The structure of the human tRNA\textsuperscript{Lys3} anticodon bound to the HIV genome is stabilized by modified nucleosides and adjacent mismatch base pairs

Yann Bilbille\textsuperscript{1}, Franck A. P. Vendeix\textsuperscript{1}, Richard Guenther\textsuperscript{2}, Andrzej Malkiewicz\textsuperscript{3}, Xavier Ariza\textsuperscript{4}, Jaume Vilarrasa\textsuperscript{4} and Paul F. Agris\textsuperscript{1,*}

\textsuperscript{1}Department of Molecular and Structural Biochemistry, North Carolina State University, Raleigh, NC 27695-7622, \textsuperscript{2}Department Plant Pathology, North Carolina State University, Raleigh, NC 27695-7616, USA, \textsuperscript{3}Institute of Organic Chemistry, Technical University, \Lódz 90-924, Poland and \textsuperscript{4}Department of Organic Chemistry, Faculty of Chemistry, University of Barcelona, Barcelona 08028, Spain

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ABSTRACT

Replication of human immunodeficiency virus (HIV) requires base pairing of the reverse transcriptase primer, human tRNA\textsuperscript{Lys3}, to the viral RNA. Although the major complementary base pairing occurs between the HIV primer binding sequence (PBS) and the tRNA’s 3’-terminus, an important discriminatory, secondary contact occurs between the viral A-rich Loop I, 5’-adjacent to the PBS, and the modified, U-rich anticodon domain of tRNA\textsuperscript{Lys3}. The importance of individual and combined anticodon modifications to the tRNA/HIV-1 Loop I RNA’s interaction was determined. The thermal stabilities of variously modified tRNA anticodon region sequences bound to the Loop I of viral sub (sero)types G and B were analyzed and the structure of one duplex containing two modified nucleosides was determined using NMR spectroscopy and restrained molecular dynamics. The modifications 2-thiouridine, s\textsuperscript{2}U\textsubscript{34}, and pseudouridine, \textsuperscript{\textPsi}C\textsubscript{9}, appreciably stabilized the interaction of the anticodon region with the viral subtype G and B RNAs. The structure of the duplex results in two coaxially stacked A-form RNA stems separated by two mismatched base pairs, U\textsubscript{162}\textsuperscript{\textPsi}C\textsubscript{39} and G\textsubscript{163}\textsuperscript{\textPsi}C\textsubscript{39}, that maintained a reasonable A-form helix diameter. The tRNA’s s\textsuperscript{2}U\textsubscript{34} stabilized the interaction between the A-rich HIV Loop I sequence and the U-rich anticodon, whereas the tRNA’s \textsuperscript{\textPsi}C\textsubscript{39} stabilized the adjacent mismatched pairs.

INTRODUCTION

Retrovirions are packaged with an enrichment of a host cell tRNA for the purpose of priming reverse transcription. Human immunodeficiency virus type 1, HIV-1 and all other lentiviruses, evolved to select and recruit the cytoplasmic tRNA\textsuperscript{Lys3} and the other tRNA\textsuperscript{Lys} isoacceptors, tRNA\textsuperscript{Lys1} and tRNA\textsuperscript{Lys2} (1). While the function of tRNA\textsuperscript{Lys1,2} in HIV-1 is not known, the selective packaging of HIV-1 reverse transcriptase primer tRNA\textsuperscript{Lys3} is required for optimizing both the annealing of tRNALys3 to viral RNA and the infectivity of the HIV-1 population (2). A considerable number of interactions are believed to occur between the human tRNA\textsuperscript{Lys3} and HIV-1, as determined by chemical and enzymatic probes (3). Eighteen nucleosides of the tRNA’s 3’-terminal sequence (Figure 1A), part of the amino-acid accepting stem (nucleosides 66–76) and the 3’-side of the T/C domain (tRNA nucleosides 59–65) (4), are bound to a complementary sequence in the viral RNA, the Primer Binding Sequence (PBS) (viral nucleosides 183–200), while the T/C domain interacts with the Primer Activation Sequence (PAS) (5) (Figure 1A). The uridine-rich anticodon and adjacent nucleosides are bound to the adenosine-rich Loop I of the viral RNA (Figure 1A and C) (4). Deletion of the A-rich Loop I of HIV-1 that is complementary to the uridine-rich anticodon domain resulted in significantly reduced levels of infectivity and reduced synthesis of viral RNA (6–8). Constitution of a virus that was able to use a host cell tRNA other than tRNA\textsuperscript{Lys3} required not only the conversion of the PBS to the complement of the investigator-selected tRNA, but also the mutation of the AAAA sequence of HIV-1.
Loop I to complement the anticodon sequence of the new tRNA (9–11). Changing one or the other of these sequences was not sufficient for maintaining the new interaction during prolonged culturing. Binding of the tRNA’s anticodon region to the A-rich Loop I region has been associated with transition of viral replication from initiation to the highly processive elongation with a possible requirement for the tRNA’s post-transcriptional modifications (12).

The tRNA\textsubscript{Lys3} anticodon domain has post-transcriptional modifications (5-methoxy carbonyl-methyl-2-thioridine, mcm\textsubscript{5}s\textsubscript{2}U\textsubscript{34}; 2-methylthio-N6-threonyl carbamoyladenosine, ms\textsubscript{2}t\textsubscript{6}A\textsubscript{37}; and pseudouridine, \Psi\textsubscript{39}) that are critical for the decoding of its cognate (AAA) and wobble codons (AAG) on the ribosome (13) and affect its interaction with the HIV Loop I (14). Fully modified, native tRNA\textsubscript{Lys3}\textsubscript{SUU} when bound to the viral RNA protected the HIV-1 Loop I AAAA sequence (viral nucleosides 169–172) from chemical reactivity and enzymatic cleavage (14). In contrast, the unmodified \textit{in vitro} transcript of the tRNA was unable to block chemical reactivity of the same sequence (14). In complex with viral RNA, the tRNA anticodon was also protected from chemical and enzymatic probes (3). In addition, the HIV nucleocapsid protein NCp7 stably denatures tRNA\textsubscript{Lys3} in the presence of the PBS and Loop I, is susceptible to chemical and enzymatic probes (3). In addition, the HIV nucleocapsid protein NCp7 stably denatures tRNA\textsubscript{Lys3} in the presence of the complementary HIV sequences (21–23). Whatever the mechanism that leads to formation of the replication initiation complex, a duplex apparently results from the annealing of the anticodon domain to the HIV Loop I. Yet, the contributions of the modified nucleosides to the stability and structure of the resulting duplex are not known.

To investigate the effect of modified nucleosides on the stability and structure of the duplex that is formed from the anticodon domain and the viral Loop I, an experimental system was designed to mimic the linear, denatured form of the RNAs. Here, we report how two of the more important individual tRNA-modified nucleosides contribute to the stabilization of the tRNA anticodon domain interaction with both the HIV sub...
(sero)type G, exhibiting the highest complementariness of the Loop I sequences with the anticodon stem and loop domain of tRNA\(^{Lys}\) (ASL, and sub(sero)type B, the most prevalent subtype in the Americas, Europe and Oceania. The modified nucleosides \(^5\)U\(_{34}\) and \(\Psi_{39}\) were most effective at stabilizing the interaction between the tRNA’s anticodon region and the viral A-loop sequence. The NMR-derived structure of one duplex containing the two modified nucleosides, \(^5\)U\(_{34}\) and \(\Psi_{39}\) resulted in two coaxially stacked A-form RNA stems separated by 12.5 Å that is comparable to that of the A-form RNA helix diameter.

**MATERIALS AND METHODS**

**Oligonucleotide synthesis**

The tRNA\(^{Lys}\) anticodon domain fragments (ADF) and HIV Loop I sequences corresponding to the subtypes G and B (VGL, VBL) were synthesized (Nucleic Acids Facility at North Carolina State University, Raleigh, NC) as dodecamers (Figure 1) with modified nucleosides \(^1\)H-31P HeteroTOCSY were collected on VGL/ADF-\(^5\)U\(_{34}\)\(\Psi_{39}\) duplex at 20°C in D\(_2\)O with the VGL/ADF-\(^5\)U\(_{34}\) and the VGL/ADF-\(^5\)U\(_{34}\)\(\Psi_{39}\) duplexes. Two-dimensional heteronuclear spectra aided in the assignment process. Natural abundance \(^1\)H-\(^15\)N HSQC experiments were performed on VGL/ADF-\(^5\)U\(_{34}\) in H\(_2\)O at 4°C and VGL/ADF-\(^5\)U\(_{34}\)\(\Psi_{39}\) in D\(_2\)O at 20°C. \(^1\)H-\(^3\)P HETCOR and a \(^1\)H-\(^3\)P HeteroTOCSY were collected on VGL/ADF-\(^5\)U\(_{34}\)\(\Psi_{39}\) in D\(_2\)O at 20°C. For samples with site-specific \(^15\)N labels, \(^1\)H-\(^15\)N HSQC spectra were obtained in H\(_2\)O at seven temperatures from 2°C to 35°C and at the concentrations used for structural analyses.

**NMR restraints generation and structure calculation**

Measurement of individual peak intensities, when possible, was performed using the box integration method in the SPARKY software. The cross-peaks were qualitatively classified as strong (0.0–3.5 Å), medium (0.0–4.5 Å), or weak (0.0–5.5 Å). For peaks that overlapped, standard A-form RNA distances were used when an A-form RNA was deduced from the NMR spectra. No restraints were used for the H3’ and H5’ protons. Intervals with wide boundaries, 0.0–5.0 or 6.0 Å, were used for restraints involving the imino protons. A total of 292 restraints were used to generate the structure (Table 1). Consistent with NMR data, the nine G\(\bullet\)C and U\(\bullet\)A base pairs were subjected to six and four hydrogen-bond restraints, respectively (1.8–2.5 Å between the hydrogen and acceptor, and 2.7–3.5 Å between the heavy atoms). The G\(\bullet\)U base pair was restrained using four hydrogen-bond distance restraints between the GO6 and the UN3H and N3 atoms, and between the UO2 and the GN1 and GN1H atoms. No hydrogen-bonds restraints were used for \(\Psi_{39}\)U\(_{162}\) and G\(_{165}\)\(\bullet\)A\(_{38}\) base pairs, but an NMR-derived, imino distance restraint between the U\(_{162}\)N3H and \(\Psi_{39}\)N3H was introduced with an interval of 0.0–6.0 Å. A total of 47 hydrogen bond distance restraints were finally used. Planarity restraints (5–50 kcal/mol Å\(^2\)) were applied to Watson–Crick and G\(\bullet\)U base pairing. Because of severe overlapping in DQF-COSY and HETCOR spectra, torsion angle restraints were set with wide ranges of ±30° and not determined from the direct measurement of coupling constants. β (P-O5’-C5’-C4’), γ (O5’-C5’-C4’-C3’), and δ (C4’-C3’-O3’-P) dihedral angles were set respectively to 178 ± 30°, 54 ± 30° and −153 ± 30°. When the presence of the regular A-form

**UV and circular dichroism spectroscopy**

Thermal denaturations and renaturations were monitored by UV absorbance at 260 nm with a Cary 3 spectrophotometer running WinUV version 3.00. Buffer conditions were identical to those for NMR. Three successive denaturations and renaturations were conducted over a temperature range of 5–55°C and a concentration range of RNA from 1 µM to 1 mM using cuvettes of 1.0 and 0.2 cm pathlengths. The temperature ramp rate was 1.0°C/min or 0.5°C/min with a data sampling interval of 0.2 min or 0.5 min. Circular dichroism spectra were collected (Jasco J600) at 4°C with 1 cm path length using a jacketed, cylindrical sample cell and RNA sample concentrations adjusted to ~0.2 absorbance units at 260 nm (28,29).
RNA structure could be determined, using both the $^{31}$P chemical shift and the expected connectivity patterns between the aromatic and sugar resonances, the $\alpha$ (O3$^\prime$-P-O5$^\prime$-C5$^\prime$: $-68 \pm 30^\circ$) and $\zeta$ (C3$^\prime$-O3$^\prime$-P-O5$^\prime$: $-71 \pm 30^\circ$) torsion angles were constrained to exclude the trans conformation. For all residues, except G163, the glycosidic torsion angles, $\chi$ [O4$^\prime$-C1$^\prime$-(N9/N1)-(C4/C2)], were restrained to the anti conformation ($-158 \pm 30^\circ$); the intensities of all the H1$^\prime$-H6 to H8 connectivities exhibited smaller volumes than the H5 to H6 connectivity. For all residues except G163, the $\delta$ angles were restrained to be in C3$^\prime$-endo conformation. A total number of 156 torsion angles restraints were used for the structure calculation (Table 1).

Molecular modeling of the VGL/ADF-s$^2$U34,$\Psi_{39}$ structure was achieved using CNS 1.1 (33). One hundred structures were calculated using standard NMR restraint annealing protocol (34). Structures with low total energy, without or with a low number of dihedral or distance restraint violations were chosen for further analysis. The helical parameters of the structure were analyzed using X3DNA software (35). To validate our structures, back calculations of the H5, H6 and H8 protons resonance chemicals shifts were conducted using MOLMOL (Johnson-Bovey model), or NUCHEMICS software (36). The output structures were visualized with MOLMOL (37) and PYMOL (38).

**RESULTS**

Thermodynamic stability and affinities of HIV-1 A-loop interactions with variously modified fragments of the tRNA$^{13}_{\text{Lys}}$ anticodon domain

The thermal stabilities and the circular dichroism spectra of the duplexes formed between the U-rich anticodon domain dodecamer (ADF) of tRNA$^{13}_{\text{Lys}}$ and the A-rich HIV Loop I indicated that the s$^2$U34 and $\Psi_{39}$ facilitated the interaction of the two RNAs. The dodecamer sequences, based on previous studies of the complex formed between tRNA$^{13}_{\text{Lys}}$ and the HIV-1 RNA (3), were constituted with substitution of C43 for A43 to reduce fraying of the duplex by forming a terminal C43$^\prime$G158 base pair. This construct allowed us to focus our studies on the contributions made by the two internal base modification chemistries, s$^2$U34 and $\Psi_{39}$, and the possible mismatch base pairs. As it would be expected for the duplexes, the melting temperatures, $T_m$, of these bimolecular interactions were concentration dependent (Figure 2A) allowing the binding affinities to be extracted from the results of the UV-monitored thermal experiments (Table 2). The contribution of modified nucleosides to the stability of the VGL/ADF complex was dependent on the tRNA$_{\text{Lys}}$ modification introduced. Relative to the unmodified ADF ($T_m = 30.0^\circ$C at 100 $\mu$M; $K_d = 320$ $\mu$M), the individual incorporations of $\Psi_{39}$ and s$^2$U34 raised the melting temperature of the VGL/ADF duplexes by +1.2 $^\circ$C and +3.9 $^\circ$C, and enhanced the affinity of the ADF for the VGL by some 8- and 72-fold, respectively. Incorporation of both the $\Psi_{39}$ and s$^2$U34 modifications significantly increased the $T_m$ (+7.2 $^\circ$C) and the affinity of the ADF for the viral sequence (Table 2). The affinity of the doubly modified ADF-s$^2$U34,$\Psi_{39}$ ($K_d = 14.2$ $\mu$M) for the VGL was significantly stronger than that of the unmodified ADF ($K_d = 324$ $\mu$M) (Table 2). Interestingly, the affinity of the fully modified anticodon stem and loop domain for its cognate codon AAA in the A-site of the *Escherichia coli* ribosome was comparable ($K_d = 3.1$ $\mu$M) to that of the ADF-s$^2$U34,$\Psi_{39}$ for the VGL, while ribosomal codon binding of the unmodified stem and loop

| Table 1. Restraints and structural statistics |
|--------------------------------------------|
| NOEs distances restraints                  | 292 |
| Internucleotide NOEs                       | 139 |
| Intranucleotide NOEs                       | 115 |
| Involving imines                           | 38  |
| Hydrogen bonds restraints                  | 47  |
| Torsion angles restraints                  | 156 |
| Planarity restraints                       | 11  |
| NOEs per residue                           | 12.2|
| Total restraints per residue               | 18.7|
| Distance violations (Å)                    | None > 0.5 |
| Dihedral violations (°)                    | None > 5.0 |
| Backbone RMSD for all atoms                | 2.55 ± 0.89 |
| Stems RMSD:                                | 0.80 ± 0.29 |
| (G158-G161/C40-C34) (Å)                    | 0.86 ± 0.39 |
| (A166-G160/C32-U31) (Å)                    | 1.01 ± 0.34 |

**Figure 2.** Thermal stability and base stacking of the VGL/ADF duplex. (A) UV-monitored, thermal stability assay of the VGL/ADF-s$^2$U34 duplex. The duplex was repeatedly denatured and renatured and monitored by UV-absorbance. The $T_m$ of a duplex is concentration dependent. Results from monitoring three concentrations of the VGL/ADF-s$^2$U34 duplex are shown: 1, 14 and 28 $\mu$M. (B) Circular dichroism spectra of the VGL/ADF duplexes. The circular dichroism spectrum of each duplex was collected with the RNA concentration of the duplex or the VGL alone adjusted to give an absorbance of 0.2 units (A260) at 25 $^\circ$C. Spectra were collected at 4 $^\circ$C and were not corrected for the background (buffer).
domain was too low to be determined (E. Gustilo and P.F. Agris, personal communication). The standard free energy values, ΔG°, from duplex formation of the VGL with the ADF-Ψ39, ADF-s2U34, and ADF-s2U34;Ψ39 were lower than that formed from the VGL and the unmodified ADF (Table 2). The ΔG° value for the doubly modified duplex was between that of the two singularly modified duplexes. We expect that the free energy of the VGL/ADF-s2U34;Ψ39 duplex is a composite of local enthalpic and entropic contributions, influenced by the effect of modified nucleosides on local structure and stability. Thus, it is not surprising that the ΔG° of the doubly modified duplex was not simply the sum of the two duplexes containing only one modification each (s2U34 or Ψ39).

A fourth duplex (VGL/ADF-C), formed from a fully complementary ADF sequence in which A39C38 substituted for U39A38, exhibited a Tm (42.2°C) that was 8.3°C higher than the VGL/ADF-s2U34;Ψ39, and 12°C higher than the duplex formed with the unmodified ADF and two mismatched pairs. In addition to increasing thermal stability, the s2U34 and Ψ39 enhanced the degree of base stacking within the VGL/ADF duplexes (Figure 2B), as monitored by circular dichroism spectroscopy (39). The spectral ellipticity of the VGL/ADF was increased with the addition of the single and combined modifications. The duplex with ADF-s2U34 exhibited the highest degree of ellipticity and presumably the greatest degree of base stacking. Surprisingly, the ellipticity of the VGL/ADF-C was comparable to that of the VGL/ADF-Ψ39, and lower than that of the VGL/ADF-s2U34 (Figure 2B). Here, we see again that the ellipticity of the doubly modified complex, VGL/ADF-s2U34;Ψ39, is not simply the sum of that of the two singularly modified complexes VGL/ADF-s2U34 and VGL/ADF-Ψ39.

The ADFs had four contiguous uridines (U13–U16) complementary to the four contiguous adenosines of the dodecamer of the HIV-1 Loop I subtype G (VGL), but were still capable of binding to the Loop I substitutions that are found in other subtypes, such as the two guanosines of the B-subtype (VBL) (Figure 1D). The affinities of the VBL for the unmodified ADF and the ADF-s2U34 were comparable to the VGL (Table 2). There are three human tRNA Lys species. Notably, an unmodified ADF with the sequence of the human tRNA Lys1,2,34 having a C34 was hardly bound by the VGL (Table 2).

### Table 2. Thermodynamic parameters of VGL and VBL duplex formation with various ADF constructs

| VGL or VBL | tRNA Lys3 | Kd (µM) | ΔG°298 | ΔAG°298 | Tm (100 µM) |
|-------------|-----------|---------|---------|----------|-------------|
| VGL ADF    | 324.0     | -4.8    | -4.5    | 14.2     | 33.9        |
| ADF-Ψ39    | 43.0      | -5.9    | 2.5     | 6.6      | 37.2        |
| ADF-s2U34  | 4.5       | -7.3    | 25.9    | 6.6      | 37.2        |
| ADF-s2U34;Ψ39 | 14.2   | -6.6    | 1.8     | 6.6      | 37.2        |
| VBL ADF    | 270.0     | -4.9    | 0.1     | 30.6     |             |
| ADF-s2U34  | 5.7       | -7.2    | 2.4     | 33.0     |             |
| VGL ADF of tRNA Lys2 >1900 |             |         |         |           |

Gibbs standard free energy in kcal/mol was calculated at 25°C from: ΔG° = -RTlnK.

NMR studies of the VGL/ADF interaction: exchangeable proton resonances identification and assignment

A doubly modified (s2U34;Ψ39) ADF bound to the VGL/ADF-s2U34;Ψ39, was studied by NMR. Three other duplexes were investigated by NMR: a fully base-paired complement (VGL/ADF-C); a duplex containing only the pseudouridine modification, ADF-Ψ39 (VGL/ADF-Ψ39); and a duplex containing only the 2-thiouridine modification, ADF-s2U34 (VGL/ADF-s2U34;Ψ39) (Figure 1D). These additional constructs aided in the assigning of NMR signals and in elucidating the effect of the different modified nucleosides of tRNA Lys1,2 on the interaction of its anticodon domain with the HIV-1 A-rich Loop I (Figure 1D). All four duplexes were studied using classical procedures (40,41). One dimensional spectra of the various duplexes exhibited exchangeable protons resonances between 12.5 and 15 ppm that were attributed to seven hydrogen-bonded imino protons involved in standard Watson–Crick base pairs for the VGL/ADF-Ψ39, VGL/ADF-s2U34 and VGL/ADF-s2U34;Ψ39 duplexes (Figure 3 and Supplementary Data). The imino proton resonance of s2U34 was shifted downfield (14.23 ppm) compared to the corresponding resonance of the similar unmodified U34 (13.85 ppm). The downfield shift of the base paired imino proton of s2U34 has been previously observed (42) and could indicate that the sulfur is contributing to a stronger hydrogen bond through a change in ring current or stacking interactions.

Imino proton resonances between 10 and 12 ppm corresponded to those protons involved in non-canonical base pairs. The characteristic chemical shifts (43) and observation of an intense NOE connectivity between the NH of G160 and N3H of U41, together with the imino–imino NOE connectivity between N3H of U41 and NH of G161 allowed the unambiguous assignment of the imino protons of G160 and U111 in the VGL/ADF-C NOESY spectrum. The formation of this base pair was observed in all four duplexes including the VGL/ADF-s2U34;Ψ39 (Supplementary Data). The imino protons resonances of Ψ39-N1H, Ψ39-N3H and U162-N1H were identified and assigned on the basis of the VGL/ADF-Ψ39 and VGL/ADF-s2U34;Ψ39 NOESY spectra at low temperatures (2°C and 4°C, respectively) and of the 15N-HSQC spectra. The chemical shifts of the Ψ39 and U162 imino protons agreements with previously published studies (44). Moreover, a weak imino–imino NOE connectivity between the Ψ39-N3H and the U162-N3H was observed on the 200 ms NOESY spectra of the VGL/ADF-Ψ39 duplex (Supplementary Data). In addition, NOE connectivities were observed between Ψ39-N1H and A38H2 and A38H3 on the NOESY spectra (data not shown). A broad imino resonance at 11.50 ppm was assigned to the imino proton of G163. Despite this proton being protected from exchange with solvent, no NOE connectivity between this proton and other exchangeable or non-exchangeable protons could be observed.

The thermal denaturations of the VGL/ADF-C, VGL/ADF-Ψ39 and VGL/ADF-s2U34;Ψ39 duplexes were followed by NMR (Supplementary Data) and were
consistent with the results of the UV-monitored melting studies. The VGL/ADF-C exhibited a Tm between 40°C and 45°C, whereas the Tm values of the VGL/ADF-Ψ39 and VGL/ADF-s2U34 and VGL/ADF-s2U34/Ψ39 were between 25°C and 30°C.

Non-exchangeable protons resonances identification and assignment

The non-exchangeable resonances of the VGL/ADF-s2U34/Ψ39 duplex were identified and assigned with a variety of homo- and heteronuclear NMR experiments using standard procedures (41) and the help of the three other duplexes. Using the different duplexes, all of the aromatics and H1’ resonances were assigned, excepted that of G163 in VGL/ADF-s2U34/Ψ39, VGL/ADF-Ψ39 and the VGL/ADF-s2U34/Ψ39 (Figure 3B and Supplementary Data). Although assignment of the G163 H8 and H1’ resonances remains tentative because of severe overlapping of signals, an NOE connectivity was observed between the U166H6 and the G163H1’ in the VGL/ADF-Ψ39 duplex, as well as a strong NOE between the U164H6 and G163H2’ in the NOESY spectra of the VGL/ADF-Ψ39 and VGL/ADF-s2U34/Ψ39 duplexes at low mixing times. The chemical shifts of the H5 and H6 protons are known to be most affected by the ring current from the 5’ adjacent base (36). The H5 and H6 chemical shifts of U164 were consistent with protons experiencing a ring current effect from its 5’-adjacent G163 (36), indicating that the G163 and U164 were stacked, and consequently that G163 is inside the helix. The U163H6 and H5 chemical shifts were also consistent with a uridine experiencing the ring current of G161 in spite of a more important displacement toward the high-field for the U165H6 chemical shift as compared to U164H6. Moreover, the volume of the U162H5 to H6 NOE connectivity was less than all of the other H5-H6 NOE connectivities indicating that the U162 was more dynamic than the other pyrimidines. An inter-strand NOE connectivity between the A163H2 and the A37H2 was observed for the VGL/ADF-s2U34/Ψ39.

The Ψ39H6 proton resonated at 6.8 ppm, characteristic of a pseudouridine (45). An aromatic to aromatic NOE connectivity was observed between Ψ39H6 and A38H8, indicating that Ψ39 and A38 were stacked. Because of overlapping peaks, we were not able to unambiguously identify the connectivities of A38 to the A37 H2. The A160H2 proton exhibited an unusual NOE connectivity with the s2U34H1’, indicating an enhanced stacking that we attributed to the 2-thio modification. The chemical shifts of A38H8 and H1’ were consistent with an adenosine involved in a typical A-form RNA. The A38H2 proton, identified and assigned by T1 relaxation experiments and confirmed through the natural abundance 1H-13C-HSQC spectra, relaxed slowly, as did other H2 resonances for the VGL/ADF-s2U34/Ψ39 duplex. Taken together along with strong sequential NOEs between the aromatic to H2’ protons, this information showed that the entire duplex adopted a conformation similar to a canonical A-form RNA. Moreover, the data indicated that all the bases were stacked and inserted into the helix.

Sugar conformations and backbone geometry

The DQF COSY spectra showed that all the residues of the VGL/ADF-s2U34/Ψ39 duplex adopted the C2 ‘endo’ sugar pucker (JH1’-H2’ < 3 Hz) characteristic of an A-form RNA helix. A narrow spread of the 31P chemical shifts (2.2 ± 0.9 ppm) around that attributed to A-RNA helix-like values indicated that on average the phosphate backbone conformed to a regular A-RNA conformation without significant deformation (Supplementary Data) (41). Each of the aromatic to H1’ connectivities of the VGL/ADF-s2U34/Ψ39 had an intensity significantly lower than that for the H5–H6 indicating that all the bases, including Ψ39, adopted the anti form of the glycosidic torsional angle (46). Nevertheless, the resonance overlaps and tentative assignment for G163 did not allow us to determine its glycosidic torsional angle.

Structure of VGL/ADF-s2U34/Ψ39

The spectral data indicated that the structure of the VGL/ADF-s2U34/Ψ39 duplex was similar to a standard double helical A-form RNA, that all the bases were inserted into the helix and that the 2 × 2 internal loop S’-U163G163:3’-Ψ39A38:5’-U34 was structurally organized. One hundred structures were calculated using a simulated annealing protocol (34). Among the 100 calculated structures, 41 converged to satisfy all of the NOE distance and dihedral angle restraints within 0.5 Å and 5°.
respectively. The U\textsubscript{162}•Ψ\textsubscript{39} and G\textsubscript{163}•A\textsubscript{38} mismatch base pairs were observed with great attention. Among the 41 accepted structures, 23 presented an imino G•A base pair and the lowest total energy. The other 18 structures presented different kinds of G•A base pairs. Of the 23 structures exhibiting an imino G•A base pair, the 10 structures with the lowest total energies were selected for further analysis. In order to validate the selected structures, a chemical shift back-calculation was conducted for the H5 and H6 chemical shifts of C\textsubscript{159} (standard), U\textsubscript{162} and U\textsubscript{164}, and for the Ψ\textsubscript{39}H6 and G\textsubscript{163}H8. Indeed, the chemical shifts of the H5 and H6 were most influenced by the 5’ bases and thus, could be used as probes to validate our structure. The back-calculated chemical shifts for the U\textsubscript{164}H5 and the H6 were in excellent agreement with the observed chemical shifts for U\textsubscript{164} (Supplementary Data). This confirmed the stacking of the U\textsubscript{164} and G\textsubscript{163} and moreover, tended to prove that the imino G•A base pair observed in the 10 selected structures was consistent with the NMR data. The predicted Ψ\textsubscript{39}H6 chemical shift was consistent with the observed chemical shift. The back-calculated and experimental chemical shifts for U\textsubscript{162}H5 were almost identical (Supplementary Data). This contrasted with U\textsubscript{162}H6 for which these chemical shift values displayed a difference of ~0.7 ppm. In this case, the position of U\textsubscript{162} in relation to G\textsubscript{161} seemed to be different from that observed in our structures.

The overall structures are well defined with a mean backbone RMSD for all residues of 2.55±0.89 Å. The structures have two canonical, A-form RNA stem regions of four and six Watson–Crick base pairs including the wobble G•U, respectively, separated by a structured 2 × 2 internal loop. Relative to the mean structure, the backbone RMSDs were 0.80±0.29 Å and 0.86±0.39 Å, respectively, for the G\textsubscript{158}-G\textsubscript{161}/C\textsubscript{40}-C\textsubscript{43} (Figure 4A) and the A\textsubscript{166}-G\textsubscript{169}/C\textsubscript{32}-U\textsubscript{35} (Figure 4B) A-form RNA stems. However, the backbone RMSD for the loop and the

![Figure 4. Structure of the VGL/ADF-s\textsubscript{2}U\textsubscript{34} duplex. The structure of the VGL/ADF-s\textsubscript{2}U\textsubscript{34} duplex was determined from NMR-restrained, molecular dynamics. Members of a family of 10 structures without distance or torsion angle violations were superimposed. Both the U\textsubscript{162}•Ψ\textsubscript{39} and imino G\textsubscript{163}•A\textsubscript{38} base pairings are within the structures. (A) Best fit for the stem residues G\textsubscript{158}-G\textsubscript{161}/C\textsubscript{40}-C\textsubscript{43}. (B) Best fit for the stem residues A\textsubscript{166}-G\textsubscript{169}/C\textsubscript{32}-U\textsubscript{35}. (C) Best fit for internal residues G\textsubscript{161}-U\textsubscript{164}/A\textsubscript{37}-C\textsubscript{40}. (D) Superposition of the ten best structures (left) and the mean structure (right). (E) The G\textsubscript{168}•A\textsubscript{38} and U\textsubscript{162}•Ψ\textsubscript{39} base stacking (top) and the geometry of the two base pairings (bottom). (F) The mean G\textsubscript{168}•A\textsubscript{38} and U\textsubscript{162}•Ψ\textsubscript{39} base stacking (top) and mean geometry of the two base pairings (bottom). (G) The imino and sheared G•A base pairs. Top: The imino G•A base pair employs the GN1H as a donor for the AN1 acceptor, whereas the carbonyl oxygen of G is the acceptor for an amino proton of A. Bottom: The sheared G•A pair employs an amino proton of G as a donor with the AN7 as the acceptor, and an amino proton of A as a donor for the N3 of G.](image-url)
immediately adjacent residues G_{161}U_{164}/A_{37}C_{40} was greater than that of the two A-form RNA stems with a value of 1.01 ± 0.34 A (Figure 4C).

Both G_{163}, being loosely restrained, and U_{162} exhibited conformational dynamics (Figure 4E and F). The average C1′–C1′ distance between U_{162} and Ψ_{39} was 11.4 ± 0.5 A, more than the conventional U•U base pair that averages a C1′–C1′ distance of ~9 A (47–49). The average hydrogen bond distance observed between the U_{162}N3H and the Ψ_{39}N3H was 3.8 ± 0.6 A. This distance is consistent with the moderately weak NOE observed between the two imino protons. In comparison, distances between the U_{162}N3 and Ψ_{39}O4 and between the U_{162}O4 and Ψ_{39}N3 were on average 5.4 ± 0.6 A and 4.4 ± 0.9 A, respectively. The former distance excluded the formation of a direct hydrogen bond, and possibly the latter as well, but did not exclude the formation of a water-mediated hydrogen bond for either. The aforementioned distance between the U_{162} and Ψ_{39} agreed with the formation of an imino base pair between G_{163} and A_{37}. Indeed, the C1′–C1′ distance between G_{163} and A_{37} was nearly 12.4 ± 0.2 A in the 10 selected structures. The average distance between Ψ_{39}N1 and the Ψ_{39}OP1 was 4.3 ± 0.6 A. Once again, this distance did not exclude the possible formation of a hydrogen bond or a water-mediated hydrogen bond between the N1H imino proton of Ψ_{39} and a phosphate atom, such as that observed in crystallographic structures (50).

**DISCUSSION**

**Modifications stabilize the interaction of the tRNA^{13x3} anticodon domain with the HIV-1 Loop I**

Replication of the human immunodeficiency virus (HIV) requires base pairing of the reverse transcriptase primer, human tRNA^{13x3}, to the viral RNA. Though conclusions from our UV-monitored studies of the thermal denaturations and structures of the duplexes are limited by the fact that the duplex sequence was altered to avoid frayed ends, the circular dichroism spectra and the NMR-derived structure underline the contributions of the 2-thio group and the pseudouridine to the stability of the interaction between the Loop I and the ADF. One of the most dramatic affects on nucleotide conformation and thermodynamic stabilization is the substitution of the carbonyl oxygen at C2 of uridine by a sulfur atom (s^2U). The van der Waals’ radius of sulfur being some 20% larger than oxygen contributes to the greatly increased stability of the 3′-endo, gauche+ ring pucker and antiperiplanar conformation of s^2U and causes the adjacent nucleotides to favor 3′-endo ring pucker and A-form geometry (14,18,51–54). The extremely stable nucleoside conformation of s^2U and that of the adjacent nucleosides stabilizes the canonical base pairings. In the NMR spectra, the downfield chemical shifts for the H5, H6 and H1′ protons relative to that of the unmodified uridine are indicative of an altered ring current that would contribute to the enhanced base pairing, as well as to base stacking. Though the N1 proton of Ψ has long been suspected to be responsible for contributing through direct hydrogen bonding to base pairing, little evidence has been found in investigations of RNAs. However, observation of the N1 proton resonance indicates that it is sufficiently protected from chemical exchange with the solvent water to invoke a stabilizing effect on base pairing through water-mediated hydrogen bonding, enhanced base stacking, or both. As with previous studies of Ψ (55,56), we saw no evidence for hydrogen bonding of the N1 proton of Ψ. Enhanced base stacking is observed when Ψ is in stems or in loops (57–59) and this could contribute to the greater stability of the U-rich anticodon domain interaction with the A-rich HIV Loop I. The modification ms^1t^6A_{37} is missing from the studies presented here. This modification excludes the formation of a canonical U•A base pair across the anticodon loop (60). The ms^1t^6A_{37} and the t^6A_{37} modifications decreased the overall T_m of the ASL (19,60). However, crystallographic studies of the ASL bound to cognate and wobble codons on the ribosome (61) indicate that the ms^1t^6A_{37} modification would act as a hydrophobic platform in stabilizing the base pairing between the tRNA anticodon and the series of As in the Loop I of HIV-1.

**The HIV-1 Loop I/tRNA^{13x3} ADF structure**

The dodecamer duplex formed between the VGL and the ADF-s^2U_{34}/Ψ_{39} exhibited an A-form RNA conformation with a relatively well-organized internal loop composed of two mismatch base pairs. The NMR data clearly demonstrated that C_{40}, Ψ_{39}, A_{38} and A_{37} are stacked and inserted into the helix. Nevertheless, despite a relatively well-organized internal loop, the VGL/ADF-s^2U_{34}/Ψ_{39} duplex exhibited a melting temperature lower than the full complement, VGL/ADF-C. The U_{162} residue was dynamic, as suggested by the weakness of the H5–H6 connectivity in comparison to other uridines or cytidines. The difference between the observed and the back-calculated chemical shifts indicated that the stacking observed in the structures between G_{161} and U_{162} does not reflect the NMR data. An intense NOE connectivity is expected between the uridine imino proton of residues involved in a U•U base pair (49,62). In those base pairs, the average distance between the two imino protons is ~2.5 A, significantly lower than the average distance observed in our structures between U_{162}N3H and Ψ_{39}N3H (3.8 A). Although a classical U•U base pairing cannot be formed, the observed distance between acceptor and donor does not exclude the formation of a water-mediated hydrogen bond stabilizing the Ψ•U interaction.

A G•A base pair can adopt more than nine conformations, but two most often observed are the imino and the sheared (43,63) (Figure 4G). The type of G•A base pair depends on the neighboring base sequences and pH (64). In the imino G•A conformation, an NOE connectivity between the GN1H and the AH2 is observed (65), whereas in the sheared G•A the GN1H proton is observed at very low temperatures (66). Conformational dynamics coupled with the presence of the neighboring weak Ψ_{39}U_{162} pair could explain the broad proton resonance attributed to the G_{163}N1H, and could explain why
the expected connectivity between G163N1H and A38H2 could not be observed. The chemical shift of the U164H5-H6 cross peak and its back-calculated chemical shift were similar and in good agreement with a uridine experiencing a 5'-guanosine (G163) ring current. This is supportive of an imino G•A base pairing. Moreover, it has been suggested that a single G•A base pairing cannot adopt the sheared G•A conformation observed in a tandem G•A pair (67). Formation of an imino base pair between G163 and A38 would result in an average C1'–C1' distance of 12.5Å. Such a base pair could prevent the formation of aΨ•U base pair with a C1'–C1' distance of ~9Å. We observed that the average C1'–C1' distance for the Ψ39 and U162 pair was 11.4Å. These results are consistent with the 31P spectrum of the VGL/ADF-s1U34Ψ39, and previous studies showing that in contrast to a sheared G•A, an imino G•A base pair resulted in little to no deformation of the backbone (68,69). A model of a 2×2 internal loop composed of 5'-UG-3'/3'-UA-5' was proposed from NMR and thermal stability studies of a model rRNA system (70). Despite a general pre-organization of the 2X2 internal loop, no hydrogen-bond was observed between the two U's, and the G was external to the helix. Nevertheless, a G•A base pair was not excluded and it was suggested that an imino G•A base pair was possible. We found that the 5'-UG-3'/3'-UA-5' was relatively well-organized with stacking of the C40, Ψ39 and A38, and that a water-mediated hydrogen-bond between Ψ39 and U162 probably existed. Furthermore, G163 was inside the helix and probably involved in an imino G•A base pair that would be supported by the NMR data.

The HIV Loop I/tRNA anticodon interaction: a ‘kissing’ loop complex?

Interactions between the A-rich Loop I and the anticodon domain of tRNA^{Lys3} are essential for priming the initiation of HIV-1 reverse transcription (8,9,71). In fact, the A-rich Loop I region or the complex formed by the A-rich Loop I with the tRNA^{Lys3} anticodon could represent promising targets for developing new therapies. Polyamides target the A-loop sequence (PNAAL), and represent promising targets for developing new therapies. Reverse transcriptase (RT) binding facilitates the annealing of the tRNA to the PBS (72). The nucleocapsid protein (NC) is known to have general unwinding and strand renaturation activities and this protein or its precursor Gag has been implicated in the placement of tRNA onto the viral genome (73,74). HIV-1 NC is a short basic protein with two zinc finger domains and functions as a nucleic-acid chaperone (75). Like RT, NC appears to destabilize base pairing in tRNA molecules, without a complete melting of the structure (76). Moreover, it has been suggested that the annealing of tRNA to PBS could open the ΨΨC stem of the tRNA, allowing residues 48–55 to interact with the PAS portion of HIV-1 (77). Our expanded duplex model was constructed with the purpose of determining the effect of modifications on the binding the HIV Loop I by tRNA^{Lys3}. The equilibrium binding constant that we observed for the s1U and Ψ-modified 12 mer to the Loop I sequence (Kd = 14.2 μM) was indicative of a higher affinity, but consistent with that found for the kissing loop interactions of the fully modified anticodon stem and loop (Kd = 71 μM) (19). There is evidence that the mechanism of duplex formation may include an initial kissing loop interaction (19,20). In an annealing experiment monitored by NMR, the anticodon stem of a ‘recombinant’ tRNA^{Lys3} produced in E. coli remained intact while the loop was reported to have undergone a conformational exchange without a directly observable loop–loop interaction (78). Chemical probing of infected cells and virions indicated that a kissing loop interaction of the anticodon and Loop I could precede and drive the annealing of the two RNAs (79). Therefore, the unwinding of the tRNA^{Lys3} anticodon stem and that of the Loop I stem may occur subsequent to a kissing loop interaction. Whatever the chronology of the steps in forming a duplex, the interaction between the U-rich anticodon loop of the primer tRNA^{Lys3} and the A-rich Loop I of HIV is stabilized by the tRNA’s natural modifications.

HIV subtypes include the A/G forms predominantly from Africa, and the B form predominantly from Europe and North America, representing almost all sequenced isolates. These isolates use tRNA^{Lys3} as the primer for reverse transcription, as do all lentiviruses. In addition, they have in common the A-rich Loop I sequence bound by the tRNA^{Lys3} anticodon with the one difference of the B form having the G166G167 substitution for the A166A167 in the A/G subtype. The VBL/ADF duplex had a G166U34 wobble pair adjacent to a modified G167ΨU35 base pair, and exhibited a stability little different from that of the ADF-s1U34 bound to the VGL. The Loop I of the viral A/G subtype was protected from chemical probing in the initiation complex for reverse transcription (79). However, the Loop I of the viral B subtype was not protected. Therefore, the interaction of the tRNA^{Lys3} anticodon with the Loop I of the viral B subtype may not be as stable as indicated by equilibrium binding experiments. It is possible that our in vitro system might not adequately reflect the in vivo assembly of the initiation complex for the B subtype of the virus.

The anticodon loop nucleoside sequences of tRNA^{Lys1,2} are identical to that of tRNA^{Lys3} except for a C34 substituting for cmc’sU34 and tA for m5tA37. Though the C34 that would pair with G167 of the VBL would be expected to improve the stability of the duplex, C34 would miss pair with A167 of the VGL and the stability of the duplex would decrease. The tRNA^{Lys1,2} are not used as the primer in the natural viral isolates. The viral primer binding site, the A-rich Loop I sequence and the recruitment of the host cell tRNA^{Lys3} by the HIV
proteins all play important roles in maintaining the virus’ dedication to tRNA_{Lys} as the primer of reverse transcription. The detailed biochemistry and structural biology of the recruitment and relaxation of the tRNA_{Lys} structure and its annealing to the HIV and other lentivirus genomes are yet to be determined.

**Accession number**

The coordinates of the 10 lowest energy structures, NMR restraints and VGL/ADF-s\(^3\)U\(_{34}\)-\(\Psi_{39}\) chemical shifts have been deposited in the Protein Data Bank (accession number: 2K7E) and in the Biological Magnetic Resonance Bank (BMRB entry: 15915). This material is available free of charge via the Internet at http://pubs.acs.org.

**SUPPLEMENTARY DATA**

Supplementary Data are available at NAR Online.

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