Mutating a Region of HIV-1 Reverse Transcriptase Implicated in tRNA\textsuperscript{Lys-3} Binding and the Consequences for (−)-Strand DNA Synthesis*

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Recently, tRNA\textsuperscript{Lys-3} was cross-linked via its anticodon loop to human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) between residues 230 and 357 (Mishima, Y., and Steitz, J. A. (1995) EMBO J. 14, 2679–2687). Scanning the surface of this region identified three basic amino acids Lys\textsuperscript{249}, Arg\textsuperscript{297}, and Lys\textsuperscript{311} flanking a small crevice on the p66 thumb subdomain outside the primer-template binding cleft. To assess an interaction of this region with the tRNA anticodon loop, these p66 residues were altered to Glu or Gln. p66 subunits containing K249Q, K311Q, K311E, and a dual R307E/p66 mutation formed a stable dimer with wild type p51. All mutants showed reduced affinity for tRNA\textsuperscript{Lys-3} and supported significantly less (−)-strand DNA synthesis from this primer than the parental heterodimer. In contrast, these variants efficiently synthesized HIV-1 (−)-strand strong-stop DNA from oligonucleotide primers and had minimal effect on RNase H activity, retaining endonucleolytic and directed cleavage of an RNA/DNA hybrid. Structural features of binary RT-tRNA\textsuperscript{Lys-3} complexes were examined by in situ footprinting, via susceptibility to 1,10-phenanthroline-copper-mediated cleavage. Unlike wild type RT, mutants p66\textsuperscript{K311Q}/p51 and p66\textsuperscript{K311Q}/p51 failed to protect the tRNA anticodon domain from chemical cleavage, indicating a significant structural alteration in the binary RT-tRNA complex. These results suggest a crevice in the p66 thumb subdomain of HIV-1 RT supports an interaction with the tRNA\textsuperscript{Lys-3} anticodon loop critical for efficient (−)-strand DNA synthesis.

Initiation of reverse transcription is a complex step in the retroviral life cycle. With human immunodeficiency virus type 1 (HIV-1), several specific events, occurring prior to virus entry, precede processive synthesis of (−)-strand DNA. The first of these is preferential packaging of tRNA\textsuperscript{Lys-3} isoacceptors and a dimer of the RNA genome into the assembling virion by gag-pol and gag precursors (p160\textsuperscript{gag-pol} and p55\textsuperscript{gag}, respectively) (2–4). tRNA\textsuperscript{Lys-3} is selectively placed on the viral RNA, serving as primer for (−)-strand DNA synthesis, by hybridization of 18 nt at its 3′ terminus to the primer binding site (PBS), a complementary sequence immediately adjacent to the unique 5′ region (U5) (5–9). Beyond this interaction, PBS-bound tRNA\textsuperscript{Lys-3} has been shown in vitro to interact with additional sequences of the viral RNA genome, most notably an -AAAA-sequence in the U5-inverted repeat (IR) loop that contacts the U-rich antizyme loop (10, 11). These events are most likely established in an immature virus particle, prior to protease-mediated maturation of p55\textsuperscript{gag} and p160\textsuperscript{gag-pol}. However, mature HIV-1 proteins are required for initiation of (−)-strand DNA synthesis from tRNA\textsuperscript{Lys-3} (12, 13). In vitro, initiation of (−)-strand synthesis involves addition of up to 6 deoxynucleotides to the tRNA\textsuperscript{Lys-3} 3′ terminus (14–16), after which such products are elongated to full-length (−)-strand strong-stop DNA containing the U5 and repeat (R) sequences of the genome. Recently, it was shown that although the initiation event, i.e. addition of two deoxynucleotides to tRNA\textsuperscript{Lys-3}, can be accomplished in the mature virus (13, 17), the level of (−)-strand strong-stop DNA in HIV-1 was ~100-fold less than the amount of tRNA\textsuperscript{Lys-3} alone or tRNA\textsuperscript{Lys-3} extended by 2 nt, suggesting further elongation of (−)-strand DNA in the virion is a rare event (12, 13).

HIV-1 proviral DNA synthesis is accomplished by reverse transcriptase (RT), a multifunctional, heterodimeric enzyme (p66/p51) containing a catalytically competent 66-kDa subunit and an inactive 51-kDa subunit (Fig. 1A). Although all retroviral RTs perform comparable functions, many complex steps, such as initiation of (−)-strand synthesis, require virus-specific substrates and catalysts (17, 18). For example, we recently observed that of several retroviral enzymes, only HIV-1 and avian myeloblastosis virus RT efficiently initiated (−)-strand DNA synthesis from unmodified or natural tRNA\textsuperscript{Lys-3} primers annealed to the HIV-1 genome (18). This finding was unexpected, since the RTs of HIV-2, simian immunodeficiency virus, feline immunodeficiency virus, and equine infectious anemia virus used in our study also exploit tRNA\textsuperscript{Lys-3} as primer for initiation of reverse transcription (19). However, disrupting the U5-IR loop-tRNA\textsuperscript{Lys-3} anticodon loop complex in the HIV-1 template-primer substrate restored the capacity for (−)-strand DNA synthesis to the heterologous enzymes (18). Interaction of this A-rich sequence 5′ to the PBS with the tRNA primer appears unique to HIV-1, since no barrier to tRNA-derived (−)-strand DNA synthesis by heterologous RTs from PBS-containing RNA fragments of the equine infectious anemia virus or feline immunodeficiency virus genome was observed (18).
Reverse Transcriptase-tRNA Interactions in HIV

We and others (14–16, 18) have demonstrated that wild type HIV-1 RT is required to disrupt the U5-IR stem and U5-IR loop-anticodon loop complex by extending tRNA\(^{\text{A\text{-}g\text{-}3}}\) by 1–6 deoxynucleotides, after which processive synthesis from the extended tRNA yields full-length (–) strand strong-stop DNA. The first HIV-1 RT mutant displaying a defect in this initiation program contained a 13-amino acid deletion at the C terminus of p51 while retaining a full-length p66 subunit. This mutant, p66/p51A13 RT, exhibits reduced affinity for the free replication primer in a binary complex and an inability to catalyze (–) strand DNA synthesis from PBS-bound tRNA while accomplishing the same event from a PBS-bound RNA primer (20, 21). In addition, the 13-residue p51 truncation indirectly influences the architecture of the p66 RNase H domain, evidenced by a dysfunctional RNase H activity, which surprisingly could be restored by addition of HIV-1 nucleocapsid protein (21, 22). For the present study, our goal was to identify amino acids in p66 HIV-1 RT that might be specifically implicated in the interaction with tRNA\(^{\text{A\text{-}g\text{-}3}}\) and subsequent initiation of (–) strand DNA synthesis without compromising any other enzymatic function.

Although HIV-1 RT has been shown to interact with tRNA\(^{\text{A\text{-}g\text{-}3}}\) at all three loops (23–26), its affinity for most tRNA isoacceptors is similar (27, 28), suggesting structure is the primary determinant of this bimolecular interaction rather than sequence. Recently, a 127-amino acid cyagenon bromide (CNBr) cleavage fragment of p66 HIV-1 RT (residues 230–357 and depicted in white in Fig. 1A) was cross-linked to synthetic tRNA\(^{\text{A\text{-}g\text{-}3}}\) thyliolate at T\(^9\) of the anticodon loop (1). By analyzing this peptide fragment in the context of the three-dimensional structural HIV-1 RT (29, 30), we tentatively identified three basic residues (Lys\(^{249}\), Arg\(^{307}\), and Lys\(^{311}\)) conserved in several HIV-1 isolates (31) and adjacent to a small hydrophobic crevice in the thumb subdomain of p66 (Fig. 1A) as targets for mutagenesis. In contrast to wild type RT, these mutants showed greatly reduced affinity for tRNA\(^{\text{A\text{-}g\text{-}3}}\) and failed to protect regions in the anticodon stem-loop from chemical cleavage, the latter of which was evaluated via a novel in situ footprinting approach. Although mutant enzymes retained the capacity to initiate (–) strand DNA synthesis from PBS-bound tRNA\(^{\text{A\text{-}g\text{-}3}}\), subsequent elongation events were severely reduced. General defects to polymerase function were ruled out by the observation that the same mutants retained their ability to (i) synthesize (–) strand DNA from PBS-bound RNA and DNA primers and (ii) catalyze synthesis-dependent and -independent modes of RNase H activity. Collectively, our findings suggest that mutating Lys\(^{249}\), Arg\(^{307}\), or Lys\(^{311}\) of the thumb subdomain of the catalytically competent p66 subunit induces structural alterations in p66/p51 HIV-1 RT which disrupt interactions with its cognate tRNA replication primer.

**EXPERIMENTAL PROCEDURES**

**Mutagenesis and Purification of Recombinant HIV-1 RT**—p66 HIV-1 RT encoded by the vector pRT (20) was selectively substituted at amino acids Lys\(^{249}\) Arg\(^{307}\) Lys\(^{311}\) by glutamine (Gln), Glu, or Arg. Mutant RT DNA templates were linearized using the Klenow fragment of E. coli DNA polymerase on a 60-nt DNA duplex, and the region surrounding the PBS in HIV-1 genomic RNA (21). 3\(^{25}\)P-capped tRNA\(^{\text{A\text{-}g\text{-}3}}\) was purified as described (21). In situ footprinting employed natural and synthetic tRNA\(^{\text{A\text{-}g\text{-}3}}\)–ligated with [\(^{32}\)P]Pcp by RNA ligase. Nucleotide sequences were obtained from NEN Life Sciences Products and molecular biology reagents from Boehringer Mannheim. Finally, a 237-nt HIV-1 PBS-containing RNA template was prepared by in vitro transcription of HgoI-linearized pH1-1 PBS vector as described (21). This template lacks the diimer initiation sequence located between the PBS and gag initiation codon.

**Gel Mobility Shift Analyses and K\(_d\) Determination**—Wild type and mutant enzymes were incubated for 10 min at room temperature with 3′-end-labeled natural, \(^32\)P internally labeled synthetic tRNA\(^{\text{A\text{-}g\text{-}3}}\) or an end-labeled tRNA/PBS RNA duplex, in binding buffer (25 mM KCl, 50 mM Tris, pH 7.4, 10% glycerol, and 1 mM diithiothreitol), then fractionated into a 5% non-denaturing polyacrylamide gel. Following electrophoresis, gels were dried and visualized by autoradiography. The stability of RT bound to free tRNA\(^{\text{A\text{-}g\text{-}3}}\) or tRNA\(^{\text{A\text{-}g\text{-}3}}\)-template complexes was evaluated using a 4.1 ratio of RT (16 μM) to tRNA (4 μM, i.e., an RT concentration below the \(K\_d\) for wild type RT binding to free tRNA\(^{\text{A\text{-}g\text{-}3}}\). Higher ratios of RT:tRNA resulted in a complete shift of free tRNA\(^{\text{A\text{-}g\text{-}3}}\) by wild type RT, whereas lower ratios prevented an analysis of tRNA\(^{\text{A\text{-}g\text{-}3}}\) binding by mutant RTs (data not shown).

In order to determine dissociation constants, the concentration of synthetic tRNA\(^{\text{A\text{-}g\text{-}3}}\) in reactions remained constant at 0.1 μM, whereas the RT concentration was increased from 0.1 μM to 1.5 μM. The binary RT:tRNA complex was separated from free tRNA on a 5% non-denaturing polyacrylamide gel and the amount of shifted complex calculated from phosphorimaging analyses of dried gels and plotted against RT concentration. A hyperbolic curve was fitted to the plots, and dissociation constants were estimated from the RT concentration resulting in 50% shifting of synthetic tRNA\(^{\text{A\text{-}g\text{-}3}}\). Dissociation constants were determined as the average of three independent experiments.

**[\(^{32}\)P]DCTP-Labeled Footprinting of RT:tRNA\(^{\text{A\text{-}g\text{-}3}}\) Complexes in Situ**—Higher concentrations of RT (200 μM) and \(^32\)P end-labeled tRNA\(^{\text{A\text{-}g\text{-}3}}\) (33 μM) were necessary to increase the amount of the binary RT:tRNA complex resolved by non-denaturing polyacrylamide gel electrophoresis as described above. Gel slices containing these bands were excised, immersed in 100 μl of 50 mM Tris, pH 7.4, and then incubated with chemical reagents according to Kuwabara and Sigman (34).

Solutions of 40 mM 1,10-ortho-phenanthroline (OP), 28 mM 2,9-dimethyl-1,10-phenanthroline, both in 100% ethanol, and aqueous solutions of 9 mM CuSO\(_4\) and 58 mM 3-mercaptopropionic acid were immediately prepared prior to chemical footprinting. 40 mM OP and 9 mM CuSO\(_4\) were mixed at equal volumes and diluted 1:10 with water. 10 μl aliquots of this solution and 3-mercaptopropionic acid were added to the solution containing the gel slice, which was incubated for 5 min at room temperature. Chemical modification was quenched with 10 μl of 28 mM 2,9-dimethyl-1,10-phenanthroline.

Modified tRNA was eluted from gel slices overnight at 37 °C after adding 270 μl of gel elution buffer (0.5 mM ammonium acetate, 1 mM EDTA, 0.1% SDS). Cleaved tRNA was precipitated with ethanol, resuspended in loading buffer (50 mM Tris, pH 7.4, and then made up to 1.0 M with water. 10 μl aliquots of this solution and 3-mercaptopropionic acid were added to the solution containing the gel slice, which was incubated for 5 min at room temperature. Chemical modification was quenched with 10 μl of 28 mM 2,9-dimethyl-1,10-phenanthroline.

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**RNA-dependent DNA Polymerase Activity**—RNA-dependent DNA polymerase activity of wild type and mutant enzymes was measured using an HIV-1 PBS-containing RNA template to which a 5′ end-labeled 18-nt DNA or RNA primer or 77-nt chimera of natural tRNA\(^{\text{A\text{-}g\text{-}3}}\) containing radiolabeled dCTP at its 3′ terminus (21) was
model of HIV-1 RT (29), we tentatively identified 5 of 13 positively charged amino acids in this cross-linked fragment (Lys249, Lys259, Lys287, Arg307, and Lys311) which might lie on the surface of the p66 thumb subdomain. Of these Lys249, Arg307, and Lys311 appear clustered around a small crevice in the thumb opposite to the primer-template binding cleft (Fig. 1). Two proposed flaps, designated flap a and flap b in Fig. 1, have hydrophilic residues on the surface (marked by dotted line), whereas the crevice floor contains mainly hydrophobic residues.

A sequence alignment of HIV-1 isolates from four different clades (or subtypes), as well as isolates of HIV-2, simian immunodeficiency virus, feline immunodeficiency virus, and equine infectious anemia virus was undertaken (data not shown). Residues Lys249, Arg307, and Lys311 are conserved in 50 HIV-1 strains, comprising every HIV-1 subtype (a mutation frequency of <3.6 × 10⁻⁵ substitutions/nt for these three residues compared with 2.5 × 10⁻³ substitutions/nt for entire pol gene) (31). These three positively charged residues are not conserved in the coding regions of other lentiviral RTs. However, hydrophilicity in the flaps and hydrophobicity in the crevice appear to be maintained by semi-conservative substitutions in HIV-1, HIV-2, and simian immunodeficiency virus. Interestingly, HIV-2 and simian immunodeficiency virus RT had reduced affinity for tRNA⁻⁹⁻³ compared with HIV-1 RT and failed to disrupt the tRNA anticodon loop-HIV-1 RNA U₅₁R loop complex for productive (−)-strand DNA synthesis (18). From this analysis, we speculated that Lys249, Arg307, and Lys311 in HIV-1 RT might be responsible for accommodating the electronegative anticodon loop of tRNA⁻⁹⁻³ and important for initiation and/or elongation of (−)-strand DNA synthesis.

Lys249, Arg307, and Lys311 were substituted with Gln or Glu in a p66 RT expression vector by site-directed mutagenesis. The hydrophilic Glu and Gln residues were designed to remove the positive charge while maintaining the flap crevice structure determined for wild type enzyme (Fig. 1). Mutated p66 subunits were reconstituted with a polyhistidine-extended, wild type p51 subunit and purified by metal chelate chromatography, which also monitors dimerization efficiency (20, 36). Mutants p66K249Q/p51, p66K249E/p51, p66K249E/p51, and p66K311E/p51/p51 RT were stable heterodimers and reconstituted efficiently with a 1:1 subunit stoichiometry. In contrast, p66K249E/K311E/p51 RT failed to efficiently reconstitute with p51, suggesting the alterations may have affected protein stability and dimerization.

Affinity of HIV-1 RT Mutants for tRNA⁻⁹⁻³.—To determine the effects of the p66 point mutations on tRNA binding, we performed gel mobility shift analyses with the wild type enzyme and the above-mentioned variants. For comparison, the selectively deleted mutant p66/p51Δ13 was included. The results of our analysis are presented in Fig. 2. A complex characteristic of wild type HIV-1 RT bound to natural tRNA⁻⁹⁻³ (23, 37) is shown in Fig. 2A. At the RT:tRNA ratio which was necessary to visualize complexes containing mutant RTs, two retarded species (labeled a and b in Fig. 2A) were identified for the wild type enzyme, the slower-migrating of which (labeled a in Fig. 2A) most likely represents a dimer of the binary complex. Reduced stability of the binary complex was clearly evident with all mutants. With p66K249E/p51RT and, to a lesser extent, p66K249E/p51 RT and, to a lesser extent, p66K311E/p51 RT and, to a lesser extent, p66K311E/p51 RT, a third species (labeled c in Fig. 2A) migrating faster than the tRNA:RT complex was observed. The migration position of this complex correlates with a binary complex of p51 RT and tRNA⁻⁹⁻³ previously described by Richter-Cook et al. (37), suggesting

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2. P. Dumas and B. Ehresmann, unpublished observations.
these mutations might induce dissociation. Although retaining a stable heterodimeric organization, data of later sections indicate p66K249Q/p51 RT has substantially reduced DNA polymerase and RNase H activity, consistent with a more pronounced structural perturbation.

In order to extend the gel mobility shift data, synthetic tRNA<sup>Lys-3</sup> was used to determine dissociation constants (K<sub>d</sub>) for each enzyme-tRNA<sup>Lys-3</sup> complex, the results of which are presented in Fig. 2B. An increase in ionic strength (25–100 mM KCl) had little effect on the amount of shifted RNA wild type RT complex. In addition, a K<sub>d</sub> of 59 ± 11 nM, determined for both synthetic tRNA-3<sup>Lys-3</sup> at 25 mM KCl, was in agreement with the value of 40 nM obtained with wild type RT (Fig. 2B). In contrast, RT mutants p66K249Q/p51 and p66K311Q/p51, which display reduced affinity for tRNA<sup>Lys-3</sup>, provide a strikingly different OP-Cu hydrolysis profile. Rather than protect the anticodon loop these p66 alterations give rise to augmented OP-Cu cleavage throughout this domain (enclosed by the upper bars in Fig. 3, A and B). Since OP-Cu does not intercalate into double-stranded RNA due to a more compact minor groove, cleavage in the anticodon stem may reflect (i) increased binding of OP-Cu at the loop-stem interface, permitting short distance diffusion of the hydroxyl radical and cleavage of the bases closest to the loop (38) or (ii) disruption of base pairing within the anticodon stem accompanying the interaction of mutant RT with tRNA<sup>Lys-3</sup>. The latter is more probable considering OP-Cu cleavage in the 3' anticodon stem of natural tRNA<sup>Lys-3</sup> does not occur immediately adjacent to the anticodon loop. Mutants p66K311Q/p51 RT and p66K311E/p51 RT also give rise to enhanced cleavage within the TψC loop but only with synthetic tRNA<sup>Lys-3</sup>. Cleavage of the TψC loop was limited to the last two bases (G<sup>59</sup> and T<sup>60</sup>) of free tRNA<sup>Lys-3</sup> or tRNA<sup>Lys-3</sup> complexed to wild type RT suggesting either a D-loop-TψC loop interaction (Fig. 3C) or protection by wild type RT, respectively. Increased cleavage in the TψC loop of synthetic tRNA<sup>Lys-3</sup> complexed with p66K311Q/p51 RT or p66K311E/p51 RT may be the result of differential binding by these mutants at the anticodon loop. Augmented OP-Cu cleavage in the anticodon stem and loop of both tRNA species complexed with p66K311Q/p51 RT and p66K311E/p51 RT suggests the mutations influence a specific interaction of RT with this region. Due to weak binding, insufficient amounts of p66K311Q/p51 RT or p66K311E/p51 RT may be recovered for in situ footprinting of tRNA<sup>Lys-3</sup> complexed to wild type RT (Fig. 3A) although less pronounced with the natural tRNA<sup>Lys-3</sup> counterpart (Fig. 3B).

In situ footprinting of tRNA<sup>Lys-3</sup> complexed to wild type RT revealed weak protection around the anticodon loop. This protection was consistently found in repeated OP-Cu footprinting experiments and in previous UV cross-linking (23) and enzymatic footprinting studies (25). Despite reduced affinity of the p66K249Q/p51 RT for free tRNA, the pattern of tRNA protection with this mutant is unaltered from that observed with wild type RT (Fig. 2B). In contrast, RT mutants p66K311Q/p51 and p66K311E/p51, which display reduced affinity for tRNA<sup>Lys-3</sup>, suggest a low level RNase A contamination. Hydrolysis was only found in single-stranded RNA regions (Fig. 3B) although less pronounced structural perturbation.

1,10-Phenanthroline Copper Cleavage—To determine how mutations in the p66 thumb subdomain might affect an interaction with the replication primer, free tRNA<sup>Lys-3</sup> and binary RT-tRNA<sup>Lys-3</sup> complexes were excised from a non-denaturing polyacrylamide gel and probed in situ with 1,10-phenanthroline copper (OP-Cu) according to the procedure of Kuwabara and Sigman (34). Cleavage products were eluted from the gel slice and fractionated by high resolution denaturing polyacrylamide gel electrophoresis, the results of which are given in Fig. 3. OP-Cu preferentially intercalates and cleaves single-stranded RNA. Both the A conformation of double-stranded RNA and bulky ribonucleoside modifications (such as those in the anticodon domain of natural tRNA<sup>Lys-3</sup>) can prevent intercalation of OP-Cu (38). Thus, enhanced OP-Cu cleavage in both the anticodon and TψC loops was observed in some single-stranded regions of synthetic tRNA<sup>Lys-3</sup> (Fig. 3A) although less pronounced with the natural tRNA<sup>Lys-3</sup> counterpart (Fig. 3B).

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In Situ Footprinting Analysis of RT-tRNA<sup>Lys-3</sup> Complexes via
p51 RT showed reduced affinity for tRNA^Lys-3^, we compared their ability to synthesize (−)-strand DNA from oligonucleotide primers and natural tRNA^Lys-3^ annealed to an HIV-1 PBS-containing RNA genome. The substrates employed in this assay and the predicted (−)-strand strong-stop products are schematically represented in Fig. 4.

As described previously, wild type HIV-1 RT supported efficient synthesis of tRNA^Lys-3^-primed (−)-strand strong-stop DNA, whereas mutant p66/p51Δ13 was considerably less active (Fig. 5A and Ref. 18). The absence of full-length (−)-strand strong-stop DNA with p66/p51Δ13 RT in our previous report (21) may be accounted for by the shorter incubation time in these experiments, whereas in the present experiment prolonged incubation and a slightly higher RT:template-primer ratio allows limited extension of prematurely terminated products. Under the same conditions, p66K249Q/p51, p66K311Q/p51, and p66K311E/p51 RT support substantially less (−)-strand strong-stop DNA synthesis from a PBS-bound tRNA^Lys-3^. In support of the data of Fig. 5A, a similar effect could be demonstrated with in vitro synthesized tRNA (data not shown). In order to determine whether this decrease in (−)-strand DNA synthesis was related to reduced affinity of mutant enzymes for the tRNA^Lys-3^-HIV-1 PBS RNA complex, we performed a gel mobility shift analysis with a pre-annealed tRNA^Lys-3^-viral RNA duplex in the absence of DNA synthesis. In contrast to the differential binding observed with mutant RTs and free tRNA^Lys-3^, the data of Figs. 2 and 5B are not necessarily contradictory. Moreover, the strength of this "pre-initiation" complex is evidenced by our observation that bound enzyme could not be displaced with an excess of tRNA^Lys-3^ or viral RNA template (data not shown).

On closer examination of Fig. 5A, DNA synthesis products migrating in the immediate vicinity of the tRNA primer indicated that, despite a reduction in full-length (−)-strand strong-stop DNA, mutant enzymes retained their capacity to initiate DNA synthesis. High resolution analysis of each (−)-strand...
DNA synthesis reaction (Fig. 5C) indicated accumulation of prematurely terminated cDNAs corresponding to addition of 3–5 deoxynucleotides to the tRNA primer. The majority of these nascent cDNAs were chased into full-length (−)-strand strong-stop DNA over 80 min by wild type p66/p51 RT (Fig. 5, A and C). However, for mutant enzymes, initiation products predominated over full-length (−)-strand DNA (Fig. 5C). Percentages of initiation and elongation products to total (−)-strand DNA products were calculated from phosphorimaging analysis and are presented in Table I. With the exception of the p66R307E/K311E/p51 RT, similar total amounts of (−)-strand DNA products were catalyzed by each enzyme at each time point. Approximately 80% of (−)-strand DNA products catalyzed by p66K249Q/p51 RT represented initiation products, whereas only 22% of tRNA Lys-3 utilized by wild type RT to initiate (−)-strand DNA synthesis was found as T13-T15 initiation products after 80 min. A similar inability to extend initiation products was observed with p66K311Q/p51, p66K311E/p51, and p66/p51Δ13 RT. As might be expected, p66R307E/K311E/p51 RT initiated substantially less (−)-strand DNA synthesis from tRNA Lys-3, none of which could be extended to full-length (−)-strand strong-stop DNA. Since very few (−)-strand DNA intermediates are detected, the results depicted in Fig. 5 suggest that altering residues
Lys$^{249}$, Arg$^{307}$, and Lys$^{311}$ of p66 HIV-1 RT compromises the transition from initiation to elongation during (−)-strand DNA synthesis. However, mutations at these sites also lead to reduced and altered affinity for the tRNA primer (Figs. 2 and 3). Based on cross-linking studies of RT to the anticodon loop of tRNA$^{\text{Lys-3}}$ (1) and OP-Cu in situ footprinting of RT-tRNA$^{\text{Lys-3}}$ complexes presented here (Fig. 3), an altered interaction of HIV-1 RT with the anticodon loop of tRNA following disruption of the viral RNA-tRNA loop-loop complex may impair the transition from initiation to elongation during (−)-strand DNA synthesis.

(−)-Strand Strong-stop Synthesis from PBS-bound RNA and DNA Primers—Although the RT mutant p66/p51 is incapable of supporting tRNA$^{\text{Lys-3}}$-primed (−)-strand strong-stop DNA synthesis, it efficiently copies the same viral RNA template when this is substituted by RNA or DNA primers (18). Since important tRNA-viral RNA loop-loop interactions outside the PBS (10, 11, 15) cannot be established under the latter conditions, substitution of oligonucleotide primers removes the intermolecular structural barrier to initiation. We therefore elected to evaluate our mutants in the context of RNA- and DNA-primed (−)-strand strong stop DNA synthesis, the results of which are presented in Fig. 6.

Under these conditions, all enzymes supported (−)-strand strong-stop DNA synthesis from an 18-nt RNA primer annealed to the PBS (Fig. 6A). Surprisingly, this included p66K249Q/p51, which was shown to be severely impaired in tRNA$^{\text{Lys-3}}$-dependent events (Figs. 3 and 5). Decreased synthesis of (−)-strand strong-stop DNA by this mutant reflects reduced initiation from the tRNA primer and not a general defect to processive DNA synthesis, since enhanced accumulation of prematurely terminated cDNAs was not observed. In addition, heterodimer instability observed in the tRNA gel shift analyses with the p66K307E/K311E/p51 RT is not evident when examining RNA-dependent DNA polymerase activity. This mutant catalyzed nearly the same amount of (−)-strand DNA from a DNA primer as the other mutant or wild type RT. These studies suggest that binding to or initiation from tRNA$^{\text{Lys-3}}$ and not DNA polymerase activity may be associated with the instability of the p66K307E/K311E/p51 dimer.

The data of Fig. 6A thus suggests that the double mutation R$^{307}$E/K$^{311}$E in p66 induces defects manifesting themselves almost exclusively in tRNA-mediated events, indicating the conformation of the reconstituted heterodimer is not globally compromised. In support of the notion that the amino acids

### Table I

| Heterodimer derivative | Time of incubation | Initiation products/total (−)-strand DNA products | Elongation products/total (−)-strand DNA products | Total amount$^a$ of (−)-strand DNA
|------------------------|--------------------|--------------------------------------------------|--------------------------------------------------|----------------------------------|
| p66/p51 WT$^b$         | min                 | %                                               | × 10$^2$ cpm                                      |                                  |
| 10                     | 53                 | 47                                              | 6.6                                              |                                  |
| 80                     | 24                 | 76                                              | 11                                               |                                  |
| 10                     | 100                | 0                                               | 2.6                                              |                                  |
| 80                     | 79                 | 21                                              | 6.2                                              |                                  |
| 10                     | 82                 | 18                                              | 4.5                                              |                                  |
| 80                     | 55                 | 45                                              | 8.5                                              |                                  |
| 10                     | 79                 | 21                                              | 4.3                                              |                                  |
| 80                     | 56                 | 44                                              | 8.3                                              |                                  |
| 10                     | 77                 | 23                                              | 1.6                                              |                                  |
| 80                     | 88                 | 12                                              | 3.8                                              |                                  |
| 10                     | 83                 | 17                                              | 5.0                                              |                                  |
| 80                     | 53                 | 47                                              | 8.6                                              |                                  |

$^a$ Total amount of (−)-strand DNA products primed from tRNA$^{\text{Lys-3}}$ (Fig. 5) was measured by phosphorimaging analysis. These values represented a relative measure of (−)-strand DNA product.

$^b$ WT, wild type.

**Evaluation of RNase H Activity—**The multifunctional nature...
of HIV-1 RT allows mutations influencing DNA polymerase function to be evaluated in the context of RNase H activity. The importance of monitoring both activities was recently evidenced by an RT mutant containing a point mutation at position 232 of the DNA polymerase catalytic center (Y232A) which directed RNase H cleavage almost exclusively to template nucleotide 28 rather than 217 (36). In light of this, the RNase H activities of all mutants were evaluated on a defined heteropolymeric RNA-DNA hybrid that monitors the hydrolysis products of endonuclease (DNA synthesis-dependent) and directional processing (DNA synthesis-independent) events. Substrate for this analysis is outlined in Fig. 7A and the cleavage products derived from each enzyme in Fig. 7B.

In our assay, the primary hydrolysis product of a reaction containing wild type p66/p51 RT is 71 nt, defining endonucleolytic cleavage at template nucleotide −17 (according to Metzger et al. (41), template nucleotide −1 is defined as that hybridized to the 3′ terminus of the tRNA primer). With prolonged incubation, this is replaced by a 62-nt fragment, reflecting directional processing as far as template nucleotide −8. This hydrolysis pattern is qualitatively preserved in RT mutants p66K249Q/p51, p66K311Q/p51, and p66K311E/p51, with only minor differences in the rates of hydrolysis. Relative amounts of the 71- and 62-nt hydrolysis products with the p66R307E/K311E/p51 RT at 30 min (lane 4, Fig. 7B) was similar to that observed with the wild type RT at an earlier time (10 s; lane 2, Fig. 7B) indicating a decrease in rate but not a change in specificity of RNase H hydrolysis. In contrast, mutant p66/p51D13 completely lacks directional processing activity. Retention of RNase H function but reduction in the rate of hydrolysis supports the contention that the R307E/K311E mutation influences primarily position of the tRNA replication primer for extension of its 3′ terminus at the DNA polymerase catalytic center.

**DISCUSSION**

Initiation of HIV-1 reverse transcription is a multi-step process, involving intricate interactions between the retroviral polymerase and its cognate tRNA primer, intertwined with the HIV-1 RNA genome at sites including and surrounding the PBS. The anticodon loop of tRNA binds to RT in a binary nucleoprotein complex (1, 23). Moreover, the anticodon loop has the capacity for intermolecular base pairing with the A-rich US-IR loop of HIV-1 RNA to provide an additional level of control/selectivity (10, 11, 15, 16, 18, 21). Although this A-rich sequence is not
phylogenetically conserved in non-primate lentiviruses or other retroviruses (18, 42), it does appear that this element is important in HIV-1 reverse transcription and replication (43). For example, a mutant virus with a deletion of these four adenosines supported substantially less (--)-strand DNA synthesis (43). Long term cultures of this mutant HIV-1 resulted in the replacement of two or three adenosines and the restoration of near wild type levels of (--)-strand DNA synthesis and virus replication (43). The significance of the tRNA anticodon loop during (--)-strand DNA synthesis is quite evident in studies showing that the use of a tRNA His isoacceptor as a replication primer on a mutated HIV-1 RNA genome (i.e. a PBS complementary to the 3′ end of tRNA His) was greatly facilitated by substitutions of U5-IR loop bases for those complementary to the tRNA His anticodon loop (44).

In this study, we have combined cross-linking (1) and crystallographic data of the p66/p51 heterodimer (29, 30, 45) to identify p66 residues potentially involved in tRNA Lys-3 binding events, beyond the duplex formed with the PBS, which promote efficient initiation of (--)-strand synthesis. Our studies have focused on three residues conserved in a large number of HIV-1 isolates, Lys249, Arg307, and Lys311, which appear to line a crevice of the p66 thumb subdomain (residues 244–322). We show here that substitutions at these positions affect the following: (a) the affinity of RT for tRNA; (b) the manner in which the tRNA primer is accommodated in a binary complex with RT; and (c) the transition from initiation to elongation of tRNA-primed (--)-strand synthesis. The same mutations have minimal impact on either oligonucleotide-primed (--)-strand synthesis or RNase H activity, suggesting their effects are localized to the manner in which tRNA Lys-3 is utilized by HIV-1 RT.

From cross-linking and biochemical studies on HIV-1 RT-tRNA Lys-3 interactions (23–26, 35), we had predicted that these mutations in the p66 component of p66/p51 RT might alter the conformation of binary RT-tRNA complexes. Indeed, enhanced reactivity over the entire anticodon domain was highlighted with two mutants via in situ footprinting with the chemical nuclease OP-Cu. Based on the reactivity of OP-Cu toward single-stranded RNA, including bulges and loops (46), the data of Fig. 3 suggest that much of the anticodon domain has been unwound following substitution of p66 residue Lys311 with either Glu or Gln. This effect is clearly evident with synthetic tRNA Lys-3 and to a lesser extent with the fully modified natural species (which may be attributable to the presence of hyper-modified bases in the latter) (38). In addition to altered protection of the anticodon domain, the TΨC loop of synthetic tRNA is rendered hypersensitive when bound to p66/p51 or p66/p51. The tRNA structure depicted in Fig. 3C indicates intramolecular pairing between bases of the TΨC and D-loops, which also appears to be disrupted following substitution of Lys311. Similarities in the OP-Cu hydrolysis profiles of mutant tRNA Lys-3 and wild type RT complexed with tRNA Lys-3 suggest this residue may not play the same role in tRNA binding, although a contribution is suggested by its 6-fold reduction in affinity for free tRNA (Fig. 2B). The inability of mutant RTs to extend PBS-bound tRNA Lys-3 into full-length (--)-strand DNA (Fig. 5A) appears unrelated to the initiation event, since T = 3 + T − 5 products accumulate in all cases (Fig. 5C). Moreover, the observation that (i) the affinity of mutant enzymes for a tRNA Lys-3-viral RNA duplex (Fig. 5D) and (ii) DNA- and RNA-directed (--)-strand synthesis (Fig. 6B) are largely unaltered, an unfavorable interaction of the tRNA anticodon domain with our RT mutants during or immediately following initiation might account for the reduced efficiency with which this primer is utilized.

A consequence of pre-annealing tRNA Lys-3 to the viral genome will be the establishment of intermolecular loop-loop interactions (10, 11, 15, 16, 18, 21) prior to RT binding. Under such conditions, viral template sequences ahead of the DNA polymerase catalytic center, together with the 18-base pair PBS/tRNA duplex, will become major determinants of the strength of the preinitiation nucleoprotein complex (evidenced by the gel mobility shift data of Fig. 5B). Thus, reduced affinity of RT for free tRNA appears unlikely to account for the inability of our mutants to overcome the tRNA-directed transition from initiation to elongation. An alternative explanation for loss of tRNA-primed synthesis would be that mutant enzymes, having initiated (--)-strand synthesis, cannot disrupt the U5-IR loop-tRNA anticodon loop interaction, with the consequence of a stalled RT. Such a general defect also seems unlikely, considering the same mutants efficiently extend a PBS-bound RNA primer through several highly structured regions of the viral genome (Ref. 47 and Fig. 6A). In light of this, an explanation for our findings might lie in the position the polymerase domain occupies on the template as the tRNA primer is extended at the DNA polymerase catalytic center.

Independent lines of evidence indicate that the p66 N-terminal fingers subdomain (residues 1–84 and 120–150) encompasses the single-stranded template overhang approximately 5–7 bases ahead of the polymerase catalytic center (48, 49). In the context of the tRNA-viral RNA structure proposed by Isel and co-workers (10, 11, 15), extending tRNA Lys-3 by three deoxyribonucleotides would translocate the fingers subdomain such that it is positioned directly over the intermolecular U5-IR loop-tRNA anticodon loop duplex. Should the fingers participate in destabilizing (“unwinding”) inter- and intramolecular template duplexes for presentation at the polymerase catalytic center, adding 3–5 dNTPs to tRNA Lys-3 3′ terminus would be accompanied by disruption of this intermolecular duplex. Once the tRNA anticodon loop is released from viral RNA within the p66 fingers, it may be involved in an unfavorable interaction with an alternate subdomain of the mutated RT (as depicted in Fig. 3), thereby inducing stalling. Such an inhibition would suggest the need for a specific interaction between wild type RT and the tRNA Lys-3 anticodon loop during initiation. Alternatively, once released from the A-rich loop of HIV-1 RNA, these mutants fail to fulfill a second interaction with the anticodon domain necessary for allosteric activation of the replication complex. While speculative, preliminary data suggest that tRNA-primed synthesis by our mutants is inhibited on a viral template lacking the U5-IR loop -AAAA- sequence, which prevents intermolecular loop-loop interactions from being established and again leaves the tRNA anticodon domain available to freely interact with RT.3 Moreover, an additional site for interaction of the tRNA anticodon loop is suggested by recent studies on the strand transfer complex (50). These hypotheses could be evaluated experimentally by determining the location of the tRNA anticodon loop in “pseudo-initiation complexes” where a chimera of tRNA containing the first 3–5 deoxyribonucleotides of (--)-strand DNA is hybridized to the viral template (18, 21). Experiments of this nature are presently underway.

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