (note the logarithmic scale used to plot Gag p24 values). At day 20 the virus-containing supernatant of the PBS-NS culture was used to infect fresh HeLa-CD4$^+$ cells. Resulting proviral sequences were amplified by PCR using the 5′ U3 region primer 5′ CE and the 30 gag primer SK39. A *Hind*III-ClaI fragment (position 77 to 376) was cloned into pSP73 (Promega) and sequenced as described in the legend of Fig. 2.

quence, which identifies tRNA^{Lys,3} as primer for HIV-1 reverse transcription in HeLa cells.

The hypothesis that the HIV-1 virus can use multiple tRNA primers in a cell-type dependent manner may have seem unlikely for several reasons. First, given the critical role for tRNA^{Lys,3} in mRNA translation, it is hard to imagine that this tRNA is not available in HeLa cells. Second, the PBS^{Lys,3} sequence is well-conserved among 20 HIV-1 and 18 HIV-2 isolates (Fig. 1A), although it should be noted that none of these viruses were isolated from HeLa cells. Third, there is little base-pair potential for other tRNAs on the HIV-1 PBS (Fig. 1B). In addition, recent data suggests that a primer tRNA can interact with retroviral RNA through additional base-pairing interactions outside the 18-bp PBS pairing, thereby increasing the selectivity of primer usage [11–13]. These arguments, combined with our experimental evidence, do strongly suggest that tRNA^{Lys,3} is the exclusive primer in HIV-1 replication in both T cells and non-T cells. For the murine leukemia viruses, however, there is some evidence that alternative tRNAs cen serve as primer in retroviral replication. Colicelli and Goff [3] described a revertant of the Moloney murine leukemia virus containing a 500 base-pair insert derived from the mouse genome. The PBS was no longer complementary to the usual tRNA^{Pro}, but had become a perfect 18-nucleotide match for tRNA^{Glu}. Furthermore, retroviral viral vectors based on the AKV murine leukemia virus were recently shown to successfully transduce their genes in the presence of a PBS specifying tRNA primers other than the normal tRNA^{Pro} molecule [14]. Perhaps there is a more selective primer usage in HIV-1 compared to the murine retroviruses. It is possible that this specificity reflects the selective binding of primer tRNA by the HIV-1 reverse transcriptase enzyme [15] when compared to its murine homologue [16].

The biochemical data reported by Jiang et al. [1] convincingly showed a dramatic different pattern of virus-associated tRNA in T cells versus HeLa cells. Most notably, no tRNA^{Lys,3} could be detected in the latter. Our results suggest that at least one tRNA^{Lys,3} molecule is present in HeLa-produced virions because it is used as primer in reverse transcription. These combined data suggest that HIV replication is not exclusively sensitive to the number of primer molecules incorporated into the virion. If we assume that the HIV-1 reverse transcriptase is responsible for the specific incorporation of the primer tRNA [15] and that HIV-1 reverse transcriptase is present in about 2–70 copies per virion as has been reported for other retroviruses [2], it follows that HIV-1 virions contain an excess of 20–70 primer molecules. This scenario reconciles the biochemical and genetic data in that a severe reduction in tRNA content does not necessarily interfere with viral replication. In other words, it is possible that HeLa cell-mediated packaging of HIV-1 virions results in the incorporation of less tRNA^{Lys,3}, without having an effect on the infectivity of the virions. Alternatively, it is possible that the reduced levels of virus-associated tRNA^{Lys,3} in HeLa cells is responsible in part for the reduced growth kinetics of HIV-1 in HeLa cells when compared to T cells. Finally, it remains of interest to identify the other non-primer tRNA species that are selectively packaged into retroviral particles [17–19], especially the abundant tRNA species in HeLa-derived HIV particles that was found in tight association with the RNA genome (1).

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