A HIV-1 Minimal Gag Protein Is Superior to Nucleocapsid at \textit{in Vitro} tRNA\textsubscript{3Lys} Annealing and Exhibits Multimerization-induced Inhibition of Reverse Transcription*

Received for publication, February 3, 2005, and in revised form, February 22, 2005
Published, JBC Papers in Press, February 24, 2005, DOI 10.1074/jbc.M501310200

Ariel Roldan‡, Otis U. Warren‡§, Rodney S. Russell‡¶, Chen Liang‡, and Mark A. Wainberg‡**

From the °McGill University AIDS Centre, Lady Davis Institute-Jewish General Hospital, Montreal, Quebec H3T 1E2, Canada and the ‡Department of Microbiology and Immunology and ¶Division of Experimental Medicine, McGill University, Montreal, Quebec H3A 2B4, Canada

HIV-1 uses tRNA\textsubscript{3Lys} to prime reverse transcription of its viral RNA. In this process, the 3’-end of tRNA\textsubscript{3Lys} must be annealed to the primer binding site of HIV-1 genomic RNA, and the two molecules together form a complex structure. During annealing, the nucleocapsid (NC) protein enhances the unwinding of tertiary structures within both RNA molecules. Moreover, the packaging of tRNA\textsubscript{3Lys} occurs prior to viral budding at a time when NC is still part of the Pr55\textsubscript{Gag} polyprotein. In contrast, Pr55\textsubscript{Gag} is able to produce virus-like particles on its own. We have recently shown that an N-terminal extended form of NC (mGag), containing all of the minimal elements required for virus-like particle formation, possesses greater affinity for HIV-1 genomic RNA than does NC alone. We have now studied the tRNA\textsubscript{3Lys} -annealing properties of mGag in comparison to those of NC and report that the former is more efficient in this regard than the latter. We have also tested each of a mutant version of mGag, an extended form of mGag, and an almost full-length form of Gag, and showed that all of these possessed greater tRNA-annealing capacity than did the viral NC protein. Yet, surprisingly, multimerization of Gag-related proteins did not abrogate this annealing process but rather resulted in dramatically reduced levels of reverse transcriptase processivity. These results suggest that the initial stages of reverse transcription may be regulated by the multimerization of Pr55\textsubscript{Gag} polyprotein at times prior to the cleavage of NC.

* This research was supported by the Canadian Institutes of Health Research. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Work performed in fulfillment of the year-out “Scholarly Pursuit” requirement toward a Doctorate of Medicine degree at the University of Pennsylvania.

** To whom correspondence should be addressed: McGill AIDS Centre, Lady Davis Institute-Jewish General Hospital, 3755 Cote Ste-Catherine, Montreal, Quebec H3T 1E2, Canada. Tel.: 514-340-8260; Fax: 514-340-7537; E-mail: mark.wainberg@mcgill.ca.

\textsuperscript{1} The abbreviations used are: RT, reverse transcriptase; HIV-1, human immunodeficiency virus, type 1 (HIV-1) uses human tRNA\textsubscript{3Lys}, one of three major tRNA\textsubscript{Lys} isoacceptors, for the priming of reverse transcription (1). tRNA\textsubscript{3Lys} is selectively incorporated into the virus particle and is annealed via 18 bases at its 3’-end to a complementary 18-base sequence near the 5’-end of viral genomic RNA termed the primer binding site (PBS) (2–4). Once annealed, tRNA\textsubscript{3Lys} primes the synthesis of minus strand cDNA. For a review, see Ref. 5.

Both tRNA\textsubscript{3Lys} and the PBS have complex three-dimensional structures, and the annealing of the two relevant sequences requires the “unwinding” of local stem-loop structures (6, 7). The mature nucleocapsid (NC) protein of HIV-1 has been shown to facilitate this annealing \textit{in vitro} (8). Presumably, NC enhances local unwinding of tertiary structures in the vicinity of the PBS to allow the annealing of tRNA\textsubscript{3Lys} (see Fig. 1a) (9). NC is a small basic protein that is cleaved from the Gag precursor during viral particle maturation. NC binds to structures in viral RNA termed stem loop 1 (SL1) through SL4 to specifically package HIV-1 genomic RNA (10–14). NC contains two Zn\textsuperscript{2+} fingers that are required for both this genomic RNA recognition as well as for its roles in strand transfer (15). However, mutational analyses of NC have not implicated the Zn\textsuperscript{2+} fingers in the tRNA\textsubscript{3Lys} annealing reaction, although it appears that the basic regions that flank the first Zn\textsuperscript{2+} finger are important in this regard (16, 17).

In current models of HIV-1 viral replication, tRNA\textsubscript{3Lys} is annealed during viral budding prior to proteolytic cleavage of the Gag precursor. Therefore, the activity of NC must occur in the context of the Pr55\textsubscript{Gag} polyprotein. Previous research showed that extracts of viral RNA from both wild-type viruses and protease-deficient HIV-1 particles were able to anneal to tRNA\textsubscript{3Lys} (18), a finding consistent with results obtained from studies on murine and avian retroviruses (19, 20). NC binds nonspecifically to tRNA\textsubscript{3Lys} \textit{in vitro} and, consequently, does not play an important role as a mature protein in the specific annealing of tRNA (21). Interestingly, other \textit{in vitro} experiments have found that both mature NC and immature Gag possess equivalent ability to anneal tRNA\textsubscript{3Lys} to a synthetic 5’-HIV-1 genomic RNA molecule (22).

Despite its roles in a variety of HIV-1 life cycle events, much of the Pr55\textsubscript{Gag} polyprotein is not required for the assembly of virus-like particles (VLPs), and, indeed, all of the required protein-protein interaction domains required for viral assembly are located in the capsid (CA) and NC regions (23–28). The minimal Gag domains necessary for VLP formation \textit{in vivo} are the myristylation signal, the C-terminal domain of CA, the SP1 spacer peptide, NC, and p6 (29, 30). It has also been proposed that SP1 and a potential α-helix that traverses the CA-SP1 boundary are involved in both Gag multimerization (31, 32).
and the specificity of HIV-1 genomic RNA packaging (33, 34). Indeed, our laboratory has shown that a purified minimal Gag (mGag) protein, which contains all of the Gag domains that support VLP formation, can bind to genomic RNA with a stronger affinity than does NC (35); this suggests that not all genomic RNA recognition by Gag is attributable to sequences in NC (36, 37). In addition, mGag was shown to possess an 10-fold greater affinity than NC for a genomic RNA template that included an extended 5'-leader region beyond the traditionally recognized "ψ" packaging signal, i.e. the SL5 and early complex platform (ECP) (35). Because tRNA\textsubscript{Lys} annealing occurs during Gag-mediated VLP assembly, this raises a number of questions. First, do other domains outside of NC but within the Gag precursor also contribute to tRNA\textsubscript{Lys} annealing? Second, what are the consequences of Gag multimerization on the efficiency of tRNA\textsubscript{Lys} annealing and the priming of reverse transcription? This report deals with the use of a mGag-purified protein (Fig. 1b), consisting of the myristylation signal, the C-terminal domain of CA, and NC to study the effect of Gag multimerization on tRNA\textsubscript{Lys} annealing as well as the possibility that Gag sequences besides NC might be involved in tRNA\textsubscript{Lys} annealing. We now show that mGag is superior to NC in its ability to anneal tRNA\textsubscript{Lys} to the PBS and also that Gag multimerization does not inhibit annealing of tRNA\textsubscript{Lys} in contrast, large multimers of mGag can inhibit reverse transcription of (−) strong stop DNA.

MATERIALS AND METHODS

**Protein Synthesis**—The synthesis and purification of the proteins used has been described previously (35). Briefly, protein was expressed in B121 (DE3) cells (Novagen) and purified under denaturing conditions by affinity with nickel agarose. Further purification was performed by anion exchange chromatography (Q-agarose). Protein was refolded by dialysis under reducing conditions, and removal of unfolded protein was accomplished by ultracentrifugation at 100,000 × g for 1 h. Proteins from several preparations were employed in each of the different experiments, and no differences among these various preparations were observed in regard to results obtained.

**Synthesis and Folding of RNA**—DNA templates were amplified from a vector containing the sequence for HIV-1 HxB2D (GenBank™ R03455) in a PCR in the following manner. A primer with the T7 promoter at its 5'-end followed by the sequence complementary to positions +1 to +24 was used as the forward primer with the reverse primer the SL5 and early complex platform (ECP) (35). Because tRNA\textsubscript{Lys} annealing occurs during Gag-mediated VLP assembly, this raises a number of questions. First, do other domains outside of NC but within the Gag precursor also contribute to tRNA\textsubscript{Lys} annealing? Second, what are the consequences of Gag multimerization on the efficiency of tRNA\textsubscript{Lys} annealing and the priming of reverse transcription? This report deals with the use of a mGag-purified protein (Fig. 1b), consisting of the myristylation signal, the C-terminal domain of CA, and NC to study the effect of Gag multimerization on tRNA\textsubscript{Lys} annealing as well as the possibility that Gag sequences besides NC might be involved in tRNA\textsubscript{Lys} annealing. We now show that mGag is superior to NC in its ability to anneal tRNA\textsubscript{Lys} to the PBS and also that Gag multimerization does not inhibit annealing of tRNA\textsubscript{Lys}. In contrast, large multimers of mGag can inhibit reverse transcription of (−) strong stop DNA.

**FIG. 1. Genomic RNA templates and proteins used.** a, top, shown schematically is the 5'-HBX2 HIV-1 genomic RNA structure predicted from M-Fold analysis and previous reports with HIV-1 \textsuperscript{tRNA\textsubscript{Lys}} from nucleotides +1 to +506, where +1 is the capping site. From 5' to 3' the structures shown are the Tat-associated region (TAR), the poly-adenylation signal (Poly(A)), the primer binding site (PBS), stem loops 1–5 (SL1–5), and the early complex platform (ECP). Bottom, primers used in PCR amplification and the resultant PCR products that were used as genomic RNA templates. Numbers correspond to the last amino acids of each peptide within the precursor. A black line denotes amino acids 2–7, which are included in mGag. An asterisk denotes the position of the M318A mutation. The gray represents the N-terminal histidine tag of the vector used. c, SDS-PAGE analysis of the different proteins used. ~5 μg of protein were loaded in each lane, separated by electrophoresis, and visualized by staining with Coomassie Brilliant Blue. Two different protein ladders are shown (first and last lanes), and the molecular masses of the bands are given on the side in kDa. The identity of the proteins employed is given below each lane.
In Vitro tRNA\textsubscript{Lys}\textsubscript{3} Annealing by mGag

**Fig. 2.** Schematic diagrams of the reactions employed. A, elongation assay in which protein, tRNA\textsubscript{Lys}\textsubscript{3}, genomic RNA, and RT were co-incubated to allow for protein-assisted tRNA\textsubscript{Lys}\textsubscript{3} annealing. After annealing, Mg\textsuperscript{2+} was added to initiate reverse transcription. B, protein annealing assay in which protein, tRNA\textsubscript{Lys}\textsubscript{3}, and genomic RNA were first co-incubated to allow for protein-assisted tRNA\textsubscript{Lys}\textsubscript{3} annealing. Then, proteinase K (PK) digestion and phenol-chloroform extraction were performed to remove any protein. Finally, the reverse transcription reaction was initiated using the protein-annealed primer/template. C, heat annealing in which tRNA\textsubscript{Lys}\textsubscript{3} was first heat-annealed to genomic RNA. In a second step, protein was incubated with the heat-annealed RNAs, and then reverse transcription reaction was initiated.

Heating to 85 °C and slowly cooling to room temperature. RNA was then stored at -80 °C. Human placental tRNA\textsubscript{Lys}\textsubscript{3} purified by high pressure liquid chromatography was purchased from Bio S&T (Montreal, Quebec), resuspended in 100 mM NaCl, 20 mM Tris, pH 7.5, and refolded as described above.

Reverse Transcription Elongation Assay—RNA transcripts and tRNA\textsubscript{Lys}\textsubscript{3} (25 nM each) were combined with appropriate concentrations of protein in the presence or absence of competitive RNA. Protein concentrations were 1–10 nM versus 0.5–1.8 µM in the presence and absence of competitive RNA, respectively. The total volume of these reactions was 14.1 µl containing 16 µM dGTP, 16 µM dTTP, 0.5 µM dCTP, 171 nM [α-32P]dCTP (3000Ci/mmol), 50 µM ddATP, 0.25 units/µl of RNase inhibitor (Invitrogen), 5 mM dithiothreitol, and 12.5 nM/µl RT (kindly supplied by Dr. M. Gérardo of our Institute) in 50 mM Tris, pH 7.5, 50 mM NaCl with or without 50 nM concentration of a 116-base competitive RNA. Mixtures were incubated at 37 °C for 10 min to allow for tRNA\textsubscript{Lys}\textsubscript{3} annealing following which MgCl\textsubscript{2} was added to a concentration of 3 mM for a final reaction volume of 15 µl. The mixtures were then incubated for an additional 30 min at 37 °C to allow reverse transcription to proceed. A positive control involved heating the tRNA\textsubscript{Lys} and template RNA together to 75 °C and slowly cooling to room temperature before inclusion in the reaction. A negative control was performed without protein. Reactions were terminated by bringing the final volume to 100 µl by addition of 0.1% SDS and 0.3 mM NaOAc. RNA was then ethanol precipitated and resuspended in 8 µl of denaturing gel loading buffer II (Ambion, Texas). 4 µl of each sample was run on a 5% polyacrylamide gel under denaturing conditions, dried, and exposed to Kodak Bio-Max films at -80 °C overnight. Results were analyzed by molecular imaging in comparison with the heat-annealed positive control. This method (shown schematically in Fig. 2c) allows us to determine the relative ability of a protein to participate in the reverse transcription reaction independently of the ability to promote tRNA\textsubscript{Lys}\textsubscript{3} annealing.

Heat Annealing Assay—25 nM each tRNA\textsubscript{Lys}\textsubscript{3} and 1-ECP template RNA were annealed by heating to 75 °C and slowly cooling to room temperature. Protein was then added at the indicated concentrations, and the mixtures were incubated for 10 min at 37 °C in the above-described reverse transcription reactions, also containing 50 nM competitive RNA but no Mg\textsuperscript{2+} in a total volume of 14.1 µl. Mg\textsuperscript{2+} was then added to a final concentration of 3 mM in a volume of 15 µl, and reverse transcription was allowed to proceed for 3 min at 37 °C. Other procedures were as indicated above for the elongation assay. This method (shown schematically in Fig. 2c) allows us to isolate the ability of a protein to participate in the reverse transcription reaction independently of the ability to promote tRNA\textsubscript{Lys}\textsubscript{3} annealing.

Statistical Analysis—Statistical analysis was performed using GraphPad Prism software, version 4.00 for Windows (San Diego, CA).

**RESULTS**

mGag Has tRNA\textsubscript{Lys} Annealing Ability Superior to NC—Previous data from our laboratory showed that mGag is superior to NC in the recognition of HIV-1 genomic RNA (35). We and others have proposed that the CA-SP1 interface plays a role in viral packaging specificity (33, 34). Accordingly, we were interested in comparing mGag with NC in its ability to anneal tRNA\textsubscript{Lys} onto the PBS. We generated a HIV-1 probe termed 1-PBS, because it contains nucleotides from positions +1 to +247, and co-incubated this transcript with either NC or mGag at the indicated concentrations together with human placental tRNA\textsubscript{Lys}. Then, we performed reverse transcription reactions using radiolabeled nucleotides. Inclusion of ddATP in the reactions as a sole source of dATP resulted in a 6-nucleotide polymorphism product (38). This method was used to determine the efficiency of tRNA\textsubscript{Lys} annealing, based on the amount of [α-32P]dCTP incorporated into DNA product (see Fig. 2a).

As shown in Fig. 3, mGag was 8–10-fold superior to NC in its ability to anneal tRNA\textsubscript{Lys} onto the PBS. Interestingly, however, the use of very high concentrations of mGag resulted in decreased incorporation of the radiolabeled nucleotide; results from experiments performed with these high concentrations of mGag were not included in our K\textsubscript{d} analyses for mGag (Fig. 3c). Many of the samples yielded a double band, which probably corresponds to incorporation of the first [α-32P]dCTP or to partial degradation of the tRNA\textsubscript{Lys} during reverse transcription. A negative control showed that RT was not able to anneal...
tRNA\textsuperscript{Lys\textsubscript{3}} on its own in the absence of a Gag-related protein. In contrast, a positive control experiment performed with heat annealing showed that RT was enzymatically active when employed in the context of previously placed tRNA\textsuperscript{Lys\textsubscript{3}}.

We next wished to determine the effects of the addition of a nonspecific competitive RNA transcript. Hence, similar reactions were carried out using serial dilutions of such a nonspecific transcript and both mGag and NC (data not shown). At ratios of nonspecific competitive RNA to HIV-1 genomic RNA above 2:1, virtually no elongation of the DNA product occurred in the presence of NC, and an -50% reduction occurred with mGag. We therefore used a ratio of 2:1 competitive RNA:viral genomic RNA in all other competitive reactions that were performed. As expected, higher concentrations of NC and mGag were required in the presence of the competitor in order for equivalent levels of annealing to take place. Interestingly, the ability of high protein concentrations to abrogate RT activity was observed for mGag in the absence of competitor RNA but was even more pronounced in cases in which competitor RNA was present. In contrast, this abrogation of RT activity was not seen when NC was used to anneal tRNA\textsuperscript{Lys\textsubscript{3}} onto the PBS (Fig. 3b). The fact that mGag, an N-terminal extended form of NC, was superior to mature NC in ability to extend tRNA\textsuperscript{Lys\textsubscript{3}} suggests that regions within mGag that are outside of NC probably contribute to tRNA\textsuperscript{Lys\textsubscript{3}} annealing, RT processivity, or both.

M318A mGag Has Similar tRNA Annealing Capabilities as Wild-type mGag but Reduced Sensitivity to the Inhibitory Effects of High Protein Concentrations in Initiation of Reverse Transcription—To investigate the possibility that mGag multimerization at high concentrations might have played an inhibitory role in our \textit{in vitro} assay, we employed a mutated mGag protein in which the methionine at position 318 had been mutated to an alanine in the C-terminal domain region of CA. This mutation has been shown to both abolish capsid dimerization and to alter virus particle formation (39, 40). The same elongation reaction conditions as those described above were employed (Fig. 2a) together with the genomic RNA template 1-ECP (Fig. 1a). The results show that mGag M318A was superior to wild-type mGag when used at high concentrations in the presence of competitive RNA (Fig. 4). Under these reaction conditions, mGag M318A behaved similarly to NC, and increased elongation was seen at higher concentrations. In contrast, in the absence of competition, wild-type mGag was superior to mGag M318A in its ability to promote elongation at low and intermediate protein concentrations (Fig. 4a, compare lanes 3–5 of the first and second panels). Thus, the mGag M318A mutation, which results in diminished multimerization, appears to have rescued the ability of mGag to anneal tRNA\textsuperscript{Lys\textsubscript{3}} and/or to initiate polymerization, even if it lost a portion of this ability in experiments performed without competition.

High Protein Concentration Does Not Abrogate tRNA Annealing onto the PBS—As shown, large mGag multimers inhibited our \textit{in vitro} tRNA\textsuperscript{Lys\textsubscript{3}} annealing assay at high protein concentrations. Therefore, we next wished to determine which step of the reaction had been blocked, i.e. either the annealing of tRNA\textsuperscript{Lys\textsubscript{3}} or reverse transcription itself. Accordingly, we performed \textit{in vitro} reactions as described above under two different sets of conditions. In the first, we performed an annealing reaction using each of the various proteins, tRNA\textsuperscript{Lys\textsubscript{3}}, the 1-ECP RNA template and competitive RNA at the indicated concentrations. After incubation at 37 °C for 10 min, we carried out a proteinase K digestion followed by phenol-chloroform extraction to remove any protein. In this circumstance, only protein-
primed RNA molecules are left and ensuing polymerization is dependent on the amount of annealed \( \text{tRNA}^{\text{Lys}} \) that was formed. Under these conditions, mGag was maximally able to anneal \( \text{tRNA}^{\text{Lys}} \) when used at high concentrations (Fig. 5a). Consistent with previous results, mGag was significantly more efficient in this regard than NC and was slightly more efficient than the mutated mGag M318A at annealing \( \text{tRNA}^{\text{Lys}} \). These results showed that \( \text{tRNA}^{\text{Lys}} \) annealing is not inhibited by high protein concentrations of mGag.

High Protein Concentrations of mGag Impairs RT—Subsequent to the above, we were interested in knowing whether polymerization might be impaired by high protein concentrations of mGag. Therefore, we pre-annealed \( \text{tRNA}^{\text{Lys}} \) to the HIV-1 template RNA termed 454-ECp by heating and slow cooling to room temperature (Fig. 2c). We next added each of the various proteins, competitive RNA and RT at the concentrations indicated and incubated to allow for protein-RNA binding. Then, Mg\(^{2+} \) was added to initiate reverse transcription (Fig. 2c). In this system, the \( \text{tRNA}^{\text{Lys}} \) is prebound to the RNA template, negating any effects of protein-facilitated \( \text{tRNA}^{\text{Lys}} \) annealing and allowing us to study the effects of protein on polymerization. Fig. 5b shows that the highest concentrations of mGag resulted in virtually no DNA product and that lower protein concentrations yielded better results in this regard. In contrast, the highest concentrations of mature NC gave rise to the highest levels of DNA. With the mutated mGag M318A, decreased efficiency of polymerization occurred at the highest concentrations of protein, but we did not witness the complete abolition of product formation as had been seen with mGag. Hence, the negative effect of mGag at high concentration may have been because of its ability to multimerize and such multimerization does not apparently inhibit Gag-mediated \( \text{tRNA}^{\text{Lys}} \) priming. These results also confirm the superiority of mGag in ability to anneal \( \text{tRNA}^{\text{Lys}} \).

The Impact of RNA Template on \( \text{tRNA}^{\text{Lys}} \) Annealing and RT Processivity—We also wished to investigate the effects of longer HIV-1 genomic RNA templates on \( \text{tRNA}^{\text{Lys}} \) annealing, given that inclusion of SL5 and ECP (Fig. 1a) seemed to promote greater protein affinity for viral genomic RNA. For this purpose, we generated two other HIV-1 RNA transcripts termed 1-SL4 and 1-ECP (Fig. 1a). In elongation RT assays, no significant differences were observed among the three different genomic RNA templates employed (1-PBS, 1-SL4, 1-ECP) (Fig. 6a). Interestingly, mGag showed higher \( \text{tRNA}^{\text{Lys}} \) annealing capacity in protein annealing assays when the 1-PBS genomic RNA template was used as opposed to either 1-SL4 or 1-ECP (Fig. 6b). This suggests that longer RNA templates can reduce the \( \text{tRNA}^{\text{Lys}} \) annealing capabilities of mGag. In contrast, abrogation of RT activity was observed in heat annealing assays at low protein concentrations when either 1-SL4 or 1-ECP was employed as template in comparison with 1-PBS (Fig. 6c). This suggests that inclusion of additional RNA sequences has the ability to enhance abrogation of RT activity in these assays. This may be due to specific RNA sequences and/or structures contained within the template or to changes in the protein:RNA ratio. It is not clear why differences among templates were not observed in ability to anneal \( \text{tRNA}^{\text{Lys}} \) onto viral RNA and to affect reverse transcription processivity in RT elongation assays, whereas we did observe differences when we specifically assessed these activities in other types of assays, i.e. protein annealing and heat annealing (Fig. 6d, see “Discussion”).

Annealing and Elongation Properties of mGag2 and GagΔp6—We were interested in understanding the effects of other domains of Pr55\(^{\text{Gag}} \) in the \( \text{tRNA}^{\text{Lys}} \) annealing and elongation process. We were particularly interested in SP2, as others have shown that this small spacer can increase some NC activities (41), and in full-length Gag, as others have found no difference in \( \text{tRNA}^{\text{Lys}} \) placement activity on the part of this protein when compared with NC (22). Several investigators have also reported that full-length Gag is relatively unstable; therefore we used a truncated GagΔp6 version (42).

With this objective, we cloned and purified GagΔp6 as well as a mGag protein containing SP2, which we termed mGag2. In protein annealing experiments, mGag2 protein did not differ significantly from mGag in its \( \text{tRNA}^{\text{Lys}} \) annealing capacity (Fig. 7, a and c), but, interestingly, mGag2 showed a greater ability to abrogate reverse transcription at low protein concentrations than did mGag in elongation assays (Fig. 7b). It is possible that the SP2 motif, included in mGag2, added another protein-protein interface to mGag, resulting in increased multimerization capabilities of this protein and an increased inhi-
FIG. 5. **Comparisons of mGag, mGag M318A, and NC in heat annealing and protein annealing experiments.**  

- **a**, protein annealing assay. Lanes 1–5 correspond to protein concentrations of 10, 7.5, 5, 2.5, and 1 μM, respectively. Lane C is a control reaction in which heat annealing was attempted in the absence of protein. mGag and mGag M318A were able to anneal tRNA3Lys maximally at their highest concentrations, whereas NC was less efficient at annealing of tRNA3Lys than mGag. These results suggest that tRNA3Lys annealing is not inhibited by protein multimerization.

- **b**, heat annealing assay. Lanes 1–5 correspond to protein concentrations of 10, 7.5, 5, 2.5, and 1 μM. Lane C is a control reaction performed in the absence of protein. At very high protein concentrations, mGag inhibited reverse transcription with maximal RT processivity occurring at a concentration of 2.5 μM. In contrast, NC resulted in maximal RT processivity when used at the highest concentration studied, i.e., 10 μM. mGag M318A possessed intermediate activity compared with either NC or mGag. All reactions were performed in the presence of competitive RNA. These findings suggest that protein multimerization can inhibit reverse transcription in heat annealing reactions.

---

**FIG. 6.** **Comparison of tRNA annealing and RT impairment by mGag using different genomic RNA templates.**

- **a**, RT elongation assay performed with different genomic RNA templates as shown. Lanes 1–5 are in the presence of competitor RNA with 1–5 representing concentrations of mGag, 10, 7.5, 5, 2.5, and 1 μM, respectively. Lanes 6–10 are in the absence of competitor RNA with 6–10 representing concentrations of mGag, 1.8, 1.5, 1.3, 1, and 0.5 μM. Note that the protein concentrations used in the competition reactions are greater than those used in the absence of competition. Lane 11 is a negative control without protein. Lane C+ is a positive control without protein but with heat-annealed tRNA3Lys placed onto the genomic RNA template employed in each panel.

- **b**, protein annealing assay performed with mGag and different genomic RNA templates as shown. Protein concentrations used are as stated on the figure in μM. Lane 0 is a negative control without protein. Lane C+ is a positive control with heat-annealed tRNA3Lys placed onto the genomic RNA template. c, heat annealing assay performed with mGag and different genomic RNA templates as shown. Protein concentrations used are as stated on the figure in μM. Lane 0 is a control without protein, which, in this case, represents the positive control and was used as a reference to calculate the percentage of RT activity at the different protein concentrations employed.

- **d**, comparative results obtained from experiments shown in a, b, and c. The Kd and R² are shown for the different RNA templates in each of the different assays, as well as the statistical differences among values observed. NS, not significantly different.
The results with GagΔp6 were surprising, because the latter protein showed similar tRNA₃Lys annealing capacities compared with mGag or mGag2 (Fig. 7, a and c), and others have reported that Pr55Gag has similar tRNA annealing activity as does NC. Unexpectedly as well, GagΔp6 in elongation assays showed almost complete abrogation of reverse transcription at almost all concentrations tested. The increased multimerization capacity of this protein appears to be the most likely explanation for these findings.

**DISCUSSION**

In this work, we used a purified mGag protein containing all relevant motifs of Pr55Gag protein for VLP formation, to study the annealing of tRNA₃Lys onto the PBS and the initiation of reverse transcription. We also employed a CA dimerization mutant, an extended version of mGag including SP2 (i.e. mGag2) and an almost full-length version of Pr55Gag (i.e. GagΔp6). We have shown that NC in the context of the precursor protein is more efficient at the annealing of tRNA₃Lys than mature NC and that tRNA₃Lys annealing is not affected by Gag multimerization, which does, however, compromise reverse transcription.

The finding that mGag is superior to NC in promoting the annealing of tRNA₃Lys was unexpected, because current belief has been that all of the tRNA₃Lys annealing ability of Gag is contained within the NC domain. Indeed, previous reports found that full-length Pr55Gag was equivalent to mature NC in this regard (22), but our results with all N-terminal extended versions of NC showed that NC in the context of the precursor has a superior ability at tRNA annealing when compared with mature NC. We have no clear explanation for the different results obtained here versus those of others. The reported instability of Pr55Gag used in previous work could be one explanation, whereas another might be the different protein purification and solubility criteria used here. In agreement with the latter idea, GagΔp6 in elongation assays showed almost complete abrogation of RT, but tRNA annealing was not affected as shown in the protein annealing assay. The known multimerization capacity of this protein appears to be the most possible and logical explanation for the results obtained. Others have also experienced protein-RNA aggregation artifacts/problems but claimed this was only observed exceptionally. In our studies, gel shift assays using mGag after 15 min of centrifugation at 16,000 x g (protein solubility criteria used by others) resulted in protein-RNA complexes that were never able to run into the gel matrix, in contrast to results obtained for NC. This problem was solved by ultracentrifugation of mGag at 100,000 x g (35).

These results suggest that domains outside of NC might improve tRNA₃Lys annealing. Here we showed that the inclusion of the C-terminal domain CA markedly improves the ability of NC to anneal tRNA₃Lys to 5'-HIV-1 genomic RNA. mGag includes a putative α-helix across the CA-SP1 cleavage site that is not present after protease cleavage. This region has been shown to be involved in the specificity of HIV-1 genomic RNA packaging (33, 34) and may play a role in HIV-1 protein-RNA interactions required for tRNA₃Lys annealing. Another possibility is that the multimerization ability of mGag itself might promote a protein conformational change that facilitates tRNA₃Lys annealing, i.e. NC annealing of tRNA₃Lys might be enhanced as a protein multimer, which occurs only in the presence of N-terminal extensions (i.e. mGag). Interestingly, we found that the mGag capsid dimer-
ization mutant, M318A, possessed a reduced but almost similar ability to anneal tRNA$^{\text{Lys}}$ than wild-type mGag. This suggests that a possible role of multimerization is overlapped by potential annealing sequences found within N-terminal extensions of NC (see below).

Previous results from our laboratory showed that affinity of mGag for HIV-1 genomic RNA was increased when longer genomic RNA templates were used to include an ECP (35). Accordingly, we expected to see a similar increase in annealing ability with the use of longer HIV-1 genomic RNA templates. Nevertheless, we found instead in RT elongations assays that genomic RNA templates that included sequences up to the PBS were equivalent in annealing ability to templates that included sequences through SL4 and the ECP. However, mGag showed higher annealing activity in protein annealing assays with 1-PBS than when 1-SL4 or 1-ECP were used as template, and, paradoxically, the abrogation of RT was clearly reduced when the shorter template was used in comparison to longer ones. One possible explanation is that the reduction of tRNA annealing by mGag when a longer genomic RNA is used, i.e. 1-SL4 or 1-ECP, is because of the fact that at least two strong binding sites for mGag are introduced by the probes (i.e. SL2 and SL3). These two elements could compete for protein binding, reducing the amount of protein available to anneal the tRNA onto the PBS.

But, then, why did we not see any difference among templates in RT elongation assays? Possibly, the tRNA-genomic RNA-mGag interaction might be able to adopt a different conformation when certain elements are present in the probe, most probably SL2 and SL3, making this interaction more stable or efficient than when these elements are not present (i.e. 1-PBS). This particular conformation would also facilitate mGag multimerization, explaining the results obtained with 1-SL4 and 1-ECP in heat annealing assays in comparison to what was observed for 1-PBS. Alternatively, the longer probes might fold differently than 1-PBS, as they lend themselves to long distance base-pairing interactions (43–46), and this differential RNA folding might explain the results obtained. Thus, a complex underlying mechanism seems to be at work that involves more than RNA coating by protein. In any case, we conclude that 1) tRNA annealing and RT abrogation involve two distinct and independent but related mechanisms, 2) longer RNA probes with complex tertiary structures, or modification of the RNA:protein ratio, is/are able to more easily promote the arrest of reverse transcription, and 3) the mechanism of abrogation of RT activity involves the multimerization ability of the protein and not simply the coating of the RNA, as more protein would be required to coat longer RNA probes.

Our results with the different templates employed (as discussed above) and with the different proteins used (i.e. wild-type mGag, M318A mGag, mGag2, and Gag$^{\text{Ap6}}$), showing that each of them possessed similar tRNA$^{\text{Lys}}$-annealing capacity but different abilities to abrogate reverse transcription, suggest that the multimerization of Gag-related proteins is what inhibits elongation. If this is true, then PR-mediated maturation of Pr$_{55}^{\text{Gag}}$ might cause a switch from a mode that favors multimerization to one that promotes disruption of the viral particle. Our results at different concentrations of mGag, suggest that Gag multimerization and the formation of VLPs might contribute to the inhibition of RT processivity and that maturation could alter Gag from being an inhibitory multimer to the facilitative role of monomeric NC. Another possibility is that PR cleavage results in the liberation of NC from other Gag motifs that promote Gag multimerization. This would leave NC tightly bound to viral genomic RNA in monomeric fashion, which is facilitative of polymerization.

It is important to note that even if mGag was superior to NC in its tRNA$^{\text{Lys}}$-annealing properties, a ratio of nonspecific RNA: HIV-1 genomic RNA >2:1 markedly reduced this ability. This is possibly because of a dilutional effect of the protein, which bound nonspecifically to the competitive RNA and hence reduced the amount of free protein available for tRNA$^{\text{Lys}}$ annealing. Current estimates are that one molecule of NC can bind to ~8 nucleotides (10–14), consistent with the protein:RNA ratios used in these experiments. This shows that other elements are also involved in the tRNA$^{\text{Lys}}$ annealing process in vivo (i.e. Lysyl-tRNA synthetase (47) and Pr$_{160}^{\text{Gag-Pol}}$ (48)). It is also worth noting that initiation of reverse transcription is modulated by interactions of tRNA$^{\text{Lys}}$ with other elements in genomic RNA (i.e. A-rich loop and primer activation signal) (6, 49–52). Our results suggest that mature NC might facilitate the interaction of tRNA$^{\text{Lys}}$ with these elements in promotion of initiation of reverse transcription, whereas NC, in the context of Pr$_{55}^{\text{Gag}}$, might not be able to efficiently facilitate these interactions, as shown by our data. However, the assays employed in this study were not designed to address how these different proteins affect interactions of relevant RNA sequences with RT and tRNA$^{\text{Lys}}$, and this represents ongoing work. Indeed, more precise biochemical tRNA annealing assays are now being developed to better address this point.

In Rous sarcoma virus, a hyperaggregative Gag protein has been identified that does not package viral genomic RNA (53). This finding, together with our own work on Gag multimerization effects on reverse transcription, points to the possibility that attempts to force linkage of Gag molecules into aggregates could lead to inhibition of viral replication.

Acknowledgments—We thank Bruno Marchand and Matthias Götte for helpful discussions. We are also grateful to Aldo and Diane Bensadoun for support of our research program.

REFERENCES
1. Wain-Hobson, S., Sonigo, P., Danos, O., Cole, S., and Alizon, M. (1985) Cell 40, 9–17
2. Kleiman, L., Caudry, S., Boulerise, F., Wainberg, M. A., and Parniak, M. A. (1991) Biochem. Biophys. Res. Commun. 174, 1272–1280
3. Litvak, S., Sarith-Cottin, L., Fournier, M., Andreola, M., and Tarrago-Litvak, L. (1994) Trends Biochem. Sci. 19, 114–116
4. Marquet, R., Isel, C., Ehresmann, C., and Ehresmann, B. (1995) Biochimie (Paris) 77, 113–124
5. Kleiman, L. (2002) IUBMB Life 53, 107–114
6. Isel, C., Ehresmann, C., Keith, G., Ehresmann, B., and Marquet, R. (1995) J. Mol. Biol. 247, 236–250
7. Isel, C., Keith, G., Ehresmann, B., Ehresmann, C., and Marquet, R. (1998) Nucleic Acids Res. 26, 1398–1404
8. Barat, C., Le Grice, S. F., and Darlix, J. L. (1991) Nucleic Acids Res. 19, 751–757
9. Isel, C., Marquet, R., Keith, G., Ehresmann, C., and Ehresmann, B. (1993) J. Biol. Chem. 268, 25689–25727
10. Sakaguchi, K., Zambrano, N., Baldwin, E. T., Shapiro, B. A., Erickson, J. W., Omichinski, J. G., Clore, G. M., Gronswenhorn, A. M., and Appella, E. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 5219–5223
11. Maki, A. H., Orozowski, A., Misra, A., Urbanaja, M. A., and Casas-Finet, J. R. (2001) Biochemistry 40, 1403–1412
12. Schindla, M. F., Paolletti, A. C., Hudson, B. S., and Borer, P. N. (2002) Biochemistry 41, 5276–5282
13. Amarsinghe, G. K., De Guzman, R. N., Turner, R. R., Chancellor, K. J., Wu, Z. R., and Summers, M. F. (2000) J. Mol. Biol. 301, 491–511
14. De Guzman, R. N., Wu, Z. R., Stalling, C. C., Papparolado, L., Borer, P. N., and Summers, M. F. (1998) Science 279, 384–388
15. Guo, J., Wu, T., Anderson, J., Kane, B. F., Johnson, D. G., Gerlich, R. J., Henderson, L. E., and Liwin, J. G. (2000) J. Virol. 74, 8890–8898
16. De Rocquigny, H., Gabus, C., Vincent, A., Fournie-Zaluski, M. C., Roques, B., and Darlix, J. L. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 6472–6476
17. Cen, S., Khoriadj, A., Gahor, J., Rong, L., Wainberg, M. A., and Kleiman, L. (1997) J. Virol. 71, 10796–10800
18. Huang, Y., Wang, J., Shalam, A., Li, Z., Khoriadj, A., Wainberg, M. A., and Kleiman, L. (1997) J. Virol. 71, 726–728
19. Crawford, S., and Goff, S. P. (1985) J. Virol. 53, 899–907
20. Stewart, L., Schats, G., and Vogt, V. M. (1990) J. Virol. 64, 5076–5092
21. Mely, Y., De Rocquigny, H., Sorinas-Jimeno, M., Keith, G., Roques, B. P., Marquet, R., and Gerard, D. (1995) J. Biol. Chem. 270, 1650–1656
22. Feng, Y. X., Campbell, S., Harvin, D., Ehresmann, B., Ehresmann, C., and Rein, A. (1999) J. Virol. 73, 4251–4256
23. Provitera, P., Goff, A., Harenberg, A., Bouman, F. C., and Scarlata, S. (2001) Biochemistry 40, 5565–5572
