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Incorporation of Excess Wild-Type and Mutant tRNA\textsubscript{Lys} into Human Immunodeficiency Virus Type 1

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Human immunodeficiency virus (HIV) particles produced in COS-7 cells transfected with HIV type 1 (HIV-1) proviral DNA contain 8 molecules of tRNA\textsubscript{Lys} per 2 molecules of genomic RNA and 12 molecules of tRNA\textsubscript{Lys}\textsuperscript{52U} per 2 molecules of genomic RNA. When COS-7 cells are transfected with a plasmid containing both HIV-1 proviral DNA and a human tRNA\textsubscript{Lys} gene, there is an increase in the amount of cytoplasmic tRNA\textsubscript{Lys} per microgram of total cellular RNA, and the tRNA\textsubscript{Lys} content in the virus increases from 8 to 17 molecules per 2 molecules of genomic RNA. However, the total number of tRNA\textsubscript{Lys} molecules per 2 molecules of genomic RNA remains constant at 20; i.e., the viral tRNA\textsubscript{Lys} content decreases from 12 to 3 molecules per 2 molecules of genomic RNA. All detectable tRNA\textsubscript{Lys} is aminocylated in the cytoplasm of inoculated cells and deaminated in the virus. When COS-7 cells are transfected with a plasmid containing both HIV-1 proviral DNA and a mutant amber suppressor tRNA\textsubscript{Lys} gene (in which the anticodon is changed from TTT to CTA), there is also a large increase in the ratio of cytoplasmic tRNA\textsubscript{Lys}. However, the number of molecules of tRNA\textsubscript{Lys} in the virus increases from 8 to 15 molecules per 2 molecules of genomic RNA, with a decrease in viral tRNA\textsubscript{Lys} from 12 to 5 molecules per 2 molecules of genomic RNA. Thus, the total number of molecules of tRNA\textsubscript{Lys} in the virion remains at 20. The alteration of the anticodon has little effect on the viral packaging of this mutant tRNA in spite of the fact that it no longer contains the modified base mcm\textsuperscript{5}S\textsuperscript{2}U at position 34, and its ability to be aminocylated is significantly impaired compared with that of wild-type tRNA\textsubscript{Lys}. Viral particles which have incorporated either excess wild-type tRNA\textsubscript{Lys} or mutant suppressor tRNA\textsubscript{Lys} show no differences in viral infectivity compared with wild-type HIV-1.

During retroviral assembly, a limited number of host cell tRNAs are selected for incorporation into the virus (33). One of these, termed the primer tRNA, is placed onto the primer-binding site of the viral genome, where it serves as a primer for the reverse transcriptase (RT)-catalyzed synthesis of minus-strand cDNA. The tRNAs most commonly used as primer tRNAs are tRNA\textsuperscript{Lys} in avian retroviruses (7, 10, 22, 27, 34) and tRNA\textsuperscript{Pr} in mammalian retroviruses. In human immunodeficiency virus type 1 (HIV-1), the primer tRNA appears to be tRNA\textsuperscript{Lys} (24), one of three major tRNA\textsubscript{Lys} isoacceptors found in mammalian cells (23).

During the assembly of HIV, the major tRNA\textsubscript{Lys} isoacceptors, tRNA\textsubscript{Lys}\textsuperscript{A15} and tRNA\textsubscript{Lys}\textsuperscript{A23}, are selectively incorporated into the virus (14). This select packaging occurs independently of genomic RNA encapsidation (17, 19, 22), and evidence implicating Pr160\textsuperscript{ gag-pol} as being the likely viral precursor protein which carries the tRNA\textsubscript{Lys} into the virus has been reported (19). In vitro studies of the interaction between mature p66/p51 RT and tRNA\textsubscript{Lys} indicate that the anticodon arm of the tRNA is involved in this interaction (1, 2, 26, 36). However, little is known about the signals on tRNA\textsubscript{Lys} which cause it to be chosen for viral incorporation from over 100 other cellular tRNAs and subsequently to be placed onto the primer-binding site of the viral genomic RNA, where it functions as the primer for HIV-1 RT. In the absence of purified functional Pr160\textsuperscript{ gag-pol}, the determination of the packaging signals on tRNA\textsubscript{Lys} must be by an in vivo approach.

Transfection of COS-7 cells with HIV-1 proviral DNA results in the production of infectious HIV-1 particles showing select incorporation of tRNA\textsubscript{Lys} (14). In this work, we have investigated whether a wild-type or mutant form of the tRNA\textsubscript{Lys} gene can be introduced into these cells along with HIV-1 proviral DNA and produce wild-type or mutant tRNA\textsubscript{Lys} which will be packaged into the virus. We find that exogenous tRNA\textsubscript{Lys} will be packaged into the virus, changing the tRNA\textsubscript{Lys}/tRNA\textsubscript{Lys} ratio, but that the total amount of tRNA\textsubscript{Lys} packaged into a virus remains at 20 molecules per 2 molecules of genomic RNA.

Possible signals on tRNA\textsubscript{Lys} which target it for interaction with the Pr160\textsuperscript{ gag-pol} precursor could include a specific base sequence or base modification, the state of aminocylation of the tRNA, or cytoplasmic proteins which complex with the tRNA, such as lysine tRNA\textsubscript{Lys} synthetase. In this work, we initiate studies on the first two parameters. COS-7 cells were transfected with a plasmid containing either the HIV-1 proviral DNA alone or the HIV-1 proviral DNA combined with either a wild-type tRNA\textsubscript{Lys} gene or an amber suppressor tRNA\textsubscript{Lys} gene in which the anticodon has been changed from TTT to CTA (11, 12). We then demonstrated the ability of the excess wild-type or mutant tRNA\textsubscript{Lys} transiently produced from these plasmids to be incorporated into HIV-1. The packaging of the tRNA\textsubscript{Lys} appears to be insensitive to alterations in the anticodon loop which remove both the thiolated U (mcm\textsuperscript{5}S\textsuperscript{2}U) and U at position 34 or 36 in the anticodon and which reduce the ability of this tRNA to be aminocylated.

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Plasmid construction. SVC21.BH10 is a simian virus 40-based vector which contains wild-type HIV-1 proviral DNA (29) and was a gift of E. Cohen, University of Montreal. pMV104-Lys and pMV104-Lys (Su') are plasmids containing, respectively, human tRNA3YS gene and an amber suppressor tRNA3YS gene (anticodon TTT changed to GTA). Both plasmids were a gift from Ye Shih Ho, Wayne State University (11, 12). SVC21.BH10Lys3 and SVC21.BH10Su+ contain the HIV-1 proviral DNA plus either the wild-type tRNA3YS gene or the amber suppressor tRNA3YS gene, respectively. Both plasmids were constructed by removing a BamHI-EcoRI fragment from the pMV104 plasmids and cloning this fragment into the HpaI site of SVC21.BH10, which is upstream of the HIV-1 proviral DNA sequence. Correct insertions were analyzed by using SpeI and Narl digestion and DNA sequencing. Restriction enzymes were used according to the specifications of the manufacturer.

Production of wild-type and mutant HIV-1 virus. Transfection of COS-7 cells with the above-described plasmids by the calcium phosphate method was as previously described (15). Virus was isolated from the cell culture medium at 63 h posttransfection. The supernatant was first centrifuged in a Beckman GS-6R rotor at 3,000 rpm for 30 min, and the virus was then pelleted from the resulting supernatant by centrifugation in a Beckman Ti45 rotor at 35,000 rpm for 1 h. The viral pellet was then purified by centrifugation at 26,500 rpm for 1 h through 15% sucrose onto a 65% sucrose cushion, using a Beckman SW41 rotor.

Isolation of COS-7 cellular and viral RNAs and human placental tRNA3YS. Total cellular and viral RNAs were extracted from viral pellets by the guanidinium isothiocyanate procedure (5). The purification of tRNA3YS from human placenta was performed as previously described (10). The tRNA species designated 1, 2, and 3 on two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) gels have been previously identified as two species of tRNA3YS and one species of tRNA2Lys (14). The term tRNA3YS refers to a population of two tRNA3YS species which differ by 1 bp in the anticodon stem (23). In this paper, 2D-PAGE spots 1 and 2 are collectively referred to as tRNA3YS since we have not characterized these two species sufficiently to distinguish tRNA3YS from tRNA3LS (14).

RNA labeling. The fractionated RNA samples were labeled by using the [32P]pCp 3' end-labeling technique (4). [32P]pCp was made as follows. First, 5 mCi of [γ-32P]ATP (specific activity, 3,000 Ci/mmol; Dupont, Canada) was dried in a microcentrifuge tube with N2. One hundred microliters of reaction solution (50 mM Tris-HCl [pH 9.2], 5 mM MgCl2, 3 mM dithiothreitol, 5% bovine serum albumin [BSA], 1 μM 3'-CMP, and 10 U of T4 kinase) was added. The reaction mixture was incubated at 37°C for 3 h, and the conversion of 3'-CMP to [32P]pCp was monitored by polyethyleneimine thin-layer chromatography in 0.8 M NH4SO4, which separates [32P]pCp from [32P]ATP.

Labeling of the RNA with [32P]pCp was as previously described (4, 16). After labeling, free [32P]pCp was removed from the labeled macromolecules either by using G-50 Sephadex (Pharmacia) homemade spin columns equilibrated with TBE buffer (10 mM Tris pH 7.5, 1 mM EDTA) or during the electrophoresis run. Before analysis by PAGE, the samples were heated at 90°C for 2 min.

1D- and 2D-PAGE. Electrophoresis of viral RNA was carried out at 4°C with the Hoeffer SE620 gel electrophoresis apparatus. The gel size was 14 by 32 cm. The first dimension was run in a 10% polyacrylamide-7 M urea gel for approximately 16 h at 800 V, until the bromphenol blue dye was beginning to elute from the bottom of the gel. After autoradiography, the piece of gel containing RNA was cut out, embedded in a second gel (20% polyacrylamide, 7 M urea), and run for 30 h (25 Watt limiting); this was followed by autoradiography. All electrophoretic runs were carried out in 0.5× TBE (1× TBE is 50 mM Tris, 5 mM boric acid, and 1 mM EDTA-Na). The electrophoretic gel patterns shown in this paper show only low-molecular-weight RNA, since the high-molecular-weight viral genomic RNA cannot enter the polyacrylamide gels. Furthermore, these patterns represent only the most abundant tRNA species present, since the high specific activities of the labeled RNAs used will reveal more minor-abundance species with longer film exposures.

The signal intensity of each radioactive low-molecular-weight RNA species in the 2D-PAGE RNA pattern was determined by Phosphor-imaging (Bio-Rad, Toronto, Canada).

Measurement of wild-type and mutant tRNA3YS by RNA-DNA hybridization. To measure the amount of tRNA3YS (wild type and mutant) present in cellular or viral RNA, we have synthesized an 18-mer DNA oligonucleotide complementary to the 3' 18 nucleotides of tRNA3LYS (5'TGGGCCCGAA CAGGGAC3'). This probe hybridizes specifically with both wild-type and mutant tRNA3LYS (14) and was hybridized to dot blots on Hybond N (Amersham) containing known amounts of purified human placental tRNA3LYS and either cellular tRNA or viral RNA produced in cells transfected with either SVC21.BH10, SVC21.BH10Lys3, or SVC21.BH10Su+. The DNA oligomer was first 5' end labeled by using T4 polynucleotide kinase and [γ-32P]ATP (3,000 Ci/mmol; Dupont Canada), and specific activities of 108 to 109 cpm/μg were generally reached. Approximately 107 cpm of oligomer was generally used per blot in hybridization reactions.

DNA probes complementary to the anticodon arms of both wild-type tRNA3LYS (5' CCCTCAGATTTAAGTCTGAT GC3') and amber suppressor tRNA3LYS (5' CCCTCAGA SGGATGGCTG3') were also synthesized. In order to specifically detect the presence of suppressor tRNA3LYS in RNA samples, blots were probed with 32P-labeled anticodon probe to the suppressor tRNA3LYS in the presence of an 8- to 25-fold excess of nonradioactive oligonucleotide complementary to the wild-type tRNA3LYS anticodon arm.

Measurement of viral genomic RNA by quantitative PCR. (i) Labeling of primer. One nanomole of sense primer was mixed with 50 μCi of [γ-32P]ATP (Dupont) and end labeled with 3 μl of T4 polynucleotide kinase (10 U/μl; Pharmacia) in kinase buffer (70 mM Tris-HCl [pH 7.5], 100 mM MgCl2, and 50 mM dithiothreitol) at 37°C for 1 h. Free radioactive label was removed by passage through a G-25 spin column.
(ii) Reverse transcription. Ten picomoles of cold antisense primer was mixed with 50 μCi of [γ-32P]ATP (Dupont) and end labeled with 3 μl of T4 polynucleotide kinase (10 U/μl; Pharmacia) in kinase buffer (70 mM Tris-HCl [pH 7.5], 100 mM MgCl2, and 50 mM dithiothreitol) at 37°C for 1 h. Free radioactive label was removed by passage through a G-25 spin column.
(iii) PCR. Seventy-five microliters of PCR master mix (10 μl...
of 10× *Tag* DNA polymerase buffer [BRL/GIBCO], 10 μl of 2 mM dNTPs, 1 μl of 32P-labeled primer mix, and 1.25 U of *Tag* DNA polymerase [BRL/GIBCO]) was added to the reaction tubes containing reverse transcription products, which were incubated at 95°C. Reaction tubes were subjected to 19 cycles of amplification (denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and elongation at 72°C for 3 min). Aliquots of PCR products were run on an 8% polyacrylamide gel with 7 M urea to separate amplification products from excess radioactive primers. The gel was dried at 80°C for 2 h and then exposed to a Phosphor-imaging screen for quantitative measurement on a Phosphor-imager.

(iv) Quantitative determination of genomic RNA in wild-type and mutant viral RNA samples. Sense primer (JS1) (5′ATTCCGTTAAGGCCAGGGG3′) and antisense primer (JA1) (5′GGGATGGTTGAGCTGTCG3′) were used for PCR amplification. A 148-bp fragment, corresponding to HIV1 RNA positions 843 to 990, was amplified.

A standard curve was established by using known amounts of in vitro-transcribed RNA for RT-PCR. A 957-base RNA fragment (sense) was made from a linearized DNA plasmid, pEA2, with T7 RNA polymerase (pEA2 plasmid was a kind gift from E. J. Arts of the McGill AIDS Centre). This RNA fragment corresponds to HIV1 DNA sequence positions 473 to 1420.

Quantitation of genomic RNA in virus containing wild-type and mutant tRNA immunodeficiency virus (HIV1) was measured by techniques previously described (11, 12, 31). To measure the extent of in vivo aminoacylation of tRNA, the isolation of cellular or viral RNA was performed under low-pH conditions required for stabilizing the aminoacyl-tRNA bond. The guanidinium thiocyanate procedure for isolating RNA (5) was modified by including 0.2 M sodium acetate (pH 4.0) in solution D, and the
TABLE 1. Viral and cytoplasmic concentrations of tRNA<sub>Lys</sub>

<table>
<thead>
<tr>
<th>RNA source</th>
<th>ng of tRNA&lt;sub&gt;Lys&lt;/sub&gt; per µg of cellular RNA</th>
<th>Molecules of genomic RNA (10&lt;sup&gt;6&lt;/sup&gt;) per µl of sample&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Molecules of tRNA&lt;sub&gt;Lys&lt;/sub&gt; (10&lt;sup&gt;6&lt;/sup&gt;) per µl of sample&lt;sup&gt;b&lt;/sup&gt;</th>
<th>tRNA&lt;sub&gt;Lys&lt;/sub&gt;/tRNA&lt;sub&gt;Lys&lt;/sub&gt; ratio in virus&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Molecules of tRNA&lt;sub&gt;Lys&lt;/sub&gt; per 2 molecules of genomic RNA</th>
<th>Molecules of tRNA&lt;sub&gt;Lys&lt;/sub&gt; per 2 molecules of genomic RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>SVC21.BH10</td>
<td>1.99</td>
<td>6.04</td>
<td>2.36</td>
<td>8</td>
<td>0.68</td>
<td>12</td>
</tr>
<tr>
<td>SVC21.BH10Lys3</td>
<td>5.96</td>
<td>8.06</td>
<td>6.79</td>
<td>17</td>
<td>6.02</td>
<td>3</td>
</tr>
<tr>
<td>SVC21.BH10Lys3a</td>
<td>5.93</td>
<td>5.58</td>
<td>4.18</td>
<td>15</td>
<td>2.94</td>
<td>5</td>
</tr>
</tbody>
</table>

<sup>a</sup> A molecular mass of 324,868 g/mol for the 957-base genomic RNA standard was used in converting nanograms of genomic RNA standard to molecules of genomic RNA.

<sup>b</sup> A molecular mass of 25,460 g/mol for the tRNA<sub>Lys</sub> was used in converting nanograms of tRNA<sub>Lys</sub> standard to molecules of tRNA<sub>Lys</sub>.

<sup>c</sup> Determined by Phosphor-image analysis of the viral 2D-PAGE patterns shown in Fig. 1.

<sup>d</sup> The number of molecules of tRNA<sub>Lys</sub> was determined from the tRNA<sub>Lys</sub>/tRNA<sub>Lys</sub> ratios.

The phenol used was equilibrated in 0.1 M sodium acetate (pH 5.0). The final isopropanol-precipitated RNA pellet was dissolved in 10 mM sodium acetate (pH 5.0) and stored at −70°C until electrophoretic analysis. RNA was mixed with 1 volume of loading buffer (0.1 M sodium acetate [pH 5.0], 8 M urea, 0.05% bromphenol blue, and 0.05% xylene cyanol) and electrophoresed in a 0.5-mm-thick polyacrylamide gel containing 8 M urea in 0.1 M sodium acetate (pH 5.0). The running buffer was 0.1 M sodium acetate (pH 5.0), and electrophoresis was carried out at 300 V and 4°C for 15 to 18 h in a Hoefer SE620 electrophoresis apparatus. RNA was electrophoretically separated onto a Hybond N filter paper (Amersham) with an electrophoretic transfer cell (Bio-Rad) at 750 mA for 15 min, using 1× TBE. Hybridization of the blots with probes for wild-type and mutant tRNA<sub>Lys</sub> was performed as described above. Decayed RNA 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.

**Infectivity assay.** The cell culture supernatant containing the virus produced by transfected COS-7 cells was centrifuged in a Beckman GS centrifuge at 3,000 rpm for 30 min, and the virus was then pelleted by centrifugation in a Beckman Ti45 rotor at 35,000 rpm for 1 h and resuspended in RPMI 1640 medium (GIBCO Laboratories). The different viral suspensions were adjusted to equal amounts of p24 per milliliter by using the commercial kit available for p24 antigen capture (Abbott Laboratories). MT-4 cells (5 × 10<sup>4</sup>) were incubated with 1 ml of virus in RPMI 1640 medium, and after 3 h of incubation at 37°C, the cells were washed twice to remove excess virus and then maintained in medium at a cell density of 3 × 10<sup>5</sup> per ml. Culture fluids were replaced at day 4 after infection. The supernatant was centrifuged in a Beckman GS centrifuge at 3,000 rpm for 30 min and stored at −70°C. The extent of infection was assessed by RT assays (3, 25).

**RESULTS**

Figure 1 shows the 2D-PAGE pattern of cytoplasmic (A to C) and viral (D to F) tRNAs produced in COS-7 cells transfected with a plasmid containing either HIV-1 proviral DNA alone (A and D) or both the HIV-1 gene and either a wild-type (B and E) or a suppressor (C and F) tRNA<sub>Lys</sub> gene. Spots 1 and 2 both represent tRNA<sub>Lys</sub>, while spot 3 is tRNA<sub>Lys</sub> (14). It can be seen in Fig. 1 that the cellular and viral tRNA<sub>Lys</sub>/tRNA<sub>Lys</sub> ratios increase when cells are transfected with either the wild-type or mutant tRNA<sub>Lys</sub> gene. The signal intensities of the viral tRNA<sub>Lys</sub> spots were determined by Phosphor-imaging, and the viral tRNA<sub>Lys</sub>/tRNA<sub>Lys</sub> ratios are listed in Table 1. These ratios increase nine- and fourfold, respectively, in HIV-1 produced from cells transfected with the wild-type or mutant tRNA<sub>Lys</sub> gene.

The amounts of tRNA<sub>Lys</sub> found in the cytoplasm and in the virus were determined by experiments profiled in Fig. 2 and 3. For Fig. 2, panel I, known amounts of cytoplasmic RNA, viral RNA, and purified tRNA<sub>Lys</sub> were blotted onto Hybond N filter

![Figure 2](image_url)

**FIG. 2.** (I) Quantitation of tRNA<sub>Lys</sub> in cellular and viral RNAs. The specificity of a tRNA<sub>Lys</sub> DNA oligomer probe has been described previously (8). Various amount of purified human placenta tRNA<sub>Lys</sub> were used as an external standard (top row). One-microgram samples of cellular RNA and 1-µl aliquots of the same viral RNA samples used to quantitate genomic RNA in Fig. 3 were used to determine the amount of tRNA<sub>Lys</sub> present. The RNA samples are given the same letter designation as in Fig. 1. (II) tRNA<sub>Lys</sub> standard curve. Hybridization signals of purified human tRNA<sub>Lys</sub> were used to construct a standard curve. Concentrations of tRNA<sub>Lys</sub> in the cellular and viral RNA samples were calculated from this curve, and these values, or values derived from them, are listed in Table 1.
paper and hybridized with a tRNA<sub>3YS</sub>-specific DNA probe complementary to the 3′-terminal 18 nucleotides of tRNA<sub>3YS</sub> (14). The letters correspond to those in Fig. 1. The microgram amounts of cellular tRNA blotted (A, B, and C) were determined by measuring the A<sub>260</sub> of the sample solutions. The amounts of viral genomic RNA in the blotted viral samples (D, E, and F) were determined by RT-PCR, as shown in Fig. 3. The DNA probe hybridizes to the 3′ end of tRNA<sub>3YS</sub> and does not distinguish between wild-type and suppressor tRNA<sub>3YS</sub>. From the tRNA<sub>3YS</sub> standard curve, we determined the relative concentrations of tRNA<sub>3YS</sub> in the cytoplasm of transfected cells and in the virus produced from these cells, and these results are listed in Table 1.

In cells not transfected with an exogenous tRNA<sub>3YS</sub> gene, the relative concentration of cellular tRNA<sub>3YS</sub> is 2 ng/μg of total cellular RNA. Assuming that approximately 10% of cellular RNA is tRNA, we calculate that about 2% of the cellular tRNA is tRNA<sub>3YS</sub>. In cells transfected with proviral DNA and either wild-type or mutant tRNA<sub>3YS</sub>, the relative concentration of tRNA<sub>3YS</sub> increases to approximately 6 ng/μg of cellular tRNA. For cells transfected with the mutant tRNA gene, this value includes both wild-type and suppressor tRNAs, since the 3′ DNA probe does not distinguish between the two tRNAs. These values are determined from measurements of RNA extracted from a plate of cells, and they represent the average amount of tRNA<sub>3YS</sub> per microgram of total RNA extracted from both transfected and nontransfected cells. Since the majority of cells are not transfected, the increase in the tRNA<sub>3YS</sub> concentration in the transfected cells will clearly be much higher than the average value indicates.

We have examined the effect of the increases in cellular tRNA<sub>3YS</sub> on the tRNA<sub>3YS</sub> concentration in virus produced from the transfected cells. In Fig. 2 and 3, we show the relative concentrations of tRNA<sub>3YS</sub> and viral genomic RNA, respectively, in the viral RNA samples, and we have used these data to determine the number of tRNA<sub>3YS</sub> molecules per two molecules of genomic RNA (Table 1). There are eight molecules of tRNA<sub>3YS</sub> per two copies of viral genomic RNA in virus produced from cells transfected with only the HIV-1 proviral DNA, and this number doubles when cells are also transfected with a gene for either wild-type or mutant tRNA<sub>3YS</sub>, again indicating that the mutant and wild-type tRNA molecules are incorporated into the virus with equal efficiency. Using the viral tRNA<sub>3YS</sub>/tRNA<sub>3YS</sub> ratios determined by 2D-PAGE analysis (Fig. 1), we can also calculate the number of tRNA<sub>3YS</sub> molecules present in the virus. It is clear that the total number of tRNA<sub>3YS</sub> molecules per virus remains constant and that increases in the number of tRNA<sub>3YS</sub> incorporated into the virus are accompanied by decreases in the number of molecules of tRNA<sub>3YS</sub> packaged, with the total number of tRNA<sub>3YS</sub> molecules per virus remaining at 20. While the tRNA<sub>3YS</sub>/tRNA<sub>3YS</sub> ratio is lower in virus containing suppressor tRNA<sub>3YS</sub> than in virus containing only wild-type tRNA<sub>3YS</sub>, this change in ratio is the result of very small differences in the actual concentration of tRNA<sub>3YS</sub> and tRNA<sub>1YS</sub> in the two viral types.

We have determined the aminoacylation states of the cellular and viral tRNA<sub>3YS</sub>. The electrophoretic mobility of acylated tRNA in acid-urea PAGE has been reported to be slower than that of the deacylated form, and this property can be used to determine the degree of tRNA aminoacylation (11, 12, 31). Figure 4 shows Northern (RNA) blots of cellular and viral RNA samples electrophoresed in acid-urea gels, blotted onto Hybond N filter paper, and hybridized with radioactive tRNA<sub>3YS</sub> DNA probes. In Fig. 4A, cellular tRNA was hybridized with the probe complementary to the 3′-18-nucleotide terminus of tRNA<sub>3YS</sub>. Lanes 1, 3, and 5 represent cytoplasmic tRNA extracted from cells transfected with a plasmid containing HIV-1 proviral DNA and either the wild-type tRNA<sub>3YS</sub> gene (lane 1), the mutant suppressor tRNA<sub>3YS</sub> gene (lane 3), or no tRNA<sub>3YS</sub> gene (lane 5). Lanes 2, 4, and 6 represent the corresponding RNA samples which have been deacylated in vitro. In cells either transfected with the wild-type tRNA<sub>3YS</sub> gene or not transfected with any tRNA gene, the tRNA<sub>3YS</sub> detected is entirely in the aminoacylated form. On the other hand, less than 40% of the mutant suppressor tRNA<sub>3YS</sub> is aminoacylated. These gels also show variable amounts of hybridizable RNA smaller than tRNA. This material is most likely degraded tRNA<sub>3YS</sub>, which is greatest in cells transfected with the suppressor tRNA<sub>3YS</sub> gene, less abundant in cells transfected with the wild-type tRNA<sub>3YS</sub> gene, and not detected at all in cells not containing an exogenous tRNA gene. The slightly lower number of suppressor tRNA<sub>3YS</sub> molecules found incorporated into virus could reflect the fact that some of these tRNA than wild-type tRNA is found in a degraded form.

The tRNA<sub>3YS</sub> isolated from cells transfected with the HIV-1 proviral DNA and the suppressor tRNA<sub>3YS</sub> gene contains both wild-type endogenous tRNA<sub>3YS</sub> and suppressor tRNA<sub>3YS</sub>, and both types of tRNA will give a signal when hybridized with the probe complementary to the 3′ terminus of tRNA<sub>3YS</sub>, which was used for Fig. 4A. A DNA probe complementary to the
cells transfected with the suppressor tRNA\textsubscript{"YS\textquotedblleft} gene or the virus made in these cells. This not only shows the specificity of this probe but also gives direct proof of the presence of the suppressor tRNA\textsubscript{"YS\textquotedblleft} in the virus.

In Fig. 4B, various cellular tRNA samples were hybridized with this anticodon probe. Cells were transfected with a plasmid containing the HIV-1 proviral DNA and either the suppressor tRNA\textsubscript{"YS\textquotedblleft} (lanes 7 and 10), no tRNA gene (lane 8), or the wild-type tRNA\textsubscript{"YS\textquotedblleft} gene (lane 9). The tRNA in lane 7 has been deacylated in vitro. Only suppressor tRNA\textsubscript{"YS\textquotedblleft} gives a signal, and Phosphor-imaging of lane 10 indicates that 64% of the suppressor tRNA\textsubscript{"YS\textquotedblleft} is in the deacylated form.

Figure 4C shows the acylation state of tRNA\textsubscript{"YS\textquotedblleft} incorporated into the virus. tRNA samples were electrophoresed onto Hybond N paper and hybridized with the 3' probe. Lanes 11 and 15 represent deacylated (in vitro) and acylated (in vivo) tRNAs, respectively, isolated from cells transfected with HIV-1 proviral DNA and the wild-type tRNA\textsubscript{"YS\textquotedblleft} gene. Lanes 12, 13, and 14 represent viral tRNA samples isolated from HIV-1 produced in H9 cells (lane 12), LTR(-) cells (lane 13), and COS-7 cells (lane 14). All viral tRNA\textsubscript{"YS\textquotedblleft} is in the deacylated form.

We have also examined the ability of the viral particles produced from COS-7 cells transfected with the three different types of plasmids to infect MT-4 cells. Figure 5 shows the viral RT activity in 50-μl aliquots of cell culture medium removed at days 1, 4, and 7. Cultures were replenished with fresh medium after sampling at day 4. These results are the average values from two separate experiments and indicate that there is no difference in the abilities of these different viruses to infect MT-4 cells. The decrease in virus production occurring between days 4 and 7 reflects the lysis of infected MT-4 cells, which occurs during this time (3, 25).

**FIG. 4.** Electrophoretic detection of acylated and deacylated tRNA\textsubscript{"YS\textquotedblleft}. Cellular or viral RNA was isolated and electrophoresed under acidic conditions as described in the text. The RNA was electrophoresed onto Hybond N and probed with wild-type or mutant tRNA\textsubscript{"YS\textquotedblleft} probes. (A) Ten-microgram samples of cellular RNA were hybridized with the DNA oligomer complementary to the 3' 18 nucleotides of both wild-type and mutant tRNA\textsubscript{"YS\textquotedblleft}. RNA samples were obtained from COS cells transfected with the following DNAs: SVC21.BH10Lys3 (lanes 1 and 2), SVC21.BH10Su+ (lanes 3 and 4), and SVC21.BH10 (lanes 5 and 6). Lanes 2, 4, and 6 contain RNA samples which have been completely deacylated in vitro. (B) Cellular RNA hybridized with the DNA oligomer complementary to the anticodon arm of suppressor tRNA\textsubscript{"YS\textquotedblleft}. RNA samples were obtained from COS cells transfected with the following DNAs: SVC21.BH10Su+ (lanes 7 and 10) (lane 7 contains RNA which has been completely deacylated in vitro), SVC21.BH10 (lane 8), and SVC21.BH10Lys (lane 9). (C) One microgram of either cellular or viral RNA was hybridized with the DNA oligomer complementary to the 3' 18 nucleotides of both wild-type and mutant tRNA\textsubscript{"YS\textquotedblleft}. Lanes 11 and 13, COS-7 cellular RNA. Lane 11 contains RNA which has been completely deacylated in vitro. Lanes 12 to 14 contain viral RNA isolated from HIV-1 produced in the following cells: chronically infected H9 (lane 12), chronically infected LTR(−) (lane 13), and COS-7 cells transfected with SVC21.BH10 (lane 14).

**FIG. 5.** Hybridization of RNA dot blots with DNA probes specific for tRNA\textsubscript{"YS\textquotedblleft} or suppressor tRNA\textsubscript{"YS\textquotedblleft}. With the samples analyzed for Fig. 2 to 4, 1 μg of the cellular RNA (A to G) and 1 μl of the viral RNA (D to F) samples analyzed were hybridized either with the 3' DNA probe specific for both wild-type and suppressor tRNA\textsubscript{"YS\textquotedblleft} (I) or with the anticodon arm probe specific for suppressor tRNA\textsubscript{"YS\textquotedblight} (II).
DISCUSSION

In this work, we show that the content of tRNA\textsubscript{\text{Lys}} in HIV-1 can be altered by altering the relative concentration of tRNA\textsubscript{\text{Lys}} in the cytoplasm. Because only a small fraction of cells in tissue culture are transduced with the tRNA\textsubscript{\text{Lys}} gene, we can only estimate the changes in the concentration of tRNA\textsubscript{\text{Lys}} in transduced cells. The 3-fold increase in the tRNA\textsubscript{\text{Lys}} to tRNA\textsubscript{\text{Lys}} microgram of cellular RNA was determined for a whole plate of cells, and it is likely that the relative concentration of tRNA\textsubscript{\text{Lys}} in the transduced cells may increase 10- to 20-fold.

In the virus produced by these cells, the number of tRNA\textsubscript{\text{Lys}} molecules incorporated doubles from 8 to 17 molecules per virion, assuming 2 molecules of genomic RNA per virion. This results in a decrease in the number of tRNA\textsubscript{\text{Lys}} molecules from 12 to 3 molecules per virion; i.e., the total amount of tRNA\textsubscript{\text{Lys}} per virus remains constant at 20. Similar results are found when COS cells are transduced with the mutant tRNA\textsubscript{\text{Lys}} gene. In another, similar set of experiments, we found that the transfection of COS cells with wild-type or mutant tRNA\textsubscript{\text{Lys}} genes resulted in the number of tRNA\textsubscript{\text{Lys}} molecules per virion increasing from 8 to 18 or 17, respectively, while the total number of molecules of tRNA\textsubscript{\text{Lys}} per virion remained at 20. Viruses containing an excess of wild-type or mutant tRNA\textsubscript{\text{Lys}} infects MT-4 cells with the same efficiency as wild-type virus. The decrease in viral tRNA\textsubscript{\text{Lys}} does not appear to affect viral infectivity, perhaps because this tRNA plays no role in this process or perhaps because the reduced number of molecules is still sufficient to support a function of tRNA\textsubscript{\text{Lys}} in viral replication. It would be of interest to decrease the viral content of tRNA\textsubscript{\text{Lys}} through the production of excess tRNA\textsubscript{\text{Lys}} in the cell and to determine the effect of this on viral infectivity. This work is in progress.

The number of tRNA\textsubscript{\text{Lys}} molecules incorporated into the virus is constant, and it could be limited by the space within the virus. However, 40% of the viral tRNA population appears to be non-tRNA\textsubscript{\text{Lys}} without any known function and probably represents the random incorporation of small amounts of cytoplasmic tRNAs (33). Excess cytoplasmic tRNA\textsubscript{\text{Lys}} molecules could be able to increase the viral tRNA\textsubscript{\text{Lys}} number beyond 20 by displacing these other tRNAs, but this does not occur. Alternatively, the limited number of tRNA\textsubscript{\text{Lys}} molecules present in the virus may reflect the limited number of Pr1600\textsuperscript{p=pol} molecules incorporated into the virus. Pr1600\textsuperscript{p=pol} is the viral protein likely to carry the tRNA\textsubscript{\text{Lys}} into HIV-1 (19). This protein, which is not processed until after viral budding (6, 35), contains the sequences for RT, a protein known to interact with tRNA\textsubscript{\text{Lys}} in vitro (1, 2, 26, 36), and mutations reducing or eliminating RT activity abolish select primer tRNA incorporation in avian (22) and murine (18) viruses and in HIV (19). However, if RT sequences within Pr1600\textsuperscript{p=pol} do interact with cytoplasmic tRNA\textsubscript{\text{Lys}}, they may do so in a manner different from the interaction occurring between mature p66/p51 RT and tRNA\textsubscript{\text{Lys}}. In vitro studies indicate that the anticodon arm of tRNA\textsubscript{\text{Lys}} is involved in the interaction between mature RT and tRNA\textsubscript{\text{Lys}} (1, 2, 26, 36), yet tRNA\textsubscript{\text{Lys}} which differs in this region from tRNA\textsubscript{\text{Lys}} by 6 of 17 bases, is incorporated into the virus with the same efficiency as tRNA\textsubscript{\text{Lys}}. Furthermore, as shown in this work, altered sequence in the anticodon loop does not appear to diminish the ability of the mutant tRNA\textsubscript{\text{Lys}} to be incorporated into the virus. It therefore seems unlikely that this region of the tRNA is involved in the select interaction which occurs between tRNA\textsubscript{\text{Lys}} and Pr1600\textsuperscript{p=pol} during packaging.

In the mutant suppressor tRNA\textsubscript{\text{Lys}}, the modified base mcm\textsuperscript{5}U, which is found in wild-type tRNA\textsubscript{\text{Lys}} at the first position in the anticodon (position 34), has been replaced with C. This does not seem to affect the ability of the tRNA to be packaged into the virus. On the other hand, this mutant tRNA may not be placed on the HIV-1 genome as efficiently as wild-type tRNA\textsubscript{\text{Lys}}. It has been reported that the anticodon loop of tRNA\textsubscript{\text{Lys}} interacts with an A-rich region upstream of the HIV-1 primer-binding site, and that destabilization of the modified nucleoside at position 34 destabilizes this interaction (13). Viruses containing suppressor tRNA\textsubscript{\text{Lys}} appears to be as infective as wild-type virus, and this may be because the mutant tRNA\textsubscript{\text{Lys}} is not an effective competitor with wild-type tRNA\textsubscript{\text{Lys}} for binding to the genomic RNA. Because the anticodon DNA probe specific for suppressor tRNA\textsubscript{\text{Lys}} hybridizes much more weakly than the DNA probe hybridizing to the 3' termini of both wild-type and suppressor tRNA\textsubscript{\text{Lys}}, we have been unable to specifically detect the extent of binding of the suppressor tRNA\textsubscript{\text{Lys}} to the viral RNA genome.

Does tRNA\textsubscript{\text{Lys}} enter the virus in the acylated or deacetylated form? It is clear that in the infected cell, all detectable cytoplasmic tRNA\textsubscript{\text{Lys}} is acylated. The tRNA\textsubscript{\text{Lys}} in the virus is deacylated, but we do not know if this is an RNA virus in vivo or is due to a spontaneous tRNA deacylation induced by the virus remaining for many hours in culture medium at neutral pH. Nevertheless, the deacylation of viral tRNA\textsubscript{\text{Lys}} is expected as a requirement for this molecule to function as a primer tRNA for RT. Does deacylation occur in the cytoplasm or in the virus? In the cytoplasm, deacylation of tRNA occurs during translation, and it may be that the cytoplasmic source of viral tRNA\textsubscript{\text{Lys}} is tRNA\textsubscript{\text{Lys}} which has just left the P site on the ribosome and which interacts with a growing Pr1600\textsuperscript{p=pol} protein still bound to the ribosome. Our data indicate that inhibiting aminoacylation of tRNA\textsubscript{\text{Lys}} by greater than 60% does not affect incorporation of the tRNA into the virus. However, we are not yet able to determine whether the acylated or deacetylated form of the mutant suppressor tRNA is more efficient in entering the virus, and resolution of this question may require studies with a mutant tRNA unable to be acylated in vivo.

The transient expression of tRNA\textsubscript{\text{Lys}} in our system cannot accurately measure the effect of excess tRNA\textsubscript{\text{Lys}} on host cell viability. However, Ho and colleagues (11, 12) created stable CHO cell lines which contained approximately 250 stably integrated human tRNA\textsubscript{\text{Lys}} genes, coding for either the wild-type tRNA\textsubscript{\text{Lys}} or the amber suppressor tRNA\textsubscript{\text{Lys}} used in this work. These cells contained approximately 50-fold more tRNA\textsubscript{\text{Lys}} than found in normal cells. As reported here for virus-infected COS-7 cells, they also found that all wild-type tRNA\textsubscript{\text{Lys}} was aminoacylated, while the suppressor tRNA\textsubscript{\text{Lys}} was only 30 to 50% aminoacylated. The cellular concentrations of tRNAs, relative to each other, mirror the relative concentrations of the major codons used (8), and this has been interpreted as a way the cell minimizes the time for placement of the correct tRNA at the most commonly used codons (30). A 50-fold increase in tRNA\textsubscript{\text{Lys}}, which normally represents 2% of the total tRNA, would halve the cellular concentration of all other tRNAs, and apparently this does not diminish cell viability.

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