Correlation Between tRNA\textsuperscript{Lys3} Aminoacylation and Its Incorporation into HIV-1*

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During human immunodeficiency virus type 1 (HIV-1) assembly, tRNA\textsuperscript{Lys} is selectively packaged into the virion, where tRNA\textsuperscript{Lys} serves as the primer for reverse transcription. Lysyl-tRNA synthetase is also selectively incorporated into HIV-1 and is therefore a strong candidate for being the signal by which viral proteins interact with tRNA\textsuperscript{Lys} isoaceptors. Previously, mutations in the tRNA\textsuperscript{Lys} anticodon have been shown to strongly inhibit the charging of tRNA\textsuperscript{Lys} by lysyl-tRNA synthetase in vitro, and we show here that in vivo aminoacylation is also inhibited by anticodon changes. The order of decreasing in vivo aminoacylation for tRNA\textsuperscript{Lys} anticodon mutants is: wild-type SUU (where S = mcm\textsuperscript{5}S\textsuperscript{U}) 100% → GGU (49%) → CGU (40%) → SGA (0%) and CGA (0%). We found that the ability of these tRNA\textsuperscript{Lys} anticodon variants to be aminoacylated in vivo is directly correlated with their ability to be packaged into HIV-1. These data showed that the anticodon is a major determinant for tRNA\textsuperscript{Lys} packaging and support the conclusion that its productive interaction with lysyl-tRNA synthetase is important for tRNA\textsuperscript{Lys} incorporation into HIV-1.

As described previously, LysRS is selectively packaged into HIV-1 (4). During HIV-1 assembly (see Ref. 5 for review), the major structural precursor protein, Gag, is assembled at the plasma membrane. Also, part of the assembly complex is viral genomic RNA and another precursor protein, Gag-Pol, which contains the sequences for the viral enzymes, protease, reverse transcriptase (RT), and integrase. During or after budding of the virion from the cell, the viral protease converts these precursor proteins to the processed proteins found in the mature virion. However, the incorporation of tRNA\textsuperscript{Lys} occurs independently of both precursor protein processing and genomic RNA packaging (6). Gag alone is sufficient to produce extracellular Gag particles, and LysRS is packaged into these particles (4), but the additional presence of Gag-Pol is required for tRNA\textsuperscript{Lys} incorporation as well (6). Gag-Pol might be stabilizing the Gag/LysRS/tRNA\textsuperscript{Lys} complex since Gag-Pol interacts with both Gag (7, 8) and tRNA\textsuperscript{Lys} (9, 10).

The recognition and binding of aminoacyl-tRNA synthetases with their cognate tRNAs involve interactions with the acceptor stem and, in most cases, the anticodon arms of the tRNAs (11). Based on studies of the human tRNA\textsuperscript{Lys}/human LysRS interaction, the anticodon of tRNA\textsuperscript{Lys} plays an important role in LysRS recognition and binding (12). In contrast, the enzyme is relatively insensitive to mutations in the acceptor stem domain both in vitro (13) and in vitro (12). Another recent report indicated that the N-terminal domain of hamster LysRS, which is adjacent to the anticodon binding domain, although not essential for aminoacylation, improves the docking of the acceptor arm of tRNA\textsuperscript{Lys} into the active site of the enzyme (14).

Previous work has indicated that a certain variability in the anticodon sequence is tolerated for tRNA\textsuperscript{Lys} packaging into virions, i.e. not only do tRNA\textsuperscript{Lys} (anticodon SUU, where S = mcm\textsuperscript{5}S\textsuperscript{U})\textsuperscript{2} and tRNA\textsuperscript{Lys}\textsuperscript{1,2} (anticodon CUU) appear to be packaged with equal efficiency, but a mutant tRNA\textsuperscript{Lys} with a CUU anticodon is packaged, although aminoacylated to only 40% of wild-type levels in vivo (15). However, mutations at U35 have been shown to have a more severe effect on aminoacylation catalytic efficiency in vitro (12, 16), and changes at this position have not yet been tested for their effect on tRNA\textsuperscript{Lys} aminoacylation in vivo nor for packaging into virions. In this report, we constructed different tRNA\textsuperscript{Lys} genes mutated at position U35 as well as at other anticodon positions, and we expressed these genes in COS7 cells also transfected with HIV-1 proviral DNA. We show that the anticodon is indeed a major determinant for tRNA\textsuperscript{Lys} packaging. Moreover, the ability of mutant tRNA\textsuperscript{Lys} molecules to be aminoacylated in vivo correlates directly with their ability to be packaged into HIV-1.

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† The abbreviations used are: HIV-1, human immunodeficiency virus type 1; BH10F, HIV-1 containing an inactive viral protease; LysRS, lysyl-tRNA synthetase; RT, reverse transcriptase; Gag, HIV-1 precursor protein containing sequences coding for HIV-1 structural proteins; Gag-Pol, HIV-1 precursor protein containing sequences coding for retroviral structural proteins and retroviral enzymes.

‡ S = hypermodified nucleotide at tRNA\textsuperscript{Lys} U34 = mcm\textsuperscript{5}S\textsuperscript{U}.
**EXPERIMENTAL PROCEDURES**

**Plasmid Construction—**SVC21.BH10, a simian virus 40-based vector containing wild-type HIV-1 proviral DNA, was a gift of E. Cohen, University of Montreal. SVC12.BH10LYs3/UGU contains the HIV-1 proviral DNA plus a wild-type tRNA^{Lys3} gene with the anticodon DNA sequence CUG. SVC12.BH10LYs3/CCG, SVC12.BH10LYs3/CCG, and SVC12.BH10LYs3/CCG contain the HIV-1 proviral DNA plus a mutant tRNA^{Lys3} gene where the anticodon DNA sequence has been changed from CTT to CGA, CGT, TGT, and TGA, respectively. Mutant tRNA^{Lys3} genes were created by PCR mutagenesis (11). The amplified products were cloned into the Hpa-I site of SVC21.BH10, which is upstream of the HIV-1 proviral DNA sequence. Mutations were confirmed by DNA sequencing.

**Production of Wild-type and Mutant HIV-1 Virus—**Transfection of COS7 cells with the above plasmids by the calcium phosphate method was as described previously (6). Viruses were isolated from COS7 cell culture medium 63 h posttransfection or from the cell culture medium of infected cell lines. The virus-containing medium was first centrifuged in a Beckman GS-6R rotor at 3,000 rpm for 30 min, and the supernatant was then filtered through a 0.2-μm filter. The viruses in the filtrate were then pelleted by centrifugation in a Beckman Ti45 rotor at 35,000 rpm for 1 h. The viral pellet was purified by centrifugation with a Beckman SW41 rotor at 26,500 rpm for 1 h through a 65% sucrose cushion. 

**RNA Isolation and Analysis—**Total cellular or viral RNA was extracted from cell or viral pellets by the guanidinium isothiocyanate procedure (17) and dissolved in 5 mM Tris buffer, pH 7.5. Dot-bLOTS of cellular or viral RNA were hybridized with DNA probes complementary to tRNALys^{3}, tRNALys^{5}, and tRNALys^{18} (1), genomic and cDNA RNA (18), and α-tubulin mRNA (DNA probe from Ambion). Two-dimensional PAGE of [32P]Cp was performed as described above.

Hybridization—Two-dimensional PAGE of [32P]Cp was performed as described above. Deacylated tRNA was produced by treating the RNA sample with 0.1 M Tris-HCl, pH 9.0, at 37 °C for 3 h to hydrolyze the aminoacyl linkage and provide an uncharged electrophoretic marker.

**Western Blotting—**Western blot analysis was performed using 300 μg of cytoplasmic or nuclear proteins as determined by the Bradford assay (21). Cytoplasmic and nuclear extracts were resolved by SDS-PAGE followed by blotting onto nitrocellulose membranes (Gelman Sciences). Detection of the nuclear transcription factor YY1 on the Western blot utilized monoclonal antibodies to YY1 (Santa Cruz Biotechnology). Western blots were analyzed by enhanced chemiluminescence (ECL kit, Amersham Biosciences) using anti-mouse (Amersham Biosciences) as a secondary antibody. The sizes of the detected protein bands were estimated using prestained high molecular weight protein markers (Invitrogen).

**Cell Fractionation—**The cytoplasmic supernatant and nuclear extract were prepared from the COS7 cells as described previously (22). Western blot analysis was performed as above using anti-YY1.

**RESULTS**

**Expression of Wild-type and Mutant tRNA^{Lys3} and Their Incorporation into Virions—**The anticodon is a major determinant for aminoacylation of human tRNA^{Lys3} by human LysRS (12). The ability of tRNA^{Lys3} to be aminoacylated in vivo was shown to be particularly sensitive to changes at anticodon position U35 (12). To determine whether a correlation exists between the ability of tRNA^{Lys3} to be aminoacylated and incorporated into HIV-1, we have transfected COS7 cells with a plasmid containing both HIV-1 proviral DNA and a wild-type or mutant tRNA^{Lys3} gene. As shown previously, this results in more tRNA^{Lys3} being synthesized in the cytoplasm, and in the case of wild-type tRNA^{Lys3} and tRNA^{Lys3} variants examined to date, increased viral packaging has also been observed (23). However, these previously described tRNAs did not contain changes at U35. Because the middle anticodon position is critical for aminoacylation, the different tRNA^{Lys3} variants examined in this work all contained a U35G mutation in addition to other possible anticodon mutations such as S34C or U36A (12).

We have measured the ability of mutant tRNA^{Lys3} to be incorporated into virions using two different hybridization probes, which are shown in Fig. 1. We have measured changes...
in total tRNA<sub>Lys</sub> packaged into virions (Fig. 2) using a hybridization probe complementary to the 3′-terminal 18 nucleotides of tRNA<sub>Lys</sub>, which detects both wild-type and mutant tRNA<sub>Lys</sub>. We have also monitored the incorporation into virions of specific mutant tRNA<sub>Lys</sub> using hybridization probes complementary to the anticodon arm, i.e. probes that are specific for each mutant tRNA<sub>Lys</sub> (see Fig. 4). As shown below, both types of probes yield similar conclusions, and both probes were used (see Fig. 6) to measure aminoacylation of either total tRNA<sub>Lys</sub> or of specific wild-type or mutant tRNA<sub>Lys</sub>.

The data in Fig. 2 show both the expression of total (wild-type and mutant) tRNA<sub>Lys</sub> in the cytoplasm of the transfected COS7 cells and their incorporation into HIV-1. Fig. 2A shows Dot-blot analysis of cellular or viral RNA hybridized with a radioactive DNA oligomer complementary to the 3′-terminal 18 nucleotides of tRNA<sub>Lys</sub>. The top strip represents increasing amounts of synthetic tRNA<sub>Lys</sub>, and the hybridization results are plotted as a standard curve in Fig. 2B. The bottom two strips in Fig. 2A show Dot-blot analysis of RNA isolated from either cell lysates containing equal amounts of β-actin mRNA (cell) or viral lysates containing equal amounts of viral genomic RNA (viral). Dot-blot analysis for determining β-actin mRNA and genomic RNA amounts are not shown. The relative total tRNA<sub>Lys</sub>/β-actin mRNA ratios are plotted in Fig. 2C, normalized to the value obtained in COS7 cells transfected with HIV-1 proviral DNA alone (BH10). Transfection with the wild-type tRNA<sub>Lys</sub> gene or the mutant tRNA<sub>Lys</sub> genes results in an approximately 2-fold increase in the cytoplasmic concentration of total tRNA<sub>Lys</sub>. However, as shown in Fig. 2D, these cytoplasmic increases in tRNA<sub>Lys</sub> did not all result in increases in tRNA<sub>Lys</sub> incorporation into virions. The maximum increase in tRNA<sub>Lys</sub> incorporation into virions occurred with excess wild-type tRNA<sub>SUU</sub> (1.85-fold). tRNA<sub>SUU</sub> and tRNA<sub>CGU</sub> increased packaging 1.4- and 1.3-fold, respectively. tRNA<sub>CGU</sub> showed no increase in tRNA<sub>Lys</sub> incorporation, and tRNA<sub>SAU</sub> actually showed a small decrease in packaging as compared with wild-type tRNA<sub>SAU</sub>.

The changes in tRNA<sub>Lys</sub> incorporation into virions upon transfection with plasmids encoding wild-type tRNA<sub>Lys</sub> or anticodon variants were also visualized by two-dimensional PAGE. Fig. 3A shows the electrophoresis pattern of low molecular weight viral RNA in wild-type virions. Previously, we have identified spot 3 as tRNA<sub>Lys</sub> and spots 1 and 2 as belonging to the tRNA<sub>Lys</sub> isoacceptor family (1). Transfection of COS7 cells with a plasmid containing both the wild-type tRNA<sub>Lys</sub> gene and HIV-1 proviral DNA results in virions containing an increase in tRNA<sub>Lys</sub> and a decrease in tRNA<sub>SAU</sub> (Fig. 3B). Similar observations were reported previously (23). When COS7 cells are transfected with HIV-1 DNA and either tRNA<sub>SUU</sub> or tRNA<sub>CGU</sub>, which have an intermediate ability to be packaged into virions (Fig. 2D), the two-dimensional PAGE patterns show an increased ratio of tRNA<sub>Lys</sub>/tRNA<sub>SAU</sub> in the virions (Fig. 3B). On the other hand, tRNA<sub>SAU</sub> and tRNA<sub>CGU</sub>, which the data indicate are not packaged into virions (Fig. 2D), do not show a significant change in the tRNA<sub>Lys</sub>/tRNA<sub>SAU</sub> ratio relative to the wild-type control (Fig. 3, panels E and F). The tRNA<sub>Lys</sub>/ARN<sub>Lys</sub> ratios, determined by phosphorimaging, are listed beneath each panel. Taken together, the data presented in Figs. 2 and 3 show that the ability of tRNA<sub>Lys</sub> anticodon variants to be packaged into HIV is directly correlated with the tRNA<sub>Lys</sub>/tRNA<sub>SAU</sub> ratio.

The experiments in Figs. 2 and 3 measure total tRNA<sub>Lys</sub> (wild-type and mutant) in the cytoplasm and in the virion. We have also used hybridization probes specific for each tRNA<sub>Lys</sub> anticodon mutant to examine their specific expression in the cytoplasm and incorporation into virions. The Dot-blot analysis shown...
FIG. 3. Two-dimensional PAGE patterns of viral low molecular weight RNA. RNA was extracted from virions produced from COS7 cells transfected with a plasmid containing HIV-1 proviral DNA and a wild-type or mutant tRNA\textsuperscript{Lys3} gene. The RNA was 3’-end-labeled \textit{in vitro} with \textsuperscript{[32P]}Cp and analyzed by two-dimensional PAGE. A, cells transfected with HIV-1 DNA alone, \textit{i.e.} containing only endogenous tRNA\textsuperscript{Lys3} (Endogenous). B–F, cells transfected with HIV-1 DNA and tRNA\textsuperscript{Lys3} genes containing the anticodon sequences listed above each panel. The numbers listed under each panel correspond to the tRNA\textsuperscript{Lys3}\!/tRNA\textsuperscript{Lys1,2} ratios and were determined by phosphorimaging.

FIG. 4. Expression of specific wild-type and mutant tRNA\textsuperscript{Lys3} in cells and viruses. COS7 cells were transfected with a plasmid containing HIV-1 proviral DNA and a wild-type or mutant tRNA\textsuperscript{Lys3} gene. For each strip in panel A, the left portion contains Dot-blots of increasing amounts of an \textit{in vitro} wild-type or mutant tRNA\textsuperscript{Lys3} transcript used to determine differences in efficiencies of hybridization for different anticodon probes. The right portion contains Dot-blots of cellular or viral RNA containing equal amounts of either \(\beta\)-actin mRNA (cellular RNA) or genomic RNA (viral RNA), which were hybridized with a DNA probe complementary to the anticodon arm of each wild-type and mutant tRNA\textsuperscript{Lys3}, as shown in Fig. 1. The control in the bottom four strips is the wild-type tRNA\textsuperscript{Lys3} \textit{in vitro} transcript, and it shows that the anticodon probes do not detect wild-type tRNA\textsuperscript{Lys3}. The letters to the left of the strips represent the anticodon sequence of the tRNA\textsuperscript{Lys3} detected. In the SUU strip, cells were transfected with HIV-1 DNA alone (BH10, also labeled as \textit{none} in panels B and C) or with HIV-1 DNA plus a wild-type tRNA\textsuperscript{Lys3} gene and then probed with a DNA probe complementary to anticodon arm of wild-type tRNA\textsuperscript{Lys3}. The normalized results are plotted in panel B (cellular) and in panel C (viral).
in Fig. 4A measure the amount of a specific tRNA<sup>Lys</sup> variant isolated from cell or viral lysates containing equal amounts of β-actin mRNA or genomic RNA, respectively. The top strip in panel A (SUU) shows the amounts of tRNA<sup>Lys</sup> in cytoplasm and viruses from cells either transfected with HIV-1 alone (BH10) or transfected with HIV-1 and a wild-type tRNA<sup>Lys</sup> gene (BH10Lys3). The remaining four strips in panel A show the amount of tRNA<sup>Lys</sup> in cytoplasm and viruses from cells transfected with HIV-1 and different mutant tRNA<sup>Lys</sup> genes whose anticodon sequence is listed to the left of each of the remaining lanes. B, graphic representation of the total cytoplasmic tRNA<sup>Lys</sup>/β-actin mRNA obtained from the data in panel A and normalized to the value obtained in HIV-1-transfected cells not transfected with a tRNA<sup>Lys</sup>-encoding gene. C, Western blot of nuclear and cytoplasmic fractions of transfected cells, labeled as in panel A. Blots were probed with antibody to YY1, a nuclear transcription factor. N, nuclear fraction; C, cytoplasmic fraction.

The relative tRNA<sup>Lys</sup>/β-actin mRNA ratios are plotted in Fig. 4B, normalized to the value found for cells transfected with HIV-1 alone (BH10). The results are very similar to those shown in Fig. 2 using a DNA hybridization probe that measures total tRNA<sup>Lys</sup>. Wild-type tRNA<sup>Lys</sup> expression is increased significantly when cells are transfected with a wild-type tRNA<sup>Lys</sup> gene. The expression of each mutant tRNA<sup>Lys</sup> in the cytoplasm is similar and results in an approximately 2-fold increase in total tRNA<sup>Lys</sup> (endogenous wild type and mutant) when probed with an oligonucleotide complementary to the 3′-end of tRNA<sup>Lys</sup> (Fig. 2C). The tRNA<sup>Lys</sup>/genomic RNA ratios in virions are shown in Fig. 4C, normalized to the value found for cells transfected with HIV-1 proviral DNA alone (BH10). The results with wild-type tRNA<sup>Lys</sup> also match the results shown in Fig. 2D. The incorporation of this tRNA into virions increased the tRNA<sup>Lys</sup>/genomic RNA ratio to 1.87, indicating a relative incorporation of exogenous tRNA<sup>Lys</sup> as
with HIV-1 DNA and tRNALys3 genes coding for the following tRNA anticodon sequence: 2, SUU; 4, SGA; 5, SGU; 6, CGU; 7, CGA. In panels B–E, the middle lanes (lanes 9, 12, 15, 18) represent the RNA from cells transfected with HIV-1 DNA alone (BH10, also referred to as none in panel F). Lanes 2 and 4–7 represents cells transfected with HIV-1 DNA and tRNA^Lys3 genes coding for the following tRNA anticodon sequence: 2, SUU; 4, SGA; 5, SGU; 6, CGU; 7, CGA. The last lane in each of these panels (lanes 10, 13, 16, and 19) represent RNA extracted from cells transfected only with HIV-1 proviral DNA. The percentage of aminoacylation, as determined from lanes 2 and 3 in panel A and the middle lanes in panels B–E, is shown in panel F.

**Fig. 7.** Electrophoretic detection of acylated and deacylated tRNA^Lys3.**

Compared with endogenous tRNA^Lys3 of 0.87. In contrast, the relative incorporation of tRNA^Lys3^SGU and tRNA^Lys3^SGA was only 0.50 and 0.37, respectively, whereas tRNA^Lys3^CGA and tRNA^Lys3^CGU showed relative incorporations of 0.013 and 0.029 (Fig. 4C). The data in Fig. 4 indicate that while wild-type or mutant tRNA^Lys3^ is expressed at approximately equal levels in the total cell lysate, they are incorporated into virions to variable extents. One explanation could be that some mutant forms of tRNA^Lys3^ are not exported out of the nucleus with equal efficiency. To test this possibility, we lysed cells and separated nuclei from cytoplasm by low speed centrifugation. A radioactive probe complementary to the 3'-terminal 18 nucleotides present in all forms of tRNA^Lys3^ studied here was hybridized to Northern blots containing both increasing amounts of an in vitro tRNA^Lys3^ transcript (Fig. 5, left side of panel A) and cytoplasmic RNA samples that contain equal amounts of β-actin mRNA (Fig. 5, right side of panel A). The standard curve generated on the left side of panel A shows that the cytoplasmic tRNA^Lys3^/β-actin mRNA obtained from these Northern blot hybridizations is shown graphically in Fig. 5B. These experiments indicate that both wild-type and tRNA^Lys3^ variants are expressed at approximately equal amounts in the cytoplasm, consistent with the data shown in Figs. 2 and 4. To ensure that effective separation of nuclear and cytoplasmic fractions was achieved in our experiments, we demonstrated that the transcription factor YY1, which concentrates in the nucleus, is only detected in the nuclear fraction (Fig. 5C).

**Aminoacylation of Wild-type and Mutant tRNA^Lys3 in Vivo.**—We next determined the aminoacylation state of the wild-type and mutant forms of tRNA^Lys3^ examined here. The electrophoretic mobility of acylated tRNA in acid-urea PAGE is reduced relative to the deacylated form, and this property can be used to determine the extent of tRNA aminoacylation (23). Fig. 6 shows Northern blots of cellular and viral RNA samples electrophoresed in acid-urea gels, blotted onto Hybond N filter paper, and hybridized with radioactive DNA probes specific for tRNA^Lys3^Aminoac. In panel A, cellular tRNA was hybridized with the 18-nucleotide DNA oligomer complementary to the 3'-18 nucleotides present in all forms of tRNA^Lys3^ studied here. The first lane in each panel (lanes 1, 8, 11, 14, and 17) represents cellular RNA that was first exposed to alkaline pH to deacylate the tRNA (see “Experimental Procedures”). Panel A, lane 3 represents cells transfected with HIV-1 DNA alone (BH10, also referred to as none in panel F). Lanes 2 and 4–7 represents cells transfected with HIV-1 DNA and tRNA^Lys3^ genes coding for the following tRNA anticodon sequence: 2, SUU; 4, SGA; 5, SGU; 6, CGU; 7, CGA. The last lane in each of these panels (lanes 10, 13, 16, and 19) represent RNA extracted from cells transfected only with HIV-1 proviral DNA. The percentage of aminoacylation, as determined from lanes 2 and 3 in panel A and the middle lanes in panels B–E, is shown in panel F.

**Fig. 7.** Graph showing the correlation between tRNA^Lys3^ aminoacylation and incorporation into HIV-1. Letters in parentheses indicate the anticodon sequence of the tRNA^Lys3^ variants tested. These data are based on the quantitative results shown in Figs. 4 (panel C) and 6 (panel F).
otides of tRNA^Lys, whereas in panels B–E, the cellular tRNA was hybridized with the anticodon probes specific for different tRNA^Lys^ wild^ mutants. Lane 1 in panel A shows the mobility of wild-type tRNA^Lys^ deacylated in vitro at alkaline pH. As reported previously (23), in cells either transfected with the wild-type tRNA^Lys^ gene (lane 2) or not transfected with any tRNA^Lys^ gene (lane 3), the tRNA^Lys^ detected is entirely in the deacylated form. As also shown in panel A, a majority of the total tRNA^Lys^ is aminoacylated in cells transfected with genes encoding tRNA^Lys^ (lane 5) and tRNA^Lys^ (lane 6). In contrast, a larger proportion of total tRNA^Lys^ is in the deacylated form in cells transfected with genes encoding tRNA^LysCA^ (lane 4) and tRNA^Lys^ (lane 7).

Although the data in Fig. 6A (lanes 4–7) suggest that the tRNA^Lys^ anticodon mutants are defective in vivo aminoacylation, the total tRNA^Lys^ probed in these experiments consists of both endogenous wild-type tRNA^Lys^ and exogenous wild-type or mutant tRNA^Lys^. Thus to more directly probe the capability of the tRNA^Lys^ mutants to be aminoacylated, we used anticodon DNA probes specific to the different tRNAs (Fig. 6, panels B–E). Lanes 8, 11, 14, and 17 represent mutant tRNA^Lys^ samples that have been deacylated in vitro at alkaline pH, whereas lanes 9, 12, 15, and 18 contain cellular RNA from cells transfected with HIV-1 proviral DNA, as well as the various tRNA genes. To demonstrate the hybridization specificity of the anticodon probes, we also probed cellular RNA from cells transfected only with HIV-1 proviral DNA (lanes 10, 13, 16, and 19). Based on the data presented in these panels, we conclude that tRNA^Lys CA^ (lane 9) and tRNA^Lys^ (lane 12) are aminoacylated to a significantly greater extent in vivo than tRNA^Lys^ (lane 15) and tRNA^Lys^ (lane 18). The latter two variants are present exclusively in the uncharged state. The percentage of wild-type or mutant tRNA^Lys^ present in the aminocytlated state is shown graphically in panel F.

**DISCUSSION**

Fig. 7 shows a plot of the relative tRNA^Lys^ incorporation into HIV-1 for each of the anticodon variants tested in this work versus the percentage of aminoacylated tRNA detected in vivo. The linear correlation is striking and shows that the ability of tRNA^Lys^ to be incorporated into HIV-1 is closely correlated with its ability to be aminoacylated. The efficiency of aminoacylation by aminoacyl-tRNA synthetases may be described by an overall specificity constant (K_m^eff)^, as well as a binding parameter (K_m). Previous studies indicated that the U35G anticodon mutation in human tRNA^Lys^ abolished in vitro aminoacylation (>3000-fold decrease in k_m^eff) and that this change affected both LysRS binding and catalysis (12). Despite this dramatic effect on the ability to be aminoacylated with lysine in vitro, we report here that mutants with changes only in U35 (tRNA^Lys^CGA^) or in both S34 and U35 (tRNA^Lys^GUA^) are still aminoacylated in vivo to significant levels (Fig. 6). These mutations change the anticodon to the sequence found in tRNA^The^.

The anticodon is a major recognition element for *Escherichia coli* threonyl-tRNA synthetase in vitro (24). Moreover, a change in the anticodon can switch the identity of *E. coli* elongator methionine tRNA^Met^ (tRNA^MetCA^) from methionine to threonine, showing that it is also a major identity determinant in vivo (25). Whether human threonyl-tRNA synthetase maintains this strong anticodon recognition, it may be that the XGU anticodon variants tested here are at least partially charged with threonine in vitro.

Based on in vitro studies, a single U36A change in human tRNA^Lys^ affects primarily the k_m^eff parameter with only a 3-fold increase in K_m, and an overall 182-fold decrease in catalytic efficiency. Coupling the U36A mutation with a U35G change abolished in vivo aminoacylation and packaging into HIV-1 (Fig. 7). Both the UGA and the CGA anticodons correspond to sequences normally found in tRNA^Ser^ isoacceptors. It has been shown that human serine-tRNA synthetase does not use the anticodon as a recognition element but instead requires a long variable extra arm (26, 27). Thus, it is unlikely that human tRNA^Lys^ or tRNA^Lys^ would be aminoacylated by serine in vitro, consistent with our observations that in vitro aminoacylation is abolished in these variants.

In this work, the data indicate that the anticodon of human tRNA^Lys^ is a major determinant for packaging into HIV-1 (Fig. 7) with the caveat that we do not yet know whether altering the anticodon sequence affects modifications elsewhere in the tRNA molecule. However, a clear correlation between aminoacylation and packaging has been established (Fig. 7). Based on these data, we cannot determine whether aminoacylation itself is a prerequisite for packaging or whether the major factor is LysRS binding. To address this question, the identity of the amino acid attached to the mutant tRNAs that are incorporated to significant extents (i.e. tRNA^Lys^CGA^ and tRNA^Lys^GUA^) must be determined, and these experiments are planned.

The data presented in this work support a model in which the tRNA^Lys^/LysRS interaction is important for tRNA^Lys^ incorporation into viruses. We also show that the anticodon is a major determinant for packaging. However, the anticodon sequence has also been shown to contribute to the in vitro binding of mature reverse transcriptase in studies using either native tRNA^Lys^ (28) or unmodified tRNA^Lys^ transcripts (29, 30). Other data suggest that RT sequences in the precursor Gag-Pol polyprotein interact with tRNA^Lys^ during its incorporation into virions (6, 9). Thus, an anticodon mutation might also weaken this tRNA^Lys^/Gag-Pol interaction. However, there is no clear evidence to date that the interaction between RT sequences in Gag-Pol and the anticodon of tRNA^Lys^ is involved in its packaging into HIV-1, and in fact, mutations in RT that prevent the enzyme from interacting with the tRNA^Lys^ anticodon in vitro (31) were reported to have no effect on viral packaging of tRNA^Lys^ (9).

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