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An RNA Complex of the HIV-1 A-Loop and tRNA^{Lys,3} Is Stabilized by Nucleoside Modifications

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The transcription initiation complex of HIV-1 nominally contains the specific viral reverse transcriptase (RT), the HIV-1 viral genome, and the specific tRNA primer, human tRNA^{Lys,3}.[1] The principal interaction between the primer and template RNAs consists of a Watson−Crick complex involving the 18 3′-nucleotides of the tRNA and the complementary primer-binding-site (PBS) in the 5′ untranslated region of the HIV genome. Additional interactions between these two RNAs have been proposed, involving either the tRNA anticodon or the T-ψ-C domains.[2,3] Extensive biochemical and genetic investigations have implicated an RNA−RNA interaction between the tRNA anticodon and an "A-rich" loop 5′ to the HIV PBS as necessary for maintenance of tRNA primer identity and progression from the initiation phase to elongation during reverse transcription.[4,5] The tRNA anticodon stem−loop (ASL) domains from both the *Escherichia coli* and human lysine tRNAs have been shown to adopt canonical U-turn structures only when the natural modified nucleosides are present.[6,7] To determine whether the hypermodified nucleosides at positions 34 and 37 stabilize the interaction between the tRNA ASL and the HIV-1 A-loop, we have measured the affinity of a model RNA complex using surface plasmon resonance. Using synthetic phosphoramidite chemistry we were able to site-specifically introduce modified nucleosides and determine that, indeed, unmodified tRNA ASLs fail to bind the HIV-1 A-loop while specific modifications provide dramatic stabilization of the tRNA/A-loop complex (Table 1). We have described methods for making RNAs containing either the ms^{2}t^{6}A, or mcm^{5}s^{2}U nucleosides,[8,9] however simultaneous incorporation of these highly functionalized nucleosides proved to be difficult. The base-labile nitrophenylethyl carboxylate-protecting group proved unworkable; therefore, the trimethylsilylethyl protecting strategy developed by our group for t^{6}A nucleoside was employed for ms^{2}t^{6}A.[10,11] An overview of our new strategy for synthesizing the protected phosphoramidite of ms^{2}t^{6}A is shown in Scheme 1. The sugar-protected phenyl carbamate **6** of ms^{2}t^{6}A nucleoside was synthesized in an overall yield of 28% from 2-methylthioadenosine and 1-*O*-acetyl-2,3,5-tri-*O*-benzoylribofuranose as described previously.[9,12] The carbamate was treated with L-threonine to furnish the sugar-protected ms^{2}t^{6}A nucleoside **7** using the method of Hong and Chheda for t^{6}A nucleoside.[10,13] The remaining synthetic transformations followed the protocol we established for t^{6}A, although the thiomethyl group surprisingly affected the chemistry such that most reaction steps needed re-optimization. A detailed description of the RNA synthesis protocol, along with the procedure we developed for maintaining the mcm^{5}s^{2}U and ms^{2}t^{6}A side chains during RNA deprotection is included in the Supporting Information.

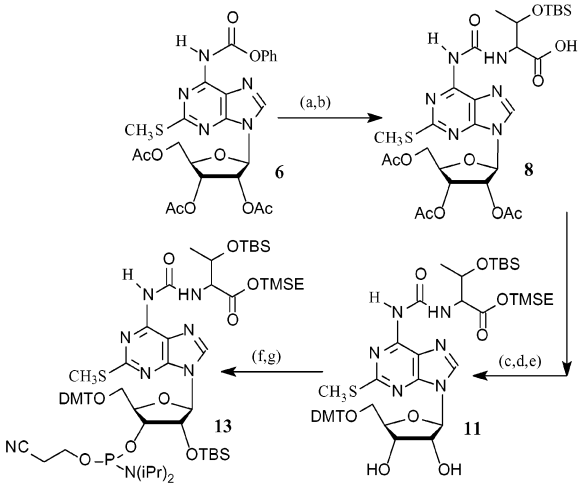
Modifications within the tRNA ASL domain are necessary for formation of the correct transcription initiation complex, but the contributions of individual nucleosides are unknown.[2,4] The 17-nucleotide A-loop hairpin from the HIV Mal isolate (Figure 1) has been shown to have a three-dimensional structure similar to that of a canonical tRNA ASL with a π-type U-turn and stacking of the 3′-adenosine nucleotides.[14] To study the interaction of the A-loop RNA hairpin with tRNA^{Lys} hairpins containing modified nucleosides from *E. coli* and human tRNA^{Lys}, we synthesized the

Table 1. Equilibrium Binding Constants of tRNA^{Lys} ASLs Complexed with the HIV-1 A-Loop[a]

tRNA^{Lys} ASL[b]	K_D (1 M NaCl)	K_D (100 mM NaCl)
mcm^{5}s^{2}U34,ms^{2}t^{6}A37,ψ39	71 μM	n.a.[c]
mnm^{5}s^{2}U34,t^{6}A37, ψ39	73 μM	150 μM
mnm^{5}s^{2}U34, ψ39	370 μM	1.48 mM
ms^{2}t^{6}A37, ψ39	350 μM	n.a.
t^{6}A37, ψ39	2.4 mM	n.d.[d]
mcm^{5}s^{2}U34, ψ39	2.6 mM	n.d.

[a] Binding reactions were done at 4 °C in 20 mM Tris buffer, pH 7.4 containing 10 mM $MgCl_2$ and either 0.1 or 1.0 M NaCl. [b] All tRNA ASLs had the primary sequence shown in Figure 1a. [c] n.a. (not acquired) These data were only collected at 1 M NaCl where the affinity is the highest to conserve material. [d] n.d. (not detected) No binding could be detected for this complex at 100 mM NaCl.

Scheme 1[a]



[a] (a) L-threonine, pyridine, 35 °C, 89%; (b) TBS-triflate, Et_3N, DCM, 82%; (c) trimethylsilylethanol, DCC, DMAP, CH_2Cl_2, 0 → 25 °C, 90%; (d) 2M NH_3/CH_3OH, 92%; (e) DMT-Cl, pyridine, 89%; (f) TBS-Cl, imidazole, pyridine, 70%; (g) N, N-diisopropylethylamine, 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite, DMAP, THF, 75%.

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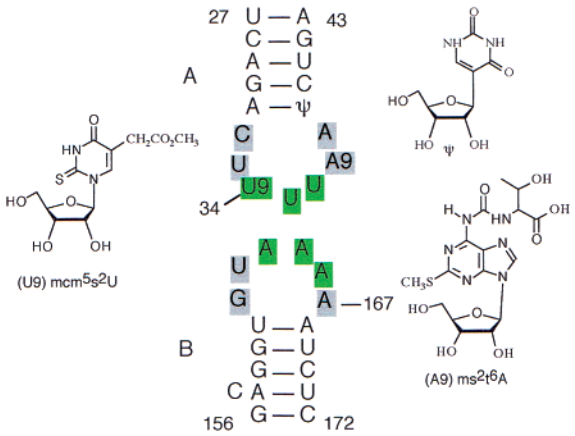

Figure 1. RNA hairpins which form the HIV primer−template complex. A dimer interface composed of three base pairs as seen in the tRNA^{Asp} crystal structure would involve pairing between the residues in green. (A) Secondary structure of the human tRNA^{Lys,3} ASL. (B) HIV-1 A-loop hairpin.

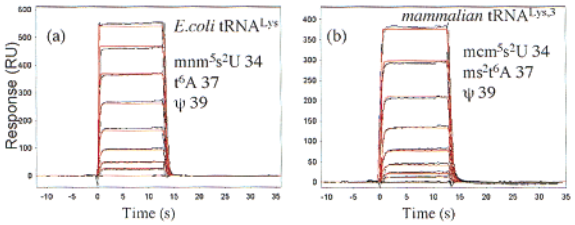

Figure 2. Biacore binding data of modified tRNA ASL 17-mers binding to an immobilized A-loop hairpin with the sequence of the Mal-1 A-loop. Global fits of the data (in black) to a 1:1 interaction model are shown in red. (a) Native *E. coli* tRNA^{Lys} bound with a $k_a = 42500 \pm 500$ M^{-1} s^{-1} and $k_d = 3.1 \pm 0.3$ s^{-1} giving a $K_D = 73$ μM. (b) Native Human tRNA^{Lys,3} bound with a $k_a = 35000 \pm 400$ M^{-1} s^{-1} and $k_d = 2.5 \pm 0.2$ s^{-1} giving a $K_D = 71$ μM.

A-loop hairpin with a 5′-biotin group and immobilized this RNA on a Biacore SA chip. Most of the binding experiments were conducted at 4 °C and 1 M NaCl to facilitate RNA−RNA interactions and detect complexes for the weaker binders. The two native tRNA ASLs had surprisingly similar affinities of ∼70 μM (Figure 2), yet the contributions of each nucleoside modification toward stabilizing the interaction were distinct. The mnm^{5}s^{2}U *E. coli* modification is strongly stabilizing, while the corresponding mcm^{5}s^{2}U modification effect is almost 10-fold weaker. The 2-thiouridine modification in both nucleosides should stabilize the 3′-endo sugar conformation of U34 and promote base-pairing with a complementary A.[15] Isel et al. selectively oxidized the wobble nucleoside in full-length tRNA^{Lys,3}, demonstrating the importance of the sulfur in the A-loop/tRNA interaction.[16] However, we have observed that the methyl ester side chain of mcm^{5}s^{2}U is relatively poor at stabilizing the tRNA U-turn compared to the amine side chain of mnm^{5}s^{2}U (not shown). It appears that the human tRNA uses the additional thiomethyl modification on A37 to compensate for the less efficient mcm^{5}U34 side chain. The thiomethyl effect on codon−anticodon interactions has also been proposed for ms^{2}i^{6}A on the basis of data from the complementary tRNA system.[17]

The effect of mcm^{5}s^{2}U and ms^{2}t^{6}A on the thermodynamic stability of the free tRNA hairpins was also measured to understand whether changes within the tRNA structure itself could be correlated with effects on the primer/template interaction (Supporting Information). As was seen for t^{6}A37, ms^{2}t^{6}A37 decreases the overall ASL T_m, but these modifications both stabilize complex formation.[7] Addition of mcm^{5}s^{2}U34 recovers some of the T_m loss caused by ms^{2}t^{6}A37 presumably by promoting stacking among the 3′-nucleosides of the anticodon loop. Preliminary NMR experiments in our laboratory indicate that the decrease in T_m from ms^{2}t^{6}A37 comes from disruption of noncanonical base-pairing interactions within the tRNA loop, which we believe then promotes the open tRNA conformation that binds to the A−loop.

The model of complementary tRNAs suggests a hypothesis for the HIV A-loop/tRNA interaction since the two native lysine tRNAs and the A-loop hairpin are known to adopt canonical tRNA conformations.[6,7,14] A complex with the topology proposed for complementary tRNAs presents a structural framework that would explain the nucleoside modification effects.[18] Such a dimer interface was observed in the crystal structure of tRNA^{Asp}.[19] The wobble modification has a key role in stabilizing the tRNA U-turn along with the threonyl side chain of t^{6}A. For ms^{2}t^{6}A, the thiomethyl group would then be positioned to stack over the adenosines of the complementary A-loop hairpin and reinforce what should already be a substantial adenosine 3′-end stacking effect.[20]

The primer−template complex is a potential target for HIV-1 therapeutics, and the rich functionality of the modified nucleosides involved in this complex may present unique binding sites for inhibitors of HIV-1 reverse transcription.

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Supporting Information Available: Synthetic procedures and characterization of nucleosides and RNA, CD spectra, Table of T_m data (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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