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Role of Pr160$^{gag\text{-}pol}$ in Mediating the Selective Incorporation of tRNA^{Lys} into Human Immunodeficiency Virus Type 1 Particles

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COS-7 cells transfected with human immunodeficiency virus type 1 (HIV-1) proviral DNA produce virus in which three tRNA species are most abundant in the viral tRNA population. These tRNAs have been identified through RNA sequencing techniques as tRNA$_3^{Lys}$ the primer tRNA in HIV-1, and members of the tRNA$_{1,2}^{Lys}$ isoacceptor family. These RNAs represent 60% of the low-molecular-weight RNA isolated from virus particles, while they represent only 6% of the low-molecular-weight RNA isolated from the COS cell cytoplasm. Thus, tRNA^{Lys} is selectively incorporated into HIV-1 particles. We have measured the ratio of tRNA$_3^{Lys}$ molecules to copies of genomic RNA in viral RNA samples and have calculated that HIV-1 contains approximately eight molecules of tRNA$_3^{Lys}$ per two copies of genomic RNA. We have also obtained evidence that the Pr160$^{gag\text{-}pol}$ precursor is involved in primer tRNA$_3^{Lys}$ incorporation into virus. First, selective tRNA^{Lys} incorporation and wild-type amounts of tRNA$_3^{Lys}$ were maintained in a protease-negative virus unable to process Pr55^{gag} and Pr160$^{gag\text{-}pol}$ precursors, indicating that precursor processing was not required for primer tRNA incorporation. Second, viral particles containing only unprocessed Pr55^{gag} protein did not selectively incorporate tRNA^{Lys}, while virions containing both unprocessed Pr55^{gag} and Pr160$^{gag\text{-}pol}$ proteins demonstrated select tRNA$_3^{Lys}$ packaging. Third, studies with a proviral mutant containing a deletion of most of the reverse transcriptase sequences and approximately one-third of the integrase sequence in the Pr160$^{gag\text{-}pol}$ precursor resulted in the loss of selective tRNA incorporation and an eightfold decrease in the amount of tRNA$_3^{Lys}$ per two copies of genomic RNA. We have also confirmed herein finding of a previous study which indicated that the primer binding site is not required for the selective incorporation of tRNA^{Lys}.

A limited number of tRNAs derived from the infected host cell are packaged into retroviruses during virus assembly (9, 11, 24, 31, 33). One of these is termed the primer tRNA because it is used to prime the reverse transcriptase (RT)-catalyzed synthesis of retroviral minus-strand DNA. All members of the avian sarcoma and leukosis virus group examined to date use tRNA^{Trp} as primer for reverse transcription (10, 14, 26, 33, 39, 40), whereas murine leukemia virus (MuLV) employs tRNA^{Pro} (13, 24, 37), and mouse mammary tumor virus utilizes tRNA$_3^{Lys}$ (25, 38). The 3′-terminal 18 nucleotides of primer tRNA are complementary to a region near the 5′ end of the 35S RNA, termed the primer binding site (PBS). The sequence of this site in human immunodeficiency virus type 1 (HIV-1) suggests that the primer tRNA in this virus is also tRNA$_3^{Lys}$, one of the three major tRNA^{Lys} isoacceptors in mammalian cells (28, 29). Retroviral tRNA is found either in a free state or associated with the viral genome (8, 10, 11, 38, 39). When the viral 70S RNA complex is used to direct DNA synthesis catalyzed by RT, one of these bound tRNAs, more tightly associated than the others, serves as a primer for DNA synthesis (11, 18, 24, 37, 39). In HIV-1, the tightly associated tRNA is tRNA$_3^{Lys}$ (16).

During viral assembly, tRNA packaging is selective, and in HIV-1 produced by transfecting COS cells with HIV-1 proviral DNA, the tRNA^{Lys} isoacceptors, tRNA$_{1,2}^{Lys}$ and tRNA$_3^{Lys}$ are the major-abundance tRNAs packaged (16). These tRNAs are selected from over 100 different tRNA isoacceptor species in the cytoplasm, and we are investigating the mechanism responsible for this process. Previous work has indicated that the reduction or absence of viral genomic RNA incorporation does not affect primer tRNA incorporation into virions (20, 26). Because retrovirus are assembled by using Pr55^{gag} and Pr160$^{gag\text{-}pol}$ precursors which are not cleaved until after viral budding (17, 41), it is likely that the selection of tRNA^{Lys} occurs by the binding of tRNA^{Lys} to either of these proteins. Pr55^{gag} contains the amino acid sequence for nucleocapsid protein (NC), and Pr160$^{gag\text{-}pol}$ contains the amino acid sequences for NC and RT. Both NC and RT (in their fully processed forms) have been shown to bind to tRNA$_3^{Lys}$ in vitro (1, 27, 32). The absence of RT has also been reported to affect the incorporation of primer tRNA in both murine (21) and avian (26) retroviruses.

In this study, we determined the number of tRNA^{Lys} molecules incorporated into wild-type HIV-1 and examined the role of the PBS, viral protease, Pr55^{gag}, and Pr160$^{gag\text{-}pol}$ on the select incorporation of tRNA^{Lys} into virus particles. To do so, we analyzed the tRNA population in wild-type and mutant HIV-1 particles produced by transfecting COS-7 cells with wild-type and mutant HIV-1 proviral DNA. The data obtained provide supporting evidence for the involvement of uncleaved Pr160$^{gag\text{-}pol}$ in tRNA^{Lys} packaging.

MATERIALS AND METHODS

Plasmid construction. Schematic representations of the plasmids used for the transfections carried out in this study are shown in Fig. 1.

SVC21 BH10 contains wild-type HIV-1 proviral DNA se-

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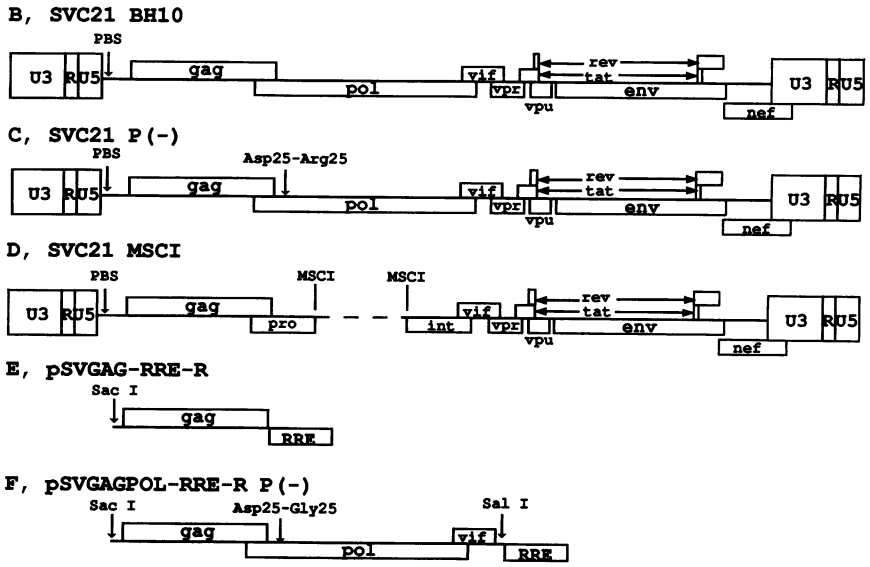

FIG. 1. Structures of wild-type and mutant HIV-1 plasmids. SVC21 BH10 (B) contains the wild-type HIV-1 proviral DNA sequence. SVC21 P(−) (C) contains a protease-deficient HIV-1 proviral DNA due to a point mutation at amino acid 25 in the protease region (12). SVC21 MSC1 (D) is an in-frame RT deletion mutant derived from SVC21 BH10 as described in Materials and Methods. The dashed line in the SVC21 MSC1 diagram indicates the deletion region. pSVGAG-RRE-R (E) and pSVGAGPOL-RRE-R P(−) (F), which serve as templates for the synthesis of $Pr55^{gag}$ and unprocessed $Pr160^{gag\text{-}pol}$, respectively, were constructed as previously described (34, 35).

quence. SVC21 P(−) differs from SVC21 BH10 by a single point mutation at position 25 of the protease region, converting Asp-25 to Arg-25. Transfection of SVC21 P(−) produces noninfectious viral particles containing wild-type genomic RNA and unprocessed precursor proteins $Pr55^{gag}$ and $Pr160^{gag\text{-}pol}$ (12). Both SVC21 BH10 and SVC21 P(−) were gifts from E. Cohen, University of Montreal.

SVC21 MSC1 contains an in-frame deletion of RT and approximately one-third of the integrase sequence and was derived from SVC21 BH10. SVC21 BH10 was completely digested with the restriction enzyme MSC1 (New England Biolabs). The resulting 12-kb fragment, containing the plasmid vector and deleted HIV-1 genome, was fractionated by agarose electrophoresis and, after extraction from the gel, further purified by using a mini-spin column and phenol-chloroform extraction. The fragment was ligated with T4 DNA ligase (Pharmacia). DNA sequencing was performed to confirm the deletion.

pSVGAG-RRE-R and pSVGAGPOL-RRE-R P(−) were previously described (34, 35). Viral production from these two plasmids requires cotransfection with the Rev protein expression vector pCMV-rev. Cotransfection of pSVGAG-RRE-R with pCMV-rev produces virus-like particles containing unprocessed $Pr55^{gag}$ precursor protein, while cotransfection of pSVGAGPOL-RRE-R P(−) with pCMV-rev produces virus-like particles containing unprocessed $Pr55^{gag}$ and $Pr160^{gag\text{-}pol}$.

All restriction and modification enzymes were used as specified by the manufacturer.

Production of wild-type and mutant HIV-1. COS-7 cells were transfected with wild-type and mutant proviral DNAs by the calcium phosphate method as previously described (17). Virus was isolated from the cell culture medium 63 h post-transfection. 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 centrifuging 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 viral RNA and human placental $tRNA^{Lys}$ isoacceptors. Total viral RNA was extracted from viral pellets by the guanidinium isothiocyanate procedure (4).

The purification of $tRNA_3^{Lys}$ and $tRNA_{1,2}^{Lys}$ from human placenta was performed as previously described (16). The term $tRNA_{1,2}^{Lys}$ refers to a population of two $tRNA^{Lys}$ species which differ by one base pair in the anticodon stem (28). In this report, spots 1 and 2 are collectively referred to as $tRNA_{1,2}^{Lys}$, since we have not sufficiently characterized these two species to distinguish $tRNA_2^{Lys}$ from $tRNA_1^{Lys}$ (16).

RNA labeling. The fractionated RNA samples were labeled by the ^{32}pCp 3′-end-labeling technique (3). To make ^{32}pCp, 5 mCi of [γ-^{32}P]ATP (specific activity, 3,000 Ci/mmol; Dupont Canada) was dried down in a microcentrifuge tube by using N_2, 100 μl of a reaction solution containing 50 mM Tris-HCl (pH 9.2), 5 mM $MgCl_2$, 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 ^{32}pCp was monitored by using polyethyleneimine thin-layer chromatography in 0.8 M NH_2SO_4, which separates ^{32}pCp from [^{32}P]ATP.

The RNA was labeled with ^{32}pCp as previously described (3, 18). After labeling, free ^{32}pCp was removed from the labeled macromolecules either by using homemade Sephadex G-50 (Pharmacia) spin columns equilibrated with TE buffer (10 mM Tris [pH 7.5], 1 mM EDTA) or during the electrophoresis run. Before analysis by polyacrylamide gel electrophoresis (PAGE), the samples were heated at 90°C for 2 min.

One-dimensional (1D) and 2D PAGE. Viral RNA was electrophoresed at 4°C in a Hoefer SE620 gel electrophoresis apparatus. 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 bromophenol 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), run for 30 h (25 W limiting), and then subjected to autoradiography. All electrophoretic runs were carried out in 0.5× TBE (1× TBE is 5 mM Tris, 5 mM boric acid, and 1 mM Na_2EDTA). The electrophoretic gel patterns shown in this report 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 tRNAs 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 with a PhosphorImager (Bio-Rad, Toronto, Ontario, Canada).

Measurement of tRNA$_3^{Lys}$ by RNA-DNA hybridization. To measure the amount of tRNA$_3^{Lys}$ in viral RNA, we synthesized an 18-mer DNA oligonucleotide complementary to the 3′ 18 nucleotides of tRNA$_3^{Lys}$ (5′-TGGCGCCCGAACAGGGAC-3′). This probe hybridizes specifically with tRNA$_3^{Lys}$ (16, 17) and was hybridized to dot blots on Hybond N (Amersham) of either purified human placental tRNA$_3^{Lys}$ or total RNA from wild-type and mutant viruses. The DNA oligomer was first 5′ end labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP (3,000 Ci/mmol; Dupont), and specific activities of 10^8 to 10^9 cpm/μg were generally reached. Approximately 10^7 cpm of oligomer was generally used per blot in hybridization reactions.

Measurement of viral genomic RNA by quantitative PCR.

(i) Labeling of primer. One nanomole of sense primer was mixed with 50 μCi of [γ-^{32}P]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 $MgCl_2$, 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 a known volume of viral RNA or in vitro-transcribed RNA in a total volume of 16.5 μl. Three drops of oil layer was placed on top of the primer-template mix to avoid evaporation. Tubes containing primer-template mix were heated at 85°C for 3 min and slowly cooled to room temperature to allow primer-template annealing. Then 8.5 μl of RT master mix (30 mM Tris-HCl [pH 8.3], 5 mM $MgCl_2$, 6 mM dithiothreitol, 1.5 μM deoxynucleoside triphosphates (dNTPs), 0.5 μg of BSA, 10 U of RNase inhibitor [Promega], 5 U of Moloney MuLV RT [Pharmacia]) was added to the primer-template mix. Reaction tubes were incubated at 37°C for 1 h. RT activity was terminated by incubation at 95°C for 5 min.

(iii) PCR. Seventy-five microliters of PCR master mix (10 μl of 10× *Taq* DNA polymerase buffer [GIBCO/BRL], 10 μl of 2 mM dNTPs, 1 μl of ^{32}P-labeled primer mix, 1.25 U of *Taq* DNA polymerase [GIBCO/BRL]) was added to the reaction tubes containing reverse transcription products, which were incubated at 95°C and subjected to 19 cycles of amplification (denaturation, 94°C, 1 min; annealing, 60°C, 1 min; elongation, 72°C, 3 min). Aliquots of PCR products were run on an 8% polyacrylamide–7 M urea gel 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 PhosphorImager.

(iv) Quantitative determination of genomic RNA in wild-type and mutant viral RNA samples. Sense primer JS1 (5′-ATTCGGTTAAGGCCAGGGGG-3′) and antisense primer JA1 (5′-GGGATGGTTGTAGCTGTCCC-3′) were used for PCR amplification. A 148-bp fragment, corresponding to HIV-1 IIIB DNA positions 843 to 990, was amplified.

A standard curve was established by using known amounts of in vitro-transcribed RNA for reverse transcription-PCR (RT-PCR). A 957-base RNA fragment (sense) was made from a linearized DNA plasmid, pEA2, with T7 RNA polymerase (pEA2 was kind gift from E. J. Arts, McGill AIDS Centre). This RNA fragment corresponds to HIV-1 IIIB DNA positions 473 to 1420.

Genomic RNA in wild-type and mutant viral RNA samples and in vitro transcript RNA were quantitated with a PhosphorImager (Bio-Rad). The relative intensities of amplified signals were used to determine the concentration of genomic RNA in the viral samples, using the standard curve.

RESULTS

We have previously shown that the tRNA^{Lys} isoacceptors, tRNA$_{1,2}^{Lys}$ and tRNA$_3^{Lys}$, are the major-abundance tRNAs that are incorporated into infectious virus particles produced from COS-7 cells transfected with HIV-1 proviral DNA (16). To identify the specific viral proteins involved in the selective packaging of these tRNA molecules, we transfected COS-7 cells with a series of wild-type and mutant HIV-1 expression plasmids. The construction and characteristics of each expression plasmid are described in Materials and Methods, and the relevant portion of each plasmid is shown in Fig. 1. The plasmids include three HIV-1 proviral clones which express (i) wild-type Pr55^{gag} and Pr160$^{gag\text{-}pol}$, which are processed later (pSVC21 BH10), (ii) unprocessed Pr55^{gag} and Pr160$^{gag\text{-}pol}$ [pSVC21 P(−)], or (iii) wild-type processed Pr55^{gag} products and a severely deleted Pr160$^{gag\text{-}pol}$ which lacks 95% of the RT domain and approximately one-third of the integrase sequence (pSVC21 MSC1). These proviral clones also express all of the viral proteins downstream of the Pr160$^{gag\text{-}pol}$ coding region. In addition, two simian virus 40 late expression plasmids that express either unprocessed Pr55^{gag} alone (pSVGAG-RRE-R) or both unprocessed Pr55^{gag} and Pr160$^{gag\text{-}pol}$ [pSVGAGPOL-RRE-R P(−)] were used.

To analyze the low-molecular-weight RNAs present in viral particles, total RNA was extracted, end labeled, and subjected to 2D PAGE as described in Materials and Methods. Figure 2 shows the 2D PAGE patterns of tRNAs extracted from normal COS-7 cells as well as from virus particles produced from the cells transfected with the different vectors. Figure 2A shows the tRNA extracted from uninfected COS-7 cells. Previous studies have identified spots 1 and 2 as tRNA$_{1,2}^{Lys}$ and spot 3 as tRNA$_3^{Lys}$ (16). To quantitate the amount of tRNA present in each spot, the gel was subjected to phosphor-imaging analysis. Table 1 shows the quantitation of the tRNA^{Lys} spots from the various gels. In untransfected COS-7 cells (Fig. 2A), approximately 6% of the cellular tRNA was represented by tRNA^{Lys}, and the tRNA$_3^{Lys}$ ratio was close to 1 (Table 1). In contrast, in wild-type virus (Fig. 2B), approximately 60% of the low-molecular-weight RNA was tRNA^{Lys}. Thus, there was a 10-fold increase in the relative concentration of tRNA^{Lys} in the virus compared with the cytoplasm of COS cells; i.e., tRNA^{Lys} was selectively incorporated into the virus. The tRNA$_{1,2}^{Lys}$/tRNA$_3^{Lys}$ ratio remained similar to that found in the cytoplasm of COS cells, indicating that the two major tRNA^{Lys} isoacceptor families are packaged with equal efficiency.

Because HIV is assembled from the precursor polypeptides Pr55^{gag} and Pr160$^{gag\text{-}pol}$, which are cleaved into multiple proteins during or after viral budding, it was of interest to determine whether the precursor molecules themselves could mediate the selective incorporation of tRNA^{Lys} or whether proteolytic cleavage was necessary to facilitate the specific incorporation. Thus, the tRNA pattern was examined in

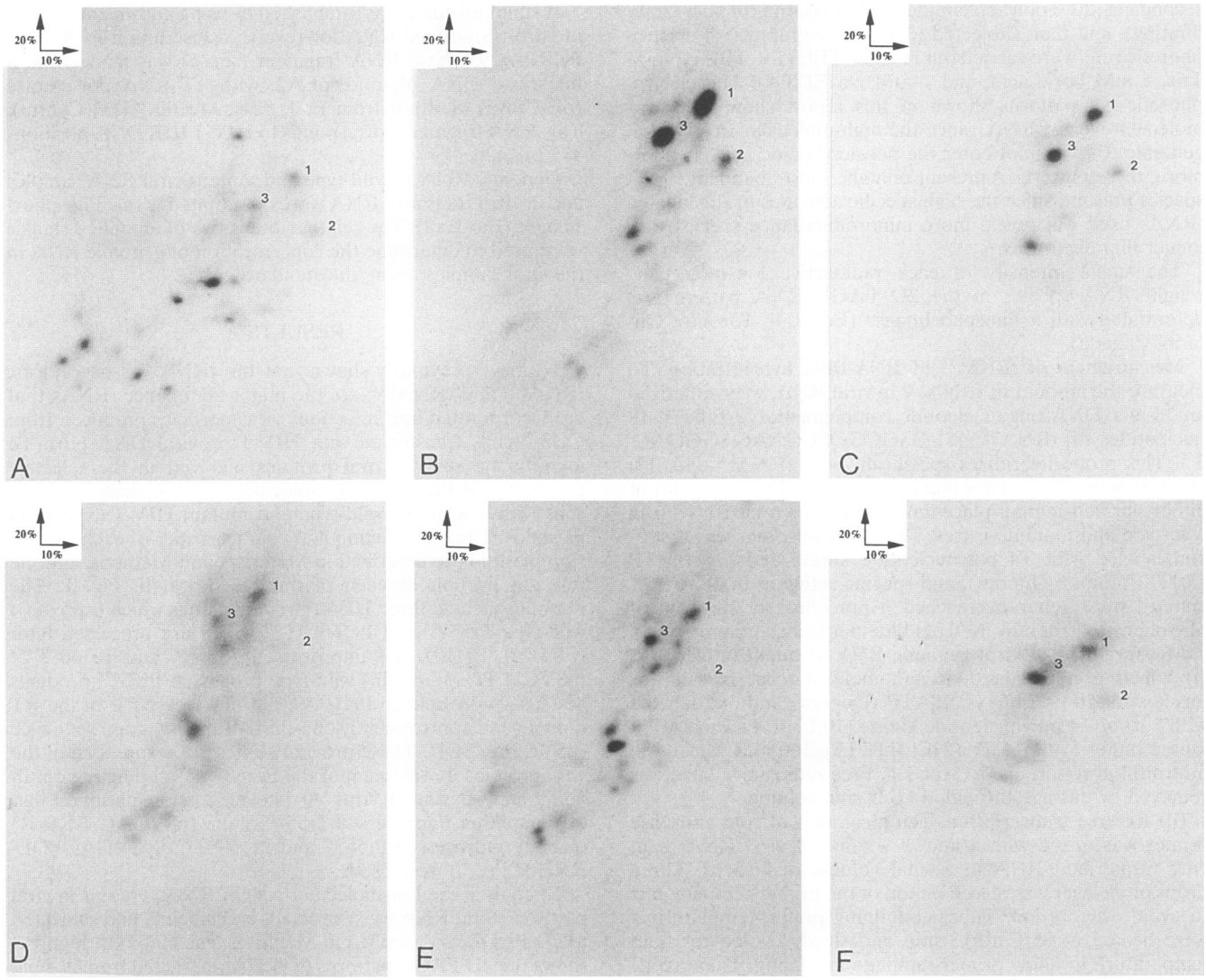

FIG. 2. 2D PAGE patterns of low-molecular-weight viral RNA. Electrophoretic conditions were as described in the text. (A) Uninfected COS cell; (B) wild-type virus (SVC21 BH10); (C to F) mutant viruses SVC21 P(−) (C), SVC21 MSC1 (D), pSVGAG-RRE-R (E), and pSVGAGPOL-RRE-R P(−) (F).

TABLE 1. Percentage of $tRNA^{Lys}$ isoacceptors in total cellular and viral $tRNA^{a}$

tRNA source	%				% $tRNA^{Lys}_{1,2}$/ %$tRNA^{Lys}_{3}$
	$tRNA^{Lys}_{1,2}$ (spot 1)	$tRNA^{Lys}_{1,2}$ (spot 2)	$tRNA^{Lys}_{3}$ (spot 3)	$tRNA^{Lys}$	
COS cells	2.1	1.0	2.6	5.7	1.2
Constructs[b]					
B	24.7	5.3	31.2	61.2	1.0
C	19.6	5.6	25.7	50.9	1.0
D	1.9	0.4	1.9	4.2	1.2
E	2.0	0.6	2.1	4.7	1.2
F	20.5	5.2	24.4	50.1	1.1

[a] Determined through phosphor-imaging analysis of the 2D PAGE patterns in Fig. 2. Spots refer to those in Fig. 2.
[b] See Fig. 1.

particles produced from a proviral clone lacking an active viral protease [pSVC21 P(−)]. This clone contains an inactive protease due to a Asp-25-to-Arg-25 change in the active site, and the particles produced from transfected cells remain in the immature form containing only unprocessed $Pr55^{gag}$ and $Pr160^{gag\text{-}pol}$ (12). As shown in Fig. 2C and Table 1, approximately 50% of the low-molecular-weight RNA is $tRNA^{Lys}$, with a $tRNA^{Lys}_{1,2}/tRNA^{Lys}_{3}$ ratio equal to 1.0.

$Pr160^{gag\text{-}pol}$ is the most likely precursor protein to be involved in $tRNA^{Lys}$ packaging because it contains the sequences for RT, a protein whose mature form has been shown to interact with $tRNA^{Lys}_{3}$ in vitro (1, 32). To investigate the role of $Pr160^{gag\text{-}pol}$ in select $tRNA^{Lys}$ packaging, we transfected COS cells with a proviral DNA (pSVC21 MSC1) which is missing 95% of RT sequences and approximately one-third of the integrase sequence (Fig. 1C). In this mutant, $Pr55^{gag}$ is processed. Figure 2D shows the low-molecular-weight RNA pattern in the virus particles produced, and the Phosphor-imaging analysis of the gel is presented in Table 1. The results indicate

that while the tRNA^{Lys} isoacceptors are present in the virus, they are not selectively packaged. The relative concentration of the tRNA^{Lys} in the virus is similar to that found in the cytoplasm of nontransfected cells, i.e., 4.2%. The deletion used in this experiment was too large to allow mapping of the precise region in Pr160$^{gag\text{-}pol}$ involved in selecting tRNA^{Lys} for incorporation into the virus, but these data clearly demonstrate the importance of Pr160$^{gag\text{-}pol}$ in the selection process.

On the other hand, viral particles containing only unprocessed Pr55^{gag} (not Pr160$^{gag\text{-}pol}$) do not selectively package tRNA^{Lys} into virus. These particles were produced by transfecting COS-7 cells with DNA construct E(pSVGAG-RRE-R; Fig. 1) (34, 35). The low-molecular-weight RNA pattern of these virus particles is shown in Fig. 2E, and phosphor-imaging analysis of this pattern (Table 1) indicates that no select tRNA^{Lys} incorporation occurs; i.e., 4.7% of the low-molecular-weight viral RNA is tRNA^{Lys}. We also produced virus particles by transfecting COS-7 cells with DNA construct F [pSVGAG-POL-RRE-R P(−)]. These particles contain both unprocessed Pr55^{gag} and Pr160$^{gag\text{-}pol}$ (34, 35). The low-molecular-weight RNA pattern of these virus particles is shown in Fig. 2F, and phosphor-imaging analysis of this pattern (Table 1) indicates that select tRNA^{Lys} incorporation occurs; i.e., 50% of the low-molecular-weight viral RNA is tRNA^{Lys}. This finding further supports the importance of Pr160$^{gag\text{-}pol}$ in tRNA^{Lys} selection. It should also be noted that construct F produces viral genomic RNA lacking the PBS (as does construct E); therefore, these experiments validate earlier results which indicated that select tRNA^{Lys} incorporation into HIV-1 does not require the presence of a PBS on the genomic RNA (16).

The results in Fig. 2 and Table 1 showed the effects of mutations in the viral proteins on the ability to selectively incorporate tRNA^{Lys} into virions, but these experiments did not address the effects of these mutations on the absolute amounts of tRNA^{Lys} incorporated into the virus particles. To investigate this, we measured the tRNA$_3^{Lys}$/genomic RNA ratio in each of the RNA samples isolated from the wild-type and mutant viruses (Fig. 3 and 4; Table 2).

We have previously demonstrated the ability of a DNA probe complementary to the terminal 3′ 18 nucleotides of tRNA$_3^{Lys}$ to hybridize specifically with this tRNA (16). Total viral RNAs from wild-type and mutant viruses were blotted onto Hybond filter paper, and the amount of tRNA$_3^{Lys}$ present in each sample was determined through hybridization both to these samples and to known amounts of purified human placental tRNA$_3^{Lys}$ (Fig. 3). The amount of genomic RNA in each sample was determined by quantitative PCR (Fig. 4). The results were quantitated by phosphor-imaging analysis (Table 2).

We found that wild-type virus contain eight molecules of tRNA$_3^{Lys}$ per two copies of genomic RNA (Table 2, RNA source B). Similar amounts of tRNA$_3^{Lys}$ were found in the protease-negative mutant virus particles (RNA source C). The virions lacking the RT and part of the integrase showed an eightfold reduction in the amount of tRNA$_3^{Lys}$ per two copies of genomic RNA (RNA source D). On the other hand, the amount of tRNA$_3^{Lys}$ per two copies of genomic RNA ratio was unexpectedly high in virus particles produced from the expression vector producing only Pr55^{gag} (RNA source E), since these particles showed no specificity of tRNA^{Lys} incorporation. The amount of tRNA$_3^{Lys}$ per two copies of genomic RNA was also unexpectedly high in virus particles produced from the expression vector producing both Pr55^{gag} and Pr160$^{gag\text{-}pol}$ (RNA source F). These particles showed specific tRNA^{Lys} incorporation, but the amount of tRNA$_3^{Lys}$ per two copies of genomic RNA was approximately 14 times higher than that found in wild-type HIV-1. These high ratios could be explained if there was a defect in constructs E and F which resulted in virus particles that had a 10-fold decrease in their ability to package genomic RNA. Recent data suggest that these constructs do produce viral particles defective in the packaging of genomic RNA (3a).

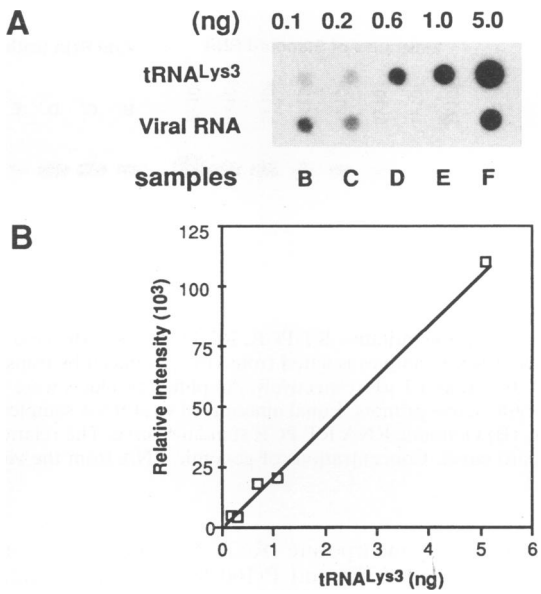

FIG. 3. (A) Quantitation of tRNA$_3^{Lys}$ in viral RNA. The specificity of the tRNA$_3^{Lys}$ DNA oligomer probe used has been described previously (15). Purified human placenta tRNA$_3^{Lys}$ was used as an external standard. Aliquots from the same viral RNA samples used to quantitate genomic RNA in Fig. 4 were used to determine the amount of tRNA$_3^{Lys}$ present. Volumes of viral RNA samples used for hybridization in lanes B to F were 2, 2, 1, 2, and 2 μl, respectively. (B) tRNA$_3^{Lys}$ standard curve. Hybridization signals of purified human tRNA$_3^{Lys}$ were used to construct a standard curve. Concentrations of tRNA$_3^{Lys}$ in viral RNA samples were calculated from this curve.

DISCUSSION

As reported above, approximately 60% of viral low-molecular-weight RNA in HIV-1 is tRNA^{Lys}, with 30% represented by the primer tRNA, tRNA$_3^{Lys}$. These results are similar to those found for the primer tRNA, tRNA^{Trp}, in avian myeloblastosis virus (AMV) by using a very different technique, aminoacylation (39). Primer tRNA^{Trp} represents 32% of free viral tRNA in AMV. We have also determined the amount of tRNA$_3^{Lys}$ and genomic RNA present in HIV-1 total viral RNA and used this information to calculate the number of tRNA$_3^{Lys}$ molecules per virion, assuming two copies of genomic RNA per virion. By these calculations, wild-type HIV-1 contains approximately eight molecules of tRNA$_3^{Lys}$ per virion. This number is similar to the value of six to eight molecules of primer tRNA per virion reported for Rous sarcoma virus (33) and AMV (39).

In this report, we provide evidence supporting the hypothesis that the unprocessed Pr160$^{gag\text{-}pol}$ is involved in selecting tRNA^{Lys} for incorporation into HIV-1. First, studies with a protease mutant clearly demonstrate that proteolysis of viral protein precursors is not required for tRNA^{Lys} incorporation. A similar conclusion was reached from studies using the MuLV (6) and avian leukemia virus (36) systems.

Second, viral particles containing only unprocessed Pr55^{gag}

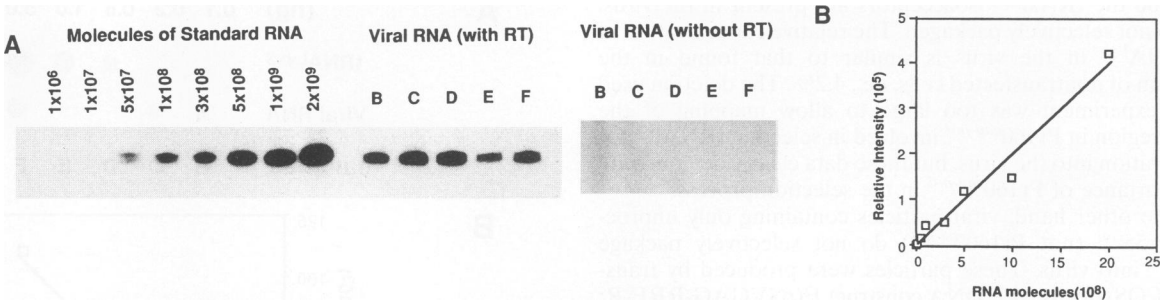

FIG. 4. (A) Quantitative RT-PCR. RT-PCR was performed on in vitro-transcribed genomic RNA (used as an external standard) or on known volumes of RNA samples isolated from virus produced by transfecting COS cells with constructs B to F (see Fig. 1), using viral RNA samples of 0.1, 0.1, 0.1, 1, and 1 μl, respectively. Amplified products were run on an 8% polyacrylamide–7 M urea gel to separate amplified products from excess radioactive primers. Equal amounts of viral RNA samples were also used for PCR amplification without reverse transcription as a negative control. (B) Genomic RNA RT-PCR standard curve. The relative intensities of amplified signals from in vitro-transcribed RNA were used to plot a standard curve. Concentrations of genomic RNA from the viral RNA samples were calculated from this curve.

do not selectively incorporate $tRNA^{Lys}$, while virions containing unprocessed $Pr55^{gag}$ and $Pr160^{gag\text{-}pol}$ do. Thus, although mature NC has been shown to bind to $tRNA_3^{Lys}$ in vitro (1, 27), the NC sequence in $Pr55^{gag}$ does not appear, by itself, to play a major role in $tRNA^{Lys}$ selection. We cannot yet rule out the possibility that $Pr55^{gag}$ interacts cooperatively with $Pr160^{gag\text{-}pol}$ to select $tRNA^{Lys}$ for incorporation. On the other hand, in construct F, the absence of the genes for viral proteins downstream of the *vif* gene shows that the protein products of these genes are not required for select $tRNA^{Lys}$ incorporation (Rev protein is made on a separate plasmid in our transfection system, and its participation in select $tRNA^{Lys}$ incorporation, while unlikely, cannot be ruled out). The ability of the virus containing unprocessed $Pr55^{gag}$ and $Pr160^{gag\text{-}pol}$ to selectively incorporate $tRNA^{Lys}$ in the absence of a PBS on the genomic RNA is also consistent with results from other retrovirus systems which indicate a lack of involvement of genomic RNA in this process (20, 26).

Finally, removal of RT and integrase sequences from $Pr160^{gag\text{-}pol}$ prevents selective $tRNA^{Lys}$ incorporated per two copies of genomic RNA. The effect of the alteration of $Pr160^{gag\text{-}pol}$ on $tRNA^{Lys}$ incorporation indicates the importance of $Pr160^{gag\text{-}pol}$ in this process. The deletion in $Pr160^{gag\text{-}pol}$ is large and could result in (i) an alteration in the conformation of the molecule, (ii) more rapid degradation of the molecule, or (iii) inability of the damaged $Pr160^{gag\text{-}pol}$ molecule to enter the virus. However, our results are consistent with the evidence that RT sequences are involved in $tRNA^{Lys}$ selection. It is known that mature RT (p66/p51) interacts with primer tRNA during reverse transcription (1, 32), and it has been shown in both the avian sarcoma virus (26) and MuLV (21) systems that a mutant virus lacking RT fails to selectively incorporate the correct tRNA. Finer mutational analysis will be required to determine whether RT sequences within the $Pr160^{gag\text{-}pol}$ protein are involved in $tRNA^{Lys}$ selection.

TABLE 2. Molecules of $tRNA_3^{Lys}$ per two molecules of genomic RNA

RNA source[a]	No. of molecules of genomic RNA/μl of sample[b]	ng of $tRNA_3^{Lys}$/μl of sample[c]	No. of molecules of $tRNA_3^{Lys}$/μl of sample[d]	No. of molecules of $tRNA_3^{Lys}$/2 molecules of genomic RNA
B	2.0×10^9	0.34	8.0×10^9	8
C	2.3×10^9	0.34	8.0×10^9	8
D	3.0×10^9	0.06	1.4×10^9	1
E	1.7×10^8	0.03	8.5×10^8	10
F	2.0×10^8	0.46	1.1×10^{10}	109

[a] See Fig. 1.
[b] Determined from Fig. 3.
[c] Determined from Fig. 4.
[d] A tRNA molecular size of 25,000 g/mol was used to calculate the number of tRNA molecules.

Wild-type HIV-1 contains approximately eight molecules of $tRNA_3^{Lys}$ per virion. Viruses unable to process precursor proteins still incorporate eight molecules of $tRNA_3^{Lys}$ per virion, while those lacking RT and integrase reduce the incorporation of $tRNA_3^{Lys}$ approximately eightfold. Our calculations assume that these mutants incorporate normal amounts of viral genomic RNA. This assumption is supported by the findings that normal amounts of viral genomic RNA are incorporated in a protease-negative avian leukemia virus mutant (36) and in RT-negative avian and murine retroviruses (21, 26).

Our results are also consistent with our unpublished data (3a) showing that RNA from constructs resembling E and F cannot be packaged into particles. This explains the unexpectedly high ratio of $tRNA_3^{Lys}$ molecules per two copies of genomic RNA observed in these cases. Particles produced from the plasmid expressing only unprocessed $Pr55^{gag}$ did not show select $tRNA^{Lys}$ incorporation yet contained 11 molecules of $tRNA_3^{Lys}$ per two copies of genomic RNA. Particles produced from the plasmid expressing both unprocessed $Pr55^{gag}$ and $Pr160^{gag\text{-}pol}$ showed selective packaging of $tRNA^{Lys}$ but contained 109 molecules of $tRNA_3^{Lys}$ per two copies of genomic RNA, a ratio 14-fold higher than found in wild-type HIV-1. The 10-fold differences in the ratio of $tRNA_3^{Lys}$ per two copies of genomic RNA seen between these two viral populations probably reflects a 10-fold difference in $tRNA^{Lys}$ selective packaging because of the presence of $Pr160^{gag\text{-}pol}$ in one and not the other. While it is not clear why the genomic RNA from these constructs fail to be packaged, these results are consistent with recent findings which indicate that the two regions missing in our constructs, the 5′ part of the genome through the PBS and the region 3′ of the *vif* gene (34, 35), are required for genomic RNA packaging (30, 37a).

The $tRNA_{1,2}^{Lys}/tRNA_3^{Lys}$ ratios are similar in the cell's cytoplasm and in the virus. This finding indicates that these $tRNA^{Lys}$ isoacceptors are packaged with equal efficiency and suggests that some common feature of these molecules is

recognized by a viral protein during packaging. Mature RT has been shown to interact in vitro with the anticodon arm of $tRNA_3^{Lys}$ (1, 2, 32, 42), yet this region is quite different between $tRNA_3^{Lys}$ and $tRNA_{1,2}^{Lys}$, differing by 6 of 17 bases. If this region of the molecule serves as a recognition signal for packaging, then the two different sequences in this arm must fold in similar conformations. On the other hand, it is possible that the interaction of $Pr160^{gag\text{-}pol}$ with $tRNA_3^{Lys}$ in vivo is quite different from the interaction studied in vitro with mature, processed RT. This difference could be due to artifacts created in the in vitro systems in which the interaction between mature RT and $tRNA_3^{Lys}$ are studied, but it is possible that the interactions involved in packaging $tRNA_3^{Lys}$ are quite different from the interaction between RT and $tRNA_3^{Lys}$ during reverse transcription. For example, it has been postulated that the tRNA binding site on RT may involve both p66 and p51 polypeptides in the RT dimer (19), but it is not known whether $Pr160^{gag\text{-}pol}$ can also form a dimer and, if so, interact with $tRNA^{Lys}$ in the dimer form. Besides the fact that $tRNA_3^{Lys}$ is interacting with two different molecules during packaging and reverse transcription, the $tRNA_3^{Lys}$ may be in different states in the cytoplasm and in the virus. For example, most cytoplasmic $tRNA_3^{Lys}$ is expected to be complexed with proteins such as lysine $tRNA^{Lys}$ synthetase or the eukaryotic elongation factor EF-1α, which carries tRNA to the ribosome (15, 23). It may therefore be that the $Pr160^{gag\text{-}pol}$ binding site on $tRNA_3^{Lys}$ is composed of protein as well as RNA.

In a retrovirus, primer tRNA is present in a higher abundance than most other tRNAs. Is this higher abundance a requirement for its efficient function as a primer tRNA, or is efficiency of function as a primer more influenced by specific structural properties of the tRNA? Colicelli and Goff (5) isolated a mutant MuLV which contained a PBS complementary to $tRNA^{Gln}$ rather than $tRNA^{Pro}$, the normal primer tRNA in this virus. These virus particles were infectious and presumably could use $tRNA^{Gln}$ as the primer tRNA. Similar results were also obtained in which a recombinant MuLV-based retroviral vector containing a PBS for $tRNA_1^{Gln}$ or $tRNA_3^{Lys}$ instead of $tRNA^{Pro}$ also appeared to be replicated, using $tRNA_1^{Gln}$ or $tRNA_3^{Lys}$ as the primer for reverse transcription (22). The concentration of $tRNA^{Lys}$ relative to other viral tRNAs is high in many retroviral systems examined, including MuLV (40), but the relative concentration of $tRNA^{Gln}$ in MuLV has not been reported, and it would be of interest to know whether the select incorporation of $tRNA^{Gln}$ into the mutant virus or in the retroviral vector particles is required for it to act efficiently as a primer for reverse transcription. As mentioned above, evidence is presented here and elsewhere that viral genomic RNA does not appear to be involved in the selective incorporation of primer tRNA, including in the MuLV system (20).

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