Structural Elements of the tRNA T Ψ C Loop Critical for Nucleocytoplasmic Transport Are Important for Human Immunodeficiency Virus Type 1 Primer Selection

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Human immunodeficiency virus type 1 (HIV-1) selects a host cell tRNA as the primer for the initiation of reverse transcription. In a previous study, transport of the intact tRNA from the nucleus to the cytoplasm during tRNA biogenesis was shown to be a requirement for the selection of the tRNA primer by HIV-1. To further examine the importance of tRNA structure for transport and the selection of the primer, yeast tRNA^{Phe} mutants were designed such that the native tRNA structure would be disrupted to various extents. The capacity of the mutant tRNA^{Phe} to complement a defective HIV-1 provirus that relies on the expression of yeast tRNA^{Phe} for infectivity was determined. We found a direct relationship between intact tRNA conformation and the capacity to be selected by HIV-1 for use in reverse transcription. tRNA^{Phe} mutants that retained the capacity for nucleocytoplasmic transport, indicative of overall intact conformation, complemented the defective provirus. The mutant tRNAs were not aminoacylated, and the levels of complementation were lower than that for wild-type tRNA^{Phe}, which did undergo transport and aminoacylation. Taken together, these results demonstrate that HIV-1 primer selection is most dependent on a tRNA structure necessary for nucleocytoplasmic transport, consistent with primer selection occurring in the cytoplasm at or near the site of protein synthesis.

Although the multistep process of reverse transcription has been well defined, the mechanism of tRNA selection to the primer-binding site (PBS) remains poorly understood. Previous studies have shown that lysyl-tRNA synthetase interacts with Pr55^{gag} and, in cooperation with Pr160^{gag-pol}, facilitates the selective incorporation of tRNA^{Lys} into human immunodeficiency virus type 1 (HIV-1) virions (4, 6, 8, 15). However, packaging of tRNA and the selection of primers to be used in reverse transcription may be separate events, since the tRNA primer enrichment in retroviral virions does not necessarily influence the selection and use of the actual primer used for initiation of reverse transcription (10, 29). Thus, the selection of the tRNA that is used as the primer might have different constraints than that for the incorporation of the tRNA into virions.

The flexibility of the primer selection process is highlighted by the fact that, in previous studies, we have found that the expression of yeast tRNA^{Phe} in mammalian cells can complement the replication of an HIV-1 provirus in which the PBS was made complementary to the 3'-terminal nucleotides of yeast tRNA^{Phe} (10, 11, 25–27). In our first series of experiments, we relied on the cotransfection of in vitro-synthesized yeast tRNA^{Phe} for infectivity (25–27). We have used this system to characterize the sequence elements of the tRNA important for selection and use in HIV-1 reverse transcription. Mutations within the T Ψ C stem-loop were found to impact on the subsequent infectivity of the virus with a PBS complementary to tRNA^{Phe} (27). A limitation of this system, though, is that in

vitro-transcribed tRNA transfected into the cytoplasm bypasses the normal cellular transport processes for tRNA and, as a result, is not used during protein synthesis. Understanding of the primer selection process should account for the normal intracellular tRNA biogenesis. Following expression and maturation in the nucleus, tRNAs are exported to the cytoplasm by exportin-t (1, 12, 14). The recognition and subsequent export of tRNAs by exportin-t are based solely on tRNA structural requirements, allowing only mature tRNAs to exit the nucleus (1). In the cytoplasm, the tRNA is aminoacylated by its cognate aminoacyl-tRNA synthetase. The interaction between the tRNA and the aminoacyl-tRNA synthetase is primarily mediated by tRNA structure; complete association and aminoacylation are facilitated by identity elements present on the tRNA. Following aminoacylation, the charged tRNA is available to the ribosome for amino acid delivery during translation.

In the current study, we have made use of a complementation system in which yeast tRNA^{Phe} is expressed endogenously. Our previous study suggested a coordination between the tRNA primer selection process and tRNA transport to the cytoplasm; tRNAs that are fully functional for use in protein synthesis are also those that are most efficiently selected by HIV-1 for use in reverse transcription (11). We have further investigated the role of tRNA conformation as it relates to intracellular transport, inclusion in the channeled tRNA cycle during translation as judged by aminoacylation, and primer selection by HIV-1 using yeast tRNA^{Phe} mutants with various degrees of structural disruptions. The results of our studies highlight the relationship between structural integrity of the tRNA and primer selection.

The 293T and HeLa H1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) plus 10% fetal calf serum

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and 1% antibiotic-antimycotic (Gibco/BRL, Gaithersburg, Maryland).

Yeast tRNA^{Phe} mutant genes were constructed via PCR extension as previously described (11). Yeast tRNA^{Phe61} wild type was created by PCR with the U6^{Phe5'} oligonucleotide (5'-AAACCTCGAGGTCCGCGATTTAGCTCAGTTGGGAGAGCGCCAGACTGAAGATCTGGAGG-3') and the U6^{Phe3'} oligonucleotide (5'-CTCCCAAGCTTCCAAAAAATGCGATTCTGTGGATCGAACACAGGACCTCCAGATCTTCA GTCT-3'). The following oligonucleotides were used for PCR extension of the yeast tRNA^{Phe61} mutant: U6^{Phe61G} 3' oligonucleotide (5'-CTCCCAAGCTTCCAAAAAATGCGATTCTGTGGATCGAACACAGGACCTCCAGATCTTCA GTCT-3') and the U6^{Phe5'} oligonucleotide. For the construction of the yeast tRNA^{Phe6153} mutant, the following oligonucleotides were used for PCR: U6^{Phe61G-G53C} 3' oligonucleotide (5'-CTCCCAAGCTTCCAAAAAATGCGATTCTGTGGATCGAACACAGGACCTCCAGATCTTCA GTCT-3') and the U6^{Phe5'} oligonucleotide. For the construction of the yeast tRNA^{Phe56} mutant, the following oligonucleotides were used for PCR: U6^{PheC56A} 3' oligonucleotide (5'-CTCCCAAGCTTCCAAAAAATGCGATTCTGTGGATCTAACACAGGACCTCCAGATCTTCA GTCT-3') and the U6^{Phe5'} oligonucleotide. For the construction of the yeast tRNA^{Phe545556} mutant the following oligonucleotides were used for PCR: U6^{PheU54A-U55A-C56G} 3' oligonucleotide (5'-CTCCCAAGCTTCCAAAAAATGCGATTCTGTGGATCTTCA CAGGACCTCCAGATCTTCA GTCT-3') and the U6^{Phe5'} oligonucleotide. Following PCR extension, PCR products were ligated into the pGEM-T Easy vector (Promega, Madison, Wisconsin). The resultant plasmids (pTA^{PheWt}, pTA^{Phe61}, pTA^{Phe6153}, pTA^{Phe56}, and pTA^{Phe545556}) were digested with HindIII and XhoI, generating fragments of approximately 100 bp containing the transcriptional units for the yeast tRNA^{Phe} mutants. These fragments were then cloned into pLS9 that had been digested with HindIII and XhoI, resulting in five plasmids identified as pU6^{PheWt}, pU6^{Phe61}, pU6^{Phe6153}, pU6^{Phe56}, and pU6^{Phe545556} (13). The correct sequence of the yeast tRNA^{Phe} mutant transcriptional units contained in the pLS9-derived vectors was verified by DNA sequencing.

The procedure used for complementation of the defective HIV provirus psHIV-Phe has been previously described (25–27). The Mammalian Transfection kit (Stratagene, San Diego, California) was used for cotransfection of plasmid DNA (psHIV-Phe, pLGRNL, and either pU6^{PheWt} or pU6^{Phe61} or pU6^{Phe6153} or pU6^{Phe56} or pU6^{Phe545556}). Virus production was determined by p24 antigen levels, using a commercially available enzyme-linked immunosorbent assay kit (Beckman Coulter, Miami, FL), and serial dilutions of the virus-containing supernatant were used to transduce HeLa H1 cells. At 12 h after infection, the cells were washed with phosphate-buffered saline and placed under selection with DMEM-XM medium (DMEM containing 10% fetal calf serum, 1% antibiotics, 20 mM HEPES [pH 7.5], 250 µg/ml xanthine, and 50 µg/ml mycophenolic acid) for 10 to 14 days for the formation of drug-resistant colonies. Cell colonies were fixed with 5% trichloroacetic acid and stained with 2% Coomassie blue, and the number of drug-resistant colonies was counted.

The Mammalian Transfection kit (Stratagene, San Diego,

California) was used to transfect HeLa H1 cells with either pU6^{PheWt} or pU6^{Phe61} or pU6^{Phe6153} or pU6^{Phe56} or pU6^{Phe545556}. For extraction of cytoplasmic RNA, 10⁷ transfected cells were washed twice with phosphate-buffered saline 48 h after transfection. The cells were then lysed on the plate on ice with 0.4 ml of lysis buffer (0.03% Triton X-100, 0.15 M NaCl, 0.01 M Tris, pH 7.8, 0.0015 M MgCl₂) for exactly 1 min. The solution was then collected and centrifuged at 1,000 × g for 3 min at 4°C. The supernatant was removed from the tube and immediately added to 0.2 ml of urea-sodium dodecyl sulfate buffer (7 M urea, 0.35 M NaCl, 0.01 M Tris, pH 7.4, 0.01 M EDTA, 1.0% sodium dodecyl sulfate), and mixed. RNA was then extracted twice with phenol-chloroform. Tri reagent (Sigma, St. Louis, Missouri) was used for the collection of nuclear RNA from transfected HeLa H1 cells following collection of cytoplasmic RNA. Prior to Northern blot analysis, residual plasmid DNA was removed by digestion with DNA Free (Ambion, Austin, Texas) according to the manufacturer's instructions. For Northern blot analysis, oligonucleotide probes were designed to be complementary to yeast tRNA^{Phe}, mammalian tRNA^{Lys}, and U6 snRNA: tRNA^{Lys} probe oligonucleotide (5'-CGCCCGAACAGGGACTTGAACCCTGGA CCCTCAGATTAAAAGTCTGATGCTCTACCGACTGAG CTATCC-3'), tRNA^{Phe} probe oligonucleotide (5'-TGCGAA TTCTGTGGATCGAACACAGGACCTCCAGATCTTCA GTCTGGCGCTCTCCCAACTGAGCTAAATCC-3'), and U6 probe oligonucleotide (5'-CGCTTACGAATTTGCGTGTC ATCCTTGCGCAGGGGCCATGCTAATCTTCTCTGTAT CGT-3'). The probes were 5' end labeled with [γ-³²P]ATP using Ready-To-Go T4 polynucleotide kinase (Amersham Pharmacia Biotech, Piscataway, New Jersey). Free nucleotides were removed by centrifugation through ProbeQuant G-50 Micro columns (Amersham Biosciences, Piscataway, New Jersey); equal amounts (15 pmol) of probes were used for each blot (10⁶ cpm/pmol). The NorthernMax-Gly kit (Ambion, Austin, Texas) was used for Northern blotting, and BrightStar-Plus (Ambion, Austin, Texas) was used as the positively charged nylon membrane. Blots were exposed on a phosphor screen and analyzed with a PhosphorImager. Isolation and blotting of aminoacylated tRNAs collected from HeLa H1 cells 48 h post-transfection with pU6^{PheWt}, pU6^{Phe61}, pU6^{Phe6153}, pU6^{Phe56}, or pU6^{Phe545556} were carried out as previously described (7).

We have previously described a complementation system using a defective HIV-1 proviral genome (psHIV-Phe) in which the PBS has been mutated to be complementary to the 18 3'-terminal nucleotides of yeast tRNA^{Phe} and the *env* gene has been replaced with an expression cassette containing a gene coding for xanthine-guanosine phosphoribosyltransferase (*gpt*) driven by the simian virus 40 early promoter (10). The virus is pseudotyped with the vesicular stomatitis virus G protein expressed from the pLGRNL plasmid. Since psHIV-Phe relies fully on the presence of yeast tRNA^{Phe} for infectivity, yet the yeast tRNA^{Phe} is not required by the cell, we have employed this system as a tool for investigating the selection of mutant tRNAs by HIV-1 for use in reverse transcription. The level of infectious virus is determined by the capacity to convert transduced HeLa H1 cells to mycophenolic acid resistance. Infectivity is then calculated by correlating the numbers

FIG. 1. Yeast tRNA^{Phe} mutants with disruption of TΨC stem secondary structure. Panel A. Schematic representation of the wild-type yeast tRNA^{Phe}. Nucleotides complementary to the primer-binding site of psHIV-Phe are highlighted in boldface. Panel B. The yeast tRNA^{Phe}61 mutant contains a nucleotide substitution at position 61 (C61G), disrupting the first base pair of the TΨC stem secondary structure. Panel C. The yeast tRNA^{Phe}6153 double mutant includes two nucleotide substitutions at positions 61 and 53 (C61G and G53C), compensating for the structural disruption of the tRNA^{Phe}61 mutant. Panel D. The tRNA^{Phe}56 mutant contains a nucleotide substitution at position 56 (C56A), ablating interloop base pairing between nucleotide 19 of the D loop and nucleotide 56 of the TΨC loop. Panel E. The tRNA^{Phe}545556 triple mutant includes nucleotide substitutions at positions 54, 55, and 56 (U54A, U55A, and C56G, respectively). These mutations disrupt interloop base pairing between nucleotides 19 and 18 of the D loop and nucleotides 56 and 55 of the TΨC loop, respectively. The reverse Hoogsteen base pairing between nucleotides 54 and 58 in the TΨC loop is also ablated. Nucleotide substitutions are denoted by closed triangles, and disruption of interloop and reverse Hoogsteen base pairs is indicated by dashed lines.

of infectious viruses with p24 antigen level for each single-round culture.

In a previous study, we analyzed the capacity of in vitro-transcribed yeast tRNA^{Phe} with mutations in the TΨC stem-loop to complement the HIV-1 provirus with a PBS complementary to yeast tRNA^{Phe} (27). The results of these studies established that a single nucleotide change (C61G) significantly compromised the capacity of the tRNA^{Phe} to complement, while a mutation in the loop (U59G) did not substan-

tially affect the capacity for complementation. The mutant tRNA^{Phe} with a single nucleotide substitution (tRNA^{Phe}61) yields a mismatch of bases adjacent to the TΨC loop, shortening the tRNA TΨC stem in the secondary structure (Fig. 1). In the tRNA tertiary structure, the ablated base pairing between nucleotides 61 and 53 is structurally important for the formation of the region at the outer corner of the tRNA L form where the D loop and TΨC loop interact, known as the D/T region (Fig. 1) (2, 20, 21). A second tRNA mutant,

tRNA^{Phe}6153, was designed in the current study to compensate for the disruption of the TΨC stem in the tRNA^{Phe}61 mutant through a substitution at position 53 (C61G and G53C). Although the second substitution allows for base pairing within the TΨC stem secondary structure, there is a minor alteration of the tertiary structure due to a disrupted hydrogen bond (2).

To determine the relationship between the structure and function of endogenously expressed tRNAs with regard to selection by HIV-1, we attempted complementation of psHIV-Phe with the yeast tRNA^{Phe} mutants expressed from a U6 polymerase III promoter. The first mutant, tRNA^{Phe}61, did not have the capacity to be selected by HIV-1 for use in reverse transcription as judged by failure to produce infectious virus (Fig. 2A). We did, however, detect levels of infectivity approaching those observed for complementation with wild-type yeast tRNA^{Phe} when tRNA^{Phe}6153 was endogenously expressed.

We next wanted to determine the nature of the defect for the yeast tRNA^{Phe} mutant with C61G. To first measure the quantities of the yeast tRNA^{Phe} mutant expression relative to wild-type yeast tRNA^{Phe} within mammalian cells, we transfected HeLa H1 cells with pU6^{Phe}Wt, pU6^{Phe}61, or pU6^{Phe}6153. Total RNA was collected 48 h posttransfection and subjected to Northern blot analysis with an oligonucleotide probe designed to be complementary to yeast tRNA^{Phe}. In preliminary studies, we determined that the oligonucleotide probe recognized both mutant tRNAs and wild-type yeast tRNA^{Phe}. We had also previously shown that the 17-nucleotide difference between yeast tRNA^{Phe} and mammalian tRNA^{Phe} was sufficient to allow highly specific binding of the yeast tRNA^{Phe} probe to yeast tRNA^{Phe} alone (10). Northern blot analysis of the two mutant tRNAs and wild-type yeast tRNA^{Phe} revealed that the mutants were expressed at lower levels than wild-type yeast tRNA^{Phe} (approximately 20% of wild-type level; data not shown). We had previously expressed mutant tRNA^{Phe} in the same manner and cell type and attained an equal level of expression (11). It is probable that the level of transcription from the mutant transgenes is equal to that of wild type, but the tRNAs are not stabilized and maintained in the cell at levels comparable to those for wild-type yeast tRNA^{Phe}.

The mutants were designed with various degrees of structural disruption in the TΨC stem that might affect binding by exportin-t and subsequent transport to the cytoplasm (1). To determine if any of the mutants were retained in the nucleus, we collected cytoplasmic RNA and performed Northern blot analysis using the probe complementary to yeast tRNA^{Phe}. The mutant tRNA^{Phe}61 did not appear in the cytoplasmic pool. The ratios of nuclear to cytoplasmic tRNA for wild type and tRNA^{Phe}6153 were similar, indicating that tRNA^{Phe}6153 was transport competent (data not shown). Analysis with an oligonucleotide probe specific for tRNA₃^{Lys} confirmed that equal amounts of RNA were used for Northern blotting of the three samples, and an oligonucleotide probe complementary to the U6 snRNA was used to ensure that the cytoplasmic fraction did not contain any nuclear RNA (Fig. 2B). Comparison of the expression levels of tRNA^{Phe}61 and tRNA^{Phe}6153 with the wild type reveals that both mutants were expressed at 10% of

the level of the wild type; the expression of tRNA^{Phe}6153 was 20% of that of the wild type in the cytoplasmic fraction.

To assess the aminoacylation status of tRNA^{Phe}6153, we collected RNA under acidic conditions from HeLa H1 cells transfected with pU6^{Phe}Wt or pU6^{Phe}6153. RNA was collected 48 h posttransfection and separated on polyacrylamide gels buffered at pH 5. Following Northern blot analysis with probes specific for yeast tRNA^{Phe}, we were unable to detect any aminoacylated tRNA^{Phe}6153 relative to wild-type yeast tRNA^{Phe} (Fig. 2C). Repeated attempts gave a pattern in which we could not distinguish between aminoacylated and nonaminoacylated tRNA. Most probably, the efficiency of aminoacylation was affected by this mutation; consistent with our previous results, aminoacylation per se is not absolutely required for selection (11). Collectively, the results from our analysis of these tRNA^{Phe} mutants demonstrate that primer selection was mainly dependent upon nuclear export.

In a previous study, we reported that in vitro-generated tRNA^{Phe} with a complete deletion in the D-loop region complemented the HIV-1 provirus infectivity (11). However, the expression of a tRNA^{Phe} D-/minus loop from endogenous promoters did not result in complementation, since the tRNA was sequestered in the nucleus. Thus, we were unable to assess the effect of minor alterations in the tRNA tertiary structure on primer selection. To further address this issue, we designed two yeast tRNA^{Phe} mutants, with various degrees of tertiary structure disruption through ablation of tertiary interactions. The first mutant, tRNA^{Phe}56, was designed to contain a single nucleotide substitution at position 56 that disrupts the inter-loop base pairing between nucleotide 56 in the TΨC loop and nucleotide 19 in the D loop (C56A) (Fig. 1). A final mutant, tRNA^{Phe}545556, was constructed that consists of three substitutions at positions 54, 55, and 56 of the tRNA (U54A, U55A, and C56G, respectively). In addition to disruption of the interaction between nucleotides 56 and 19, the tRNA^{Phe}545556 mutant has a less tertiary structure relative to tRNA^{Phe}56, due to disrupted interloop base pairing of nucleotides 55 and 18 and because the reverse Hoogsteen base pair, 54-58, is also disrupted. Studies indicate that the reverse Hoogsteen base pair may be more important than interloop base pairs with regard to intracellular tRNA function (28) (Fig. 1).

We first analyzed the capacity of the tRNAs to complement the defective HIV-1 proviral genome with a PBS complementary to tRNA^{Phe}. Although we did detect complementation with tRNA^{Phe}56, the levels were less (approximately 75%) than that observed for wild-type tRNA^{Phe} (Fig. 3A). Further disruption of the tRNA tertiary structure, as found in tRNA^{Phe}545556, resulted in a tRNA that did not complement infectivity of psHIV-Phe over background.

To determine the nature of the defect of tRNA^{Phe}545556, we first examined the expression of tRNAs following transfection. Similar to the other mutant tRNA^{Phe}, we found that the expression levels of both tRNA^{Phe}56 and tRNA^{Phe}545556 were reduced compared to that of the wild-type tRNA^{Phe} (Fig. 3B). The levels of tRNA^{Phe}56 compared to tRNA^{Phe}545556, though, were comparable, indicating that the lack of complementation by tRNA^{Phe}545556 was not due to lower levels of expression. We next analyzed the intracellular distribution of the mutant tRNAs. Here, we found a clear difference between tRNA^{Phe}56 and tRNA^{Phe}545556. In the case of tRNA^{Phe}56,

FIG. 2. Analysis of yeast tRNA^{Phe}61 and tRNA^{Phe}6153 mutants. Panel A. Complementation of psHIV-Phe by yeast tRNA^{Phe} mutants. Numbers of CFU from infection of psHIV-Phe pseudoviruses complemented by either yeast tRNA^{Phe}Wt (diamonds), tRNA^{Phe}61 (boxes), or tRNA^{Phe}6153 (triangles) expressed from designated quantities of cotransfected pU6^{Phe}Wt, pU6^{Phe}61, or pU6^{Phe}6153, respectively. The represented data are from three independent transfections with standard deviations. Panel B. Intracellular distribution to yeast tRNA^{Phe} and mutants. Northern blots of 2 μ g of cytoplasmic RNA (top) or total RNA (bottom) collected from HeLa H1 cells after transfection with pU6^{Phe}Wt, pU6^{Phe}61, or pU6^{Phe}6153. Lanes 1: RNA from HeLa cells transfected with pU6^{Phe}Wt. Lanes 2: RNA from HeLa cells transfected with pU6^{Phe}61. Lanes 3: RNA from HeLa cells transfected with pU6^{Phe}6153. The Northern blot was probed for yeast tRNA^{Phe} and mammalian tRNA^{Lys} as depicted; the U6 snRNA probe served as a control for nuclear and cytoplasmic fractionation. Panel C. Aminoacylation of yeast tRNA^{Phe} mutants expressed in mammalian cells. North-

we were able to demonstrate cytoplasmic expression, indicating that this tRNA mutant had retained the capacity to interact with exportin-t necessary for transport from the nucleus to the cytoplasm. Similar to the tRNA^{Phe}6153 mutant, the nucleus-to-cytoplasmic ratio of tRNA^{Phe}56 was similar to wild-type tRNA^{Phe} (data not shown). In contrast, the tRNA mutant with the greatest disruption of tertiary function, tRNA^{Phe}545556, was retained in the nucleus, indicating that it was unable to interact with exportin-t for transport. Comparison of the expression levels of tRNA^{Phe}56 and tRNA^{Phe}545556 with the wild type revealed that both mutants were present at about 10% of the level of wild-type tRNA^{Phe}. The tRNA^{Phe}56 mutant was present at 20% of the wild-type level in the cytoplasmic fractions. Finally, we found that tRNA^{Phe}56 was not efficiently aminoacylated following export to the cytoplasm, suggesting that the conformational requirements for tRNA undergoing transport and efficient aminoacylation are different (Fig. 3C). The fact that this tRNA was still selected by HIV-1 as a primer for replication demonstrates that aminoacylation, per se, is not an absolute requirement for selection, which is consistent with our previous study in which we found that mutations within the anticodon loop that precluded aminoacylation, due to ablated identity elements, did not eliminate the capacity of this tRNA to be selected by HIV as a primer for replication (11).

Although the step at which the tRNA primer is selected during the retroviral life cycle remains unresolved, studies from our laboratory and others suggest that the tRNA primer is selected to the PBS prior to encapsidation of the viral genome into progeny virions (9–11). In fact, a recent study proposed that the selection of tRNA to the PBS might act as a checkpoint triggering conformational changes in the 5' untranslated region and subsequent shift from translation of the viral genome to packaging (3). Both the tRNA primer selection and the incorporation of primers into virions must be regulated to assure negligible interference with protein synthesis needed for the production of viral proteins. The results of our current study extend our previous report in which we demonstrated that a tRNA^{Phe} D-loop-minus mutant, which had the capacity to complement HIV-1 replication if provided in the cytoplasm, was unable to complement infectivity since it was retained in the nucleus (11). The tertiary structure of the D-loop-minus mutant is drastically altered as a result of the deletion of approximately one-fourth of the tRNA. The exportin-t/tRNA interaction is needed for the efficient transport of tRNA from the nucleus to the cytoplasm (1). Since exportin-t is known to interact with conformationally intact tRNAs, it is not surprising the D-loop-minus tRNA would not undergo transport. The specificity of the exportin-t/tRNA interaction is highlighted by the fact that two additional mutants, tRNA^{Phe}61

ern blot analysis of yeast tRNA^{Phe} and tRNA^{Lys} from cells transfected with pU6^{Phe}Wt (yeast tRNA^{Phe}) or pU6^{Phe}6153 (yeast tRNA^{Phe}6153). All lanes contain 2 μ g RNA isolated under acidic conditions; the RNA loaded in lane 2 was adjusted to pH 9.0 and incubated at 37°C for 1 h to serve as the deacylated control. The migration of aminoacylated tRNA and deacylated control tRNA is denoted as AA and DA, respectively. The blots were probed for tRNA^{Lys} or tRNA^{Phe}. The majority of wild-type tRNA^{Phe} and tRNA^{Lys} is present in the aminoacylated form; however, aminoacylated tRNA^{Phe}6153 was not evident.

FIG. 3. Analysis of yeast tRNA^{Phe}56 and tRNA^{Phe}545556. Panel A. Complementation of psHIV-Phe by intracellularly expressed yeast tRNA^{Phe} mutants containing ablated tertiary interactions. Numbers of CFU from infection of psHIV-Phe pseudoviruses complemented by either yeast tRNA^{Phe}Wt (diamonds), tRNA^{Phe}56 (circles), or tRNA^{Phe}545556 (multiplication signs) expressed from designated quantities of cotransfected pU6^{Phe}Wt, pU6^{Phe}56, or pU6^{Phe}545556, respectively. The represented data are from three independent transfections with standard deviations. Panel B. Intracellular distribution of tRNA^{Phe} mutants. Northern blots of 2 μ g of cytoplasmic RNA (top) or total RNA (bottom) collected from HeLa H1 cells after transfection with pU6^{Phe}Wt, pU6^{Phe}56, or pU6^{Phe}545556. Lanes 1: RNA from HeLa cells transfected with pU6^{Phe}Wt. Lanes 2: RNA from HeLa cells transfected with pU6^{Phe}56. Lanes 3: RNA from HeLa cells transfected with pU6^{Phe}545556. The Northern blot was probed for yeast tRNA^{Phe} and mammalian tRNA^{Lys} as depicted; the U6 snRNA probe served as a control for nuclear/cytoplasmic fraction. Panel C. Aminoacylation of

and tRNA^{Phe}545556, known to have conformational defects, were not transported and thus did not complement psHIV-Phe, establishing a link between nuclear export and selection. One would predict that only those tRNAs that retain sufficient conformation to interact with exportin-t would be targets for capture by HIV-1 as a primer. Why is this important? In the process of biogenesis and transport, the tRNA interacts with a myriad of proteins as it is channeled into translation (1). Thus, HIV-1 is in competition with host cell proteins for the selection of the tRNA primer. Since the nucleus probably contains a large number of aberrant tRNAs, HIV-1 might have evolved to capture these aberrant tRNAs, ostensibly to avoid competition with host cell protein synthesis apparatus as a means to acquire tRNA primers. In fact, analysis of tRNA^{Phe}61 and tRNA^{Phe}545556 using in vitro-synthesized tRNA transfected directly into the cytoplasm, which bypasses the need for nuclear transport, revealed that both mutants could complement psHIV-Phe, although the levels were lower than the wild-type tRNA^{Phe} (approximately 15%) (27). It is possible that the inability of psHIV-Phe to effectively use these mutant tRNAs might be a result of the block in reverse transcription downstream from the selection step; possibly in a later step of reverse transcription, the reverse transcriptase copies the tRNA primer to generate a plus-strand copy of the PBS. The use of aberrant tRNAs could impact negatively on this step, which would result in an overall reduction of successful reverse transcription. Additional experiments will be needed to address this issue. It is now clear, though, that under normal cellular conditions the exportin-t checkpoint for nucleus to cytoplasmic transport, which is dependent upon tRNA conformation, also functions as a checkpoint to ensure selection of tRNAs competent for reverse transcription.

Once the tRNA is transported from the nucleus to the cytoplasm, the tRNA is rapidly incorporated into the translation. At any one time, the tRNA is associated with numerous proteins involved in the channeled tRNA cycle during protein synthesis (22, 24). For the most part, the tRNA is found in a ternary complex with EF1A-GTP prior to interaction with the ribosome. The tRNA in the complex is aminoacylated with the cognate amino acid. Following participation in protein synthesis, the tRNA is released from the E site of the ribosome and reacquired by either EF1A-GDP or the appropriate synthetase for reaminoacylation (17). In our system, at any one time, the majority, if not all, of wild-type tRNA^{Phe} was found to be aminoacylated, similar to what was seen for tRNA^{Lys} (11), indicating participation in host cell translation. The results from our current study are consistent with our previous work in which we found that the most efficient complementation occurred with the wild-type yeast tRNA^{Phe} that was present in a

yeast tRNA^{Phe} mutants, with ablated tertiary interactions, expressed in mammalian cells. Northern blot analysis of yeast tRNA^{Phe} and tRNA^{Lys} from cells transfected with pU6^{Phe}Wt or pU6^{Phe}56. All lanes contain 2 μ g RNA isolated under acidic conditions; the RNA loaded in lane 2 was adjusted to pH 9.0 and incubated at 37°C for 1 h to serve as the deacylated control. The migration of aminoacylated tRNA and deacylated control tRNA is denoted as AA and DA, respectively. The blots were probed for tRNA^{Lys} or tRNA^{Phe}. The majority of wild-type tRNA^{Phe} and tRNA^{Lys} is present in the aminoacylated form; however, no aminoacylated tRNA^{Phe}56 was detected.

fully aminoacylated form (11). A mutation in the identity elements that precluded aminoacylation (but not transport) resulted in a tRNA that complemented the infectivity of psHIV-Phe at levels lower than that for the wild-type, fully aminoacylated yeast tRNA^{Phe}. Similarly, both the tRNA^{Phe56} and tRNA^{Phe6153} mutants complemented psHIV-Phe and were not aminoacylated following transport to the cytoplasm. While it is clear that HIV-1 can select primers that do not maintain the capacity to be charged with an amino acid, interaction with the aminoacyl-tRNA synthetase might be important for efficient selection to the PBS, a point that is highlighted upon examination of the structural differences between the tRNA^{Phe56} mutant and the tRNA^{Phe6153} mutant. Since phenylalanyl-tRNA synthetase displays a higher degree of selectivity at the level of binding than at the level of catalysis and is sensitive to variances in tRNA conformation, the tRNA^{Phe56} and tRNA^{Phe6153} mutants would be expected to interact with the synthetase with different affinities (18, 23). Thus, the distinct levels of complementation by tRNA^{Phe56} and tRNA^{Phe545556} may be a result of their affinities for the synthetase enzyme. It is possible that tRNA^{Phe6153} and tRNA^{Phe56} might be defective for aminoacylation *in vivo* due to a lowered capacity to compete with the wild-type (mammalian) tRNA^{Phe} for interaction with EF1A or the synthetase. The inability to effectively interact with these proteins could result in a loss of intracellular stability, manifested as the lower levels of tRNAs that we detected in our analysis. If one takes into consideration the relative levels of the tRNA^{Phe56} and tRNA^{Phe6153} mutants compared to wild-type tRNA^{Phe} (mutants expressed at levels approximately 20% of wild type), the amounts of complementation were similar.

We speculate that the tRNA^{Phe} mutants, by virtue of their competency for nucleocytoplasmic transport, are found within the same intracellular pools as the wild-type tRNA^{Phe}. This would be consistent with the concept of a nebula of proteins and substrates (including tRNA) required for protein synthesis that is around polyribosomes during translation (16). If this is the case, selection of the tRNA might occur from this nebula in conjunction with translation of the viral genome. A relationship between translation and encapsidation of genomic HIV-1 RNA has recently been described (19). Furthermore, consistent with this proposal, studies have shown that lysyl-tRNA synthetase (4) or EF1A (5) is found in HIV-1 virions. Thus, the lines between protein synthesis specific for HIV-1, such as -1 frameshifting, usage of internal ribosomal entry sites, and even codon preference on selection of primer tRNA by HIV-1, will need to be further explored to resolve the relationships with primer selection.

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REFERENCES

- Arts, G.-J., S. Kuersten, P. Romby, B. Ehresmann, and I. W. Mattaj. 1998. The role of exportin-t in selective nuclear export of mature tRNAs. *EMBO J.* **17**:7430–7441.
- Behlen, L. S., J. R. Sampson, A. B. DiRenzo, and O. C. Uhlenbeck. 1990. Lead-catalyzed cleavage of yeast tRNA^{Phe} mutants. *Biochemistry* **29**:2515–2523.
- Berkhout, B., M. Ooms, N. Beerens, H. Huthoff, E. Southern, and K. Verhoeve. 2002. *In vitro* evidence that the untranslated leader of the HIV-1 genome is an RNA checkpoint that regulates multiple functions through conformational changes. *J. Biol. Chem.* **277**:19967–19975.
- Cen, S., A. Khorchid, H. Javanbakht, J. Gabor, T. Stello, K. Shiba, K. Musier-Forsyth, and L. Kleiman. 2001. Incorporation of lysyl-tRNA synthetase into human immunodeficiency virus type 1. *J. Virol.* **75**:5043–5048.
- Cimarelli, A., and J. Luban. 1999. Translation elongation factor 1- α interacts specifically with the human immunodeficiency virus type 1 *gag* polyprotein. *J. Virol.* **73**:5388–5401.
- Halwani, R., S. Cen, H. Javanbakht, J. Saadatmand, S. Kim, K. Shiba, and L. Kleiman. 2004. Cellular distribution of lysyl-tRNA synthetase and its interaction with *gag* during human immunodeficiency virus type 1 assembly. *J. Virol.* **78**:7553–7564.
- Ho, Y.-S., and Y. W. Kan. 1987. *In vivo* aminoacylation of human and *Xenopus* suppressor tRNAs constructed by site-specific mutagenesis. *Proc. Natl. Acad. Sci. USA* **84**:2185–2188.
- Javanbakht, H., R. Halwani, S. Cen, J. Saadatmand, K. Musier-Forsyth, H. Gottlinger, and L. Kleiman. 2003. The interaction between HIV-1 *gag* and human lysyl-tRNA synthetase during viral assembly. *J. Biol. Chem.* **278**:27644–27651.
- Kanevsky, I., N. Vasilenko, H. Dumay-Odelot, and P. Fosse. 2003. *In vitro* characterization of a base pairing interaction between the primer binding site and the minimal packaging signal of avian leukosis virus genomic RNA. *Nucleic Acids Res.* **31**:7070–7082.
- Kelly, N. J., and C. D. Morrow. 2003. Yeast tRNA^{Phe} expressed in human cells can be selected by HIV-1 for use as a reverse transcription primer. *Virology* **313**:354–363.
- Kelly, N. J., M. T. Palmer, and C. D. Morrow. 2003. Selection of retroviral reverse transcription primer is coordinated with tRNA biogenesis. *J. Virol.* **77**:8695–8701.
- Kutay, U., G. Lipowsky, E. Izaurralde, F. R. Bischoff, P. Schwarzmaier, E. Hartmann, and D. Gorlich. 1998. Identification of a tRNA-specific nuclear export receptor. *Mol. Cell* **1**:359–369.
- Lobo, S. M., and N. Hernandez. 1989. A 7 bp mutation converts a human RNA polymerase II snRNA promoter into an RNA polymerase III promoter. *Cell* **58**:55–67.
- Lund, E., S. Guttinger, A. Calado, J. E. Dahlberg, and U. Kutay. 2004. Nuclear export of microRNA precursors. *Science* **303**:95–98.
- Mak, J., M. Jiang, M. A. Wainberg, M.-L. Hammariskjold, D. Rekosh, and L. Kleiman. 1994. Role of Pr160^{gag-pol} in mediating the selective incorporation of tRNA^{Lys} into human immunodeficiency virus type 1 particles. *J. Virol.* **68**:2065–2072.
- Mirande, M. 1991. Aminoacyl-tRNA synthetase family from prokaryotes and eukaryotes: structural domains and their implications. *Prog. Nucleic Acid Res.* **40**:95–142.
- Negrutskii, B. S., and M. P. Deutscher. 1991. Channeling of aminoacyl-tRNA for protein synthesis *in vivo*. *Proc. Natl. Acad. Sci. USA* **88**:4991–4995.
- Perret, V., C. Florentz, J. D. Puglisi, and R. Giege. 1992. Effect of conformational features on the aminoacylation of tRNAs and consequences on the permutation of tRNA specificities. *J. Mol. Biol.* **226**:323–333.
- Poon, D. T. K., E. N. Chertova, and D. E. Ott. 2002. Human immunodeficiency virus type 1 preferentially encapsidates genomic RNAs that encode Pr55^{Gag}: functional linkage between translation and RNA packaging. *Virology* **293**:368–378.
- Quigley, G. J., and A. Rich. 1976. Structural domains of transfer RNA molecules. The ribose 2' hydroxyl which distinguishes RNA from DNA plays a key role in stabilizing tRNA structure. *Science* **194**:796–806.
- Romby, P., P. Carbon, E. Westhof, C. Ehresmann, J. P. Ebel, B. Ehresmann, and R. Giege. 1987. Importance of conserved residues for the conformation of the T-loop in tRNAs. *J. Biomol. Struct. Dyn.* **5**:669–687.
- Stapulionis, R., and M. P. Deutscher. 1995. A channeled tRNA cycle during mammalian protein synthesis. *Proc. Natl. Acad. Sci. USA* **92**:7158–7161.
- Vasil'eva, I. A., V. N. Ankilova, O. I. Lavrik, and N. A. Moor. 2002. tRNA discrimination by T. thermophilus phenylalanyl-tRNA synthetase at the binding step. *J. Mol. Recognit.* **15**:188–196.
- Wolin, S. L., and A. G. Matera. 1999. The trials and travels of tRNA. *Genes Dev.* **13**:1–10.
- Yu, Q., and C. D. Morrow. 1999. Complementarity between 3' terminal nucleotides of tRNA and primer binding site is a major determinant for selection of the tRNA primer used for initiation of HIV-1 reverse transcription. *Virology* **254**:160–168.

26. **Yu, Q., and C. D. Morrow.** 2000. Essential regions of the tRNA primer required for HIV-1 infectivity. *Nucleic Acids Res.* **28**:4783–4789.
27. **Yu, Q., and C. D. Morrow.** 2001. Identification of critical elements in the tRNA acceptor stem and TΨC loop necessary for human immunodeficiency virus type 1 infectivity. *J. Virol.* **75**:4902–4906.
28. **Zagryadskaya, E. I., F. R. Doyon, and S. V. Steinberg.** 2003. Importance of the reverse Hoogsteen base pair 54–58 for tRNA function. *Nucleic Acids Res.* **31**:3946–3953.
29. **Zhang, Z., S. M. Kang, A. LeBlanc, S. L. Hajduk, and C. D. Morrow.** 1996. Nucleotide sequences within the U5 region of the viral RNA genome are the major determinants for a human immunodeficiency virus type 1 to maintain a primer binding site complementary to tRNA^{His}. *Virology* **226**:306–317.