HIV-1 Primer Binding Site:Lysyl-tRNA Synthetase Interaction Affinity Diminishes Upon tRNA Primer Annealing and Extension

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ABSTRACT

The retrovirus, human immunodeficiency virus type 1 (HIV-1), possesses a positive sense RNA genome (gRNA) that is reverse transcribed into proviral DNA upon infection. In order for reverse transcription to occur, HIV-1 co-opts cellular $tRNA^{Lys3}$, whose 3′ 18 nucleotides are perfectly complimentary to a region with the gRNA, to serve as the primer. $tRNA^{Lys3}$ is selectively packaged into virions through its interaction with the cellular enzyme lysyl-tRNA synthetase (LysRS), which in turn interacts with the viral protein responsible for orchestrating virus assembly, Gag. However, the mechanism of $tRNA^{Lys3}$ transfer from the packaged LysRS to the primer-binding site (PBS) remains incompletely understood. The PBS is harbored in the 5′ untranslated region (5′UTR) of the gRNA, a highly conserved segment of the HIV-1 genome. We have recently found that a U-rich stem loop immediately upstream of the PBS mimics the anticodon loop nucleotides of $tRNA^{Lys3}$, a critical LysRS recognition element. This tRNA-like element (TLE) specifically binds to LysRS, and can competitively displace $tRNA^{Lys3}$ from the synthetase. Furthermore, small angle X-ray scattering analysis revealed that the whole PBS domain (PBS_{105}) mimics the overall 3D shape of tRNA. Overall, these data suggest

a mechanism where structural and functional tRNA mimicry by the TLE in the PBS domain facilitate primer release from LysRS and targeting to the 18 nucleotide PBS.

An observation from the structural analysis was that both apoPBS and PBS annealed to a DNA oligonucleotide corresponding to the 18 complementary nucleotides in $tRNA^{Lys3}$ ($antiPBS_{18}$) mimicked the overall tRNA shape to a similar degree. In order to further investigate the function of the PBS/TLE domain, we performed a fluorescence anisotropy-based binding study examining LysRS interactions with the PBS domain in various functionally relevant states. We find LysRS has similar affinities for both the $apoPBS_{105}$ and PBS_{105}:$antiPBS_{18}$ complex, confirming the SAXS structure indicating both complexes mimic tRNA shape. In order to investigate if the additional tRNA-gRNA contacts outside of the 18 nucleotides of complementarity affected the LysRS interaction, we tested PBS_{105}:primer complexes containing full-length and 3′-half tRNAs, finding that PBS:tRNA primer complexes displayed reduced affinities for LysRS under certain conditions. Also, when progressively extended $antiPBS_{18}$ primers were annealed to PBS_{105}, mimicking the initial steps of reverse transcription, we observed a concomitant drop in LysRS affinity. These data further elucidate the role that LysRS plays in the evolution of the reverse transcription initiation complex.

INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1) possesses a positive-sense RNA genome (gRNA) that is reverse transcribed into cDNA upon infection of susceptible lymphocytes[1]. This process is carried out by HIV-1 reverse transcriptase which, in order to initiate polymerization, requires a primer. In the case of HIV-1, cellular $tRNA^{Lys3}$ is co-opted by the virus to serve in this regard[2]. Interestingly, the primer $tRNA^{Lys3}$ is not recruited in the actively infected cell, and rather originates from the virus progenitor cell. While only $tRNA^{Lys3}$ is able to serve as the primer for reverse transcription, all three $tRNA^{Lys}$ isoacceptors are incorporated into virions at levels significantly enriched over those found in the cytoplasm[3,4].

The canonical role for $tRNA^{Lys}$ is in protein synthesis, where a $tRNA^{Lys}$ molecule covalently linked to the amino acid lysine is used by the ribosome to decode lysine codons in mRNA transcripts[5]. Correct aminoacylation of $tRNA^{Lys}$ is carried out by lysyl-tRNA synthetase (LysRS), and LysRS is the only cellular factor known to bind all three $tRNA^{Lys}$ isoacceptors at equivalent levels[4]. It was demonstrated that LysRS is selectively packaged into virions, and its upregulation and downregulation correlate positively and negatively with virus viability, respectively. Furthermore, LysRS downregulation leads to diminished packaging of $tRNA^{Lys}$. LysRS has been shown to interact specifically with the capsid domain of the viral protein Gag, both *in vitro* and *in vivo*[4]; Gag is the primary driver of virion formation, and its expression alone is sufficient to make virus like particles[6]. While Gag alone is sufficient to incorporate LysRS into virions, both Gag and GagPol are required for tRNA packaging[4]. Taken together, these data support a model whereby LysRS is responsible for mediating the interaction between the viral proteins

principally responsible for assembly (i.e. Gag and GagPol) and primer $tRNA^{Lys3}$, ensuring its selective incorporation.

The primer binding site (PBS) that $tRNA^{Lys3}$ anneals to in order to initiate revere transcription is found in the 5′ untranslated region (5′UTR) of the gRNA (Figure 1). It is still not known precisely at what point during the viral life cycle $tRNA^{Lys3}$ associates with the PBS. Another poorly understood aspect of this mechanism is the means by which LysRS:$tRNA^{Lys3}$ correctly localize to the PBS and transfer tRNA from LysRS to the PBS, as well as what happens to LysRS once this transfer is complete. Recently, we have shown that a tRNA-like element (TLE) immediately upstream of the PBS shares a similar sequence to the anticodon of $tRNA^{Lys3}$, and is capable of competitively displacing tRNA from LysRS[7]. We also showed that the whole PBS domain mimics the overall 3D shape of a tRNA[8]. Interestingly, even upon annealing of a DNA oligonucleotide corresponding to the 18 complementary nucleotides in $tRNA^{Lys3}$ (antiPBS_{18}), the PBS domain maintained its tRNA-like structure. The implications of this result on the presence/absence of LysRS in the PBS:primer complex remain unknown.

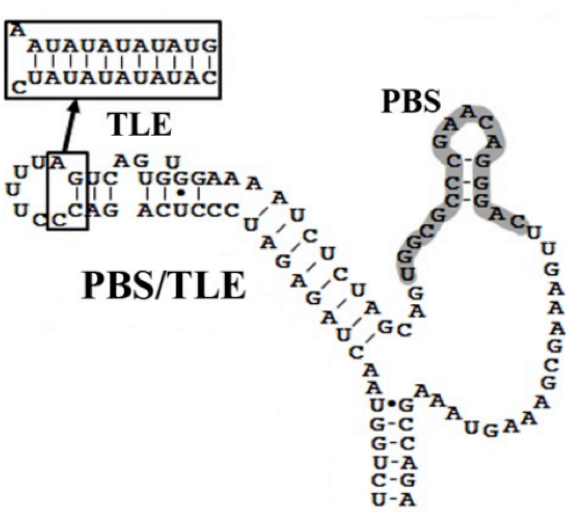

Figure 1: Predicted secondary structure of the 105-nucleotide PBS domain (PBS_{105}). The actual 18-nucleotide site of tRNA primer annealing is indicated in grey, and the location of the 10 base pair extension of the TLE stem loop (PBS_{105}-TLE extension) is indicated in the inset.

In the present work, we use a fluorescence anisotropy-based binding assay to further investigate the interaction of LysRS with the PBS domain in various functionally relevant

states. We find that, consistent with the structural observations, there is no significant difference in the affinity of LysRS for apo vs. antiPBS$_{18}$ primer annealed PBS domains. However, when we use tRNA primers or primers meant to mimic the initial steps of reverse transcription, we observe a drop in LysRS affinity for the complex. The implications of this finding regarding the presence of LysRS in the initiation complex are discussed.

MATERIALS AND METHODS

Preparation of proteins and nucleic acids

Human LysRS[9] was prepared as previously described[7]. Briefly, LysRS contains a His$_6$ tag and was purified using Ni^{2+} or Co^{2+} resins. Protein concentration was determined using a Bradford assay (Bio-Rad).

All DNA constructs used in this study (antiPBS$_{18}$, antiPBS$_{18}$+3, antiPBS$_{18}$+6, and antiPBS$_{18}$+11) were purchased from IDT (Coralville, IA), and used without further purification. All RNAs used—PBS$_{105}$, PBS$_{105}$-TLE extension, tRNA$^{Lys3}_{44}$, and tRNA$^{Lys3}_{76}$—were prepared by *in vitro* transcription using T7 RNA polymerase and purified using denaturing (8 M urea) polyacrylamide gel electrophoresis. The genes encoding these RNAs have previously been described[7], and all RNAs, except tRNA$^{Lys3}_{44}$, were transcribed from FokI-digested plasmid that generated the appropriately sized DNA template. In the case of tRNA$^{Lys3}_{44}$, primers containing the T7 promoter were used to amplify the desired DNA sequence from the tRNA$^{Lys3}_{76}$ gene, and then used as template for *in vitro* transcription. Prior to any experiment, the RNA or RNA:RNA/DNA complex

was folded by heating to 80˚C for 2 min, 60˚C for 4 min, adding $MgCl_2$ to 10 mM, incubating at 37˚C for 6 min, and cooling on ice for at least 30 min.

Fluorescently labeled RNAs were generated essentially as previously described[7,10], except that 1.5-2.5 nmol RNA was treated with 20-fold excess sodium periodate, and 5 mM fluorescein-5-semithiocarbazide was used. Labeling efficiencies were typically greater than 80%.

Native gel shift annealing assays

Fluorescently labeled PBS_{105} RNA (568 nM) was folded as described above in the presence of varying amounts of $antiPBS_{18}$, $tRNA^{Lys3}{}_{44}$, or $tRNA^{Lys3}{}_{76}$, (relative stoichiometries in Figure 2 legend). This was then diluted to final solution conditions closely approximating those of the anisotropy binding assays (20 mM Tris-HCl pH 8, 10 mM $MgCl_2$, 35 mM KCl, 15 mM NaCl, and 10 mM HEPES pH 7.5) plus native loading dye (50% glycerol, xylene cyanol, and bromophenol blue), and resolved on a 12% native polyacrylamide gel. Gels were then imaged on a Typhoon Trio phosphorimager (GE Healthcare) using fluorescein dye specific excitation and emission wavelengths as described[7]. Band intensities were quantified using ImageJ software.

Fluorescence anisotropy equilibrium binding assays

Fluorescently labeled PBS_{105} or PBS_{105}-TLE extension RNA was refolded as described above in the absence or presence of other nucleic acids (at varying stoichiometric ratios, indicated in results section). Reactions were then diluted to final conditions (20 mM Tris-HCl pH 8, 10 mM $MgCl_2$ **or** 1 mM $MgCl_2$, 35 mM KCl, 15 mM NaCl, 5 mM HEPES pH

7.5, 30 nM RNA or RNA:RNA/DNA complex, and various protein concentrations), and incubated at room temperature for at least 30 min. Resulting fluorescence intensity and anisotropy was read in 384-well plates using a SpectraMax M5 plate reader (Molecular Devices) by exciting at 485 nm, and measuring emission at 525 nm with a emission cutoff of 515 nm. For each protein titration point, three (20 μL) aliquots were read to control for device measurement variation, and each titration itself was repeated in at least three independent trials. Binding curves were fit to a 1:1 binding model as previously described to obtain the apparent equilibrium dissociation constant (K_d) for the interaction[11].

RESULTS

Mammalian LysRS proteins contain a 65 amino acid N-terminal domain that has been shown to contribute non-specifically to RNA binding. Given that mammalian LysRS is more sensitive to differences in specific RNA interactions without this domain (LysRSΔN65)[12], and that we have previously shown that both human LysRS-wt and human LysRSΔN65 bind to PBS/TLE RNA[7], we elected to carry out all further studies using the human LysRSΔN65 construct (which will henceforth simply be referred to as LysRS).

We used a fluorescence anisotropy assay to measure the binding affinity of LysRS to the various nucleic acids and nucleic acid complexes examined in this work. We began by testing LysRS affinity for the apo form of the PBS domain (apoPBS_{105}), and observed an apparent K_d of 423 nM at 1 mM [$MgCl_2$] (plus other solution conditions listed in materials and methods section). We wanted to see how increasing the stringency of the

salt conditions would affect the strength of the observed K_d, so we performed the same experiment except that we increased [$MgCl_2$] to 10 mM. Under these conditions we observed approximately two-fold increase in K_d up to 866 nM. Moving forward we tested most interactions both at 1 mM and 10 mM [$MgCl_2$] conditions (results of all binding data are summarized in Table 1).

Table 1: Apparent equilibrium dissociation constants (K_d) for human LyRSΔN65 binding to RNAs or RNA complexes.

	LysRSΔN65	
RNA	**K_d, nM (1 mM $MgCl_2$)**	**K_d, nM (10 mM $MgCl_2$)**
apoPBS_{105}	423 ± 108	866 ± 57
PBS_{105}-TLE extension	593 ± 84	1450 ± 241
PBS_{105}:antiPBS_{18} (1:1.5)[a]	ND	787 ± 143
PBS_{105}:antiPBS_{18}	374 ± 24	760 ± 260
PBS_{105}:$tRNA^{Lys3}_{44}$	961 ± 81	724 ± 71
PBS_{105}:$tRNA^{Lys3}_{76}$	643 ± 59	666 ± 178
PBS_{105}:antiPBS_{18}+3	402 ± 212	ND
PBS_{105}:antiPBS_{18}+6	704 ± 78	ND
PBS_{105}:antiPBS_{18}+11	1320 ± 223	ND

All measurements were performed with 30 nM RNA/RNA complex in 15 mM NaCl, 35 mM KCl, 20 mM Tris-HCl pH 8, and either 1 or 10 mM $MgCl_2$ (indicated above).
Results are the average of at least three trials ± the standard deviation.
[a]All RNA complexes were tested at 1:1 stoichiometric ratios, except this trial which was tested at 1:1.5 PBS:primer ratios.
ND = not determined

In order to test the importance of the tRNA-like shape of the PBS domain for binding to LysRS, independent of its anticodon sequence mimicry, we extended the TLE stem loop by 10 base pairs while keeping the anticodon loop the same (PBS_{105}-TLE extension, Figure 1), and determined LysRS affinity. We find that there is a negligible drop in affinity under the 1 mM [$MgCl_2$] conditions (423 nM for PBS_{105} to 593 nM for PBS_{105}-TLE extension), while there is a more significant two-fold decrease under the more stringent 10 mM [$MgCl_2$] conditions (866 nM for PBS_{105} to 1450 nM for PBS_{105}-TLE extension). This suggests that the overall tRNA-like shape of PBS is contributing, at least in part, to LysRS binding.

Given our previous structural observation that annealing of antiPBS_{18} to the PBS domain did not lead to a significant structural change in the TLE stem[8], we wanted to test if LysRS affinity for the complex would remain similarly unchanged. First, however, we

wanted to confirm that we were achieving complete complex formation under our assay conditions. Thus, we performed native polyacrylamide gel shift-annealing assays to determine the optimal stoichiometries at which to carry out the LysRS binding studies. We examined the formation of PBS_{105}:$antiPBS_{18}$, PBS_{105}:$tRNA^{Lys3}_{44}$, and PBS_{105}:$tRNA^{Lys3}_{76}$ complexes in these experiments, and found that at PBS:primer stoichiometric ratios of 1:1 there was ~90% homogenous complex formation and at 1:1.5 ratios there was ~100% homogenous complex formation (Figure 2). We suspected that using either the 1:1 or 1:1.5 PBS:primer conditions would yield similar results, so we tested PBS_{105}:$antiPBS_{18}$ at both ratios for their affinity towards LysRS under the 10 mM [$MgCl_2$] conditions, finding that they possess nearly indistinguishable K_d values (760 nM at 1:1 ratios and 787 nM at 1:1.5 ratios, Table 1). In order to alleviate any concern about the excess primer present in the 1:1.5 stoichiometric condition, we elected to carry out all further experiments at the 1:1 PBS:primer ratios. Importantly, we show that regardless of [$MgCl_2$], there is little difference between measured K_d's of $apoPBS_{105}$ and PBS_{105}:$antiPBS_{18}$ (Table 1), confirming the structural observation that the TLE stem loop and overall tRNA-like shape of the PBS domain remain unchanged upon $antiPBS_{18}$ primer annealing.

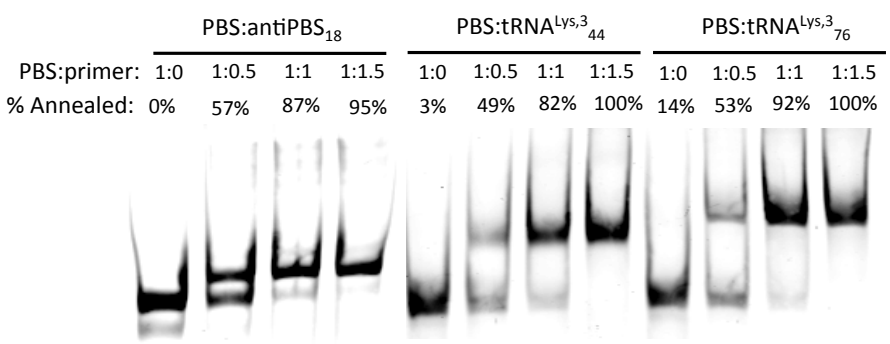

Figure 2: Native-PAGE analysis of various PBS:primer complexes, revealing homogenous complex formation and high amounts of annealed complex.

We next wanted to investigate if the annealing of primers more closely resembling the authentic $tRNA^{Lys3}$ primer would lead to differences in LysRS affinity. Indeed, additional

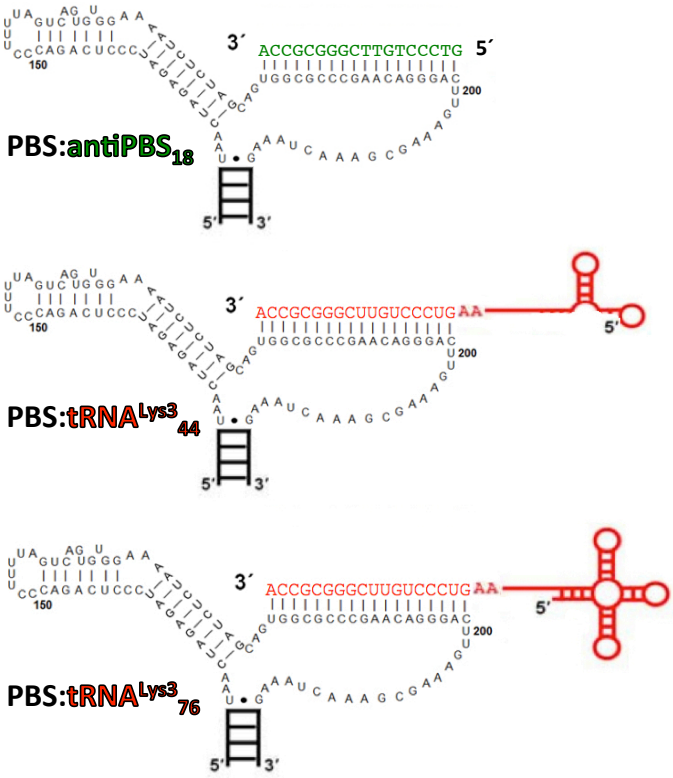

Figure 3: Figure illustrating the PBS domain in complex with the $antiPBS_{18}$, $tRNA^{Lys3}{}_{44}$, and $tRNA^{Lys3}{}_{76}$ primers. $tRNA^{Lys3}{}_{44}$ and $tRNA^{Lys3}{}_{76}$ are predicted to make additional contacts with the PBS domain not shown here.

contacts between the tRNA and viral RNA outside of the 18 nucleotides of complementarity have been proposed to occur[13,14]. Our $tRNA^{Lys3}{}_{44}$ construct is composed of the final 44 nucleotides on the 3′ half of $tRNA^{Lys3}$, and possesses all of the nucleotides theoretically required to make these additional interactions with the PBS domain (Figure 3). We began by testing PBS_{105}:$tRNA^{Lys3}{}_{44}$ complexes at 1:1 stoichiometric ratios for their affinity to LysRS. Our results show that under the 10 mM [$MgCl_2$] conditions there is little difference between no primer and $antiPBS_{18}$ or $tRNA^{Lys3}{}_{44}$ as the primer (866 nM, 787 nM, 760 nM, respectively), while at the 1 mM [$MgCl_2$] condition there is a ~3-fold drop in affinity (to 961 nM) when $tRNA^{Lys3}{}_{44}$ is used as the primer compared to the apo or $antiPBS_{18}$ primer-annealed states (423 nM and 374 nM, Figure 3 and Table 1). When

full-length $tRNA^{Lys3}_{76}$ is used as the primer, we similarly observe little change in K_d at 10

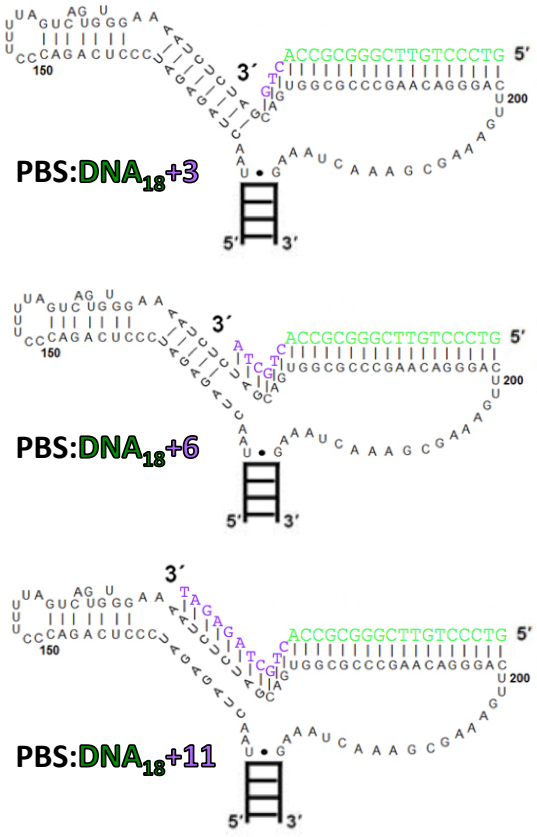

Figure 4: Figure illustrating the PBS domain in complex with the $antiPBS_{18}$+3, $antiPBS_{18}$+6, and $antiPBS_{18}$+11 primers. The additional base pairs that these DNAs make with the TLE stem are also indicated.

mM [$MgCl_2$] (666 nM), while under the 1 mM [$MgCl_2$] conditions LysRS binds PBS_{105}:$tRNA^{Lys3}_{76}$ more weakly by about 2-fold (643 nM) vs. apoPBS105 or PBS_{105}:$antiPBS_{18}$ (423 nM and 374 nM, Table 1). These results indicate that LysRS affinity for the PBS:primer complex does decrease upon primer annealing if a tRNA construct capable of making additional contacts with the PBS is used, but only under the less stringent solution conditions. This result is further discussed later on.

We next wanted to investigate how the early steps of reverse transcription would affect LysRS affinity for the PBS:primer complex. In order replicate this situation, we annealed a series of DNA primers containing the $antiPBS_{18}$ sequence plus varying numbers of additional nucleotides complimentary to the PBS sequence at the 3′ end of the primer (Figure 4). The set of primers extended progressively further into the TLE stem. These

experiments were only carried out under the 1 mM [$MgCl_2$] conditions. We found that PBS_{105}:antiPBS$_{18}$+3 bound LysRS with similar affinity as apoPBS_{105} or PBS_{105}:antiPBS$_{18}$ (all around ~400 nM, Table 1). However, PBS_{105}:antiPBS$_{18}$+6 bound LysRS more weakly (K_d = 704 nM), and PBS_{105}:antiPBS$_{18}$+11 even more weakly (K_d = 1320 nM). This result is consistent with the TLE being the main site of LysRS interaction, as it is *only* when TLE integrity is disrupted by the +6 and +11 primers that we observe a drop in LysRS affinity.

DISCUSSION

In this work we have shown that LysRS interacts with the PBS domain due at least in part to tRNA structural mimicry. Furthermore, we have found that the identity of the primer annealed to the PBS domain affects the strength of the LysRS interaction, and that those primers mimicking the early steps of reverse transcription lead to diminished LysRS binding. The fact that annealing of primers more closely resembling authentic $tRNA^{Lys3}$ lead to reduced LysRS affinity makes sense as once the tRNA primer is annealed, LysRS's presence would seem to be dispensable in the reverse transcription initiation complex. It is interesting to note that LysRS affinity only decreases under the less stringent $MgCl_2$ conditions. This could be caused by the higher [$MgCl_2$] leading to incomplete formation of the additional contacts made in the PBS:tRNA complex. Indeed, the formation of these additional contacts appears to be more difficult to make, and unmodified $tRNA^{Lys3}$ does not make them as efficiently[14]. One of the additional PBS-$tRNA^{Lys3}$ contacts occurs between the TLE stem loop of PBS and the variable loop/anticodon loop of tRNA[15]. Therefore, it is plausible that the reason that $tRNA^{Lys3}{}_{44}$

and $tRNA^{Lys3}_{76}$ only cause a decrease in LysRS affinity under the 1 mM $MgCl_2$ conditions is because it is only under these conditions that the additional PBS-tRNA contacts in the TLE region are made, thus disrupting the putative LysRS binding site. The importance of the TLE stem loop as the *bona fide* LysRS binding site is further supported by the evidence that only $antiPBS_{18}$+6 and +11 primers (i.e. the two primers that would disrupt TLE stem integrity) led to a decrease in LysRS affinity. This result also further raises the possibility that if LysRS does remain bound after primer annealing, it is likely remodeled out of the complex once reverse transcription initiates.

This study has helped to further our understanding of how and when human LysRS interacts with the HIV-1 PBS domain. However, further studies in cell culture will be required to confirm the precise timing of tRNA placement, and the viral life cycle stages in which LysRS is present in the HIV-1 reverse transcription initiation complex.

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