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A comparative binding study of modified Bovine Immunodeficiency Virus TAR RNA against its Tat peptide

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Abstract—Besides generating novel binding peptides or small molecules to their RNA target, successful design of chemically modified RNA constructs capable of tighter binding with their binding peptides is also of significant importance. Herein, the synthesis and binding studies of a series of both wt and mutant bovine immunodeficiency virus (BIV) TAR RNA constructs against its Tat peptide are reported. Understanding the requirements that enable RNA construct binding properties, especially at the hairpin loop or internal bulge, would afford potential therapeutic approaches to control the BIV life cycle.

1. Introduction

The recognition event between RNA and peptide is integral to many biological events, such as in the proper regulation of gene expression, and in ensuring successful RNA splicing and protein translation. A huge amount of information has been generated pertaining to how peptides specifically recognize RNA over the last decade.[1–4] These information are extremely important in unraveling the paradigm of RNA–peptide interaction, since macromolecules such as peptides and RNA are able to fold into numerous unpredictable tertiary conformations. Understanding these various molecular structural complexes would then render valuable information to design high-affinity peptide binders to their nucleic acid targets.

The most prevalent mode of peptide–RNA complexes reported to date involves insertion of α-helical peptides into either the minor or major grooves of a double helical RNA.[5] One well-studied example of this particular recognition mode is the interaction of the human immunodeficiency virus (HIV) transactivation response (TAR) RNA construct with its Tat protein.[6,7] The Tat proteins are especially important in ensuring successful virus replication, in which they were observed to bind to the TAR sequences located within the long terminal repeats at the 5′-ends of the viral mRNA.[4] The recognition of Tat peptides with its TAR RNA requires the prebinding of an accessory protein (cyclin T1) to the TAR RNA hairpin loop, which presumably stabilizes the TAR RNA tertiary structure for optimum binding affinity against Tat peptide.[8] Recently, a related yet contrary system to HIV was reported in the bovine immunodeficiency virus (BIV) system.[9,10] Instead of adopting an α-helical conformation, the BIV Tat peptide (AA 65-81) assumes a β-sheet upon binding to its target BIV TAR RNA construct.[6,9–12] To date, other reported cases in which β-sheet conformation peptide or protein conformation is adopted is being observed is $tRNA^{asp}$ synthetase and U1–U1A complexes.[13,14] Previous published structural data from NMR experiments have indicated that the 17 AA BIV Tat peptide adopts a β-sheet conformation upon binding to the 28 nucleotides (nt) TAR RNA.[6,11] Within the TAR RNA (construct **A**), there are two unpaired RNA nucleotides in U10 and U12 (labeling scheme is shown in Fig. 1a). It is through these two unpaired uridine nucleotides that enable the opening of the major groove of an otherwise regular RNA helix, subsequently allowing insertion of the β-ribbon Tat peptide structure (Fig. 1b). The conformation of the two stranded anti-parallel β-ribbons loosely matches that of a double-stranded nucleic acid, which allows

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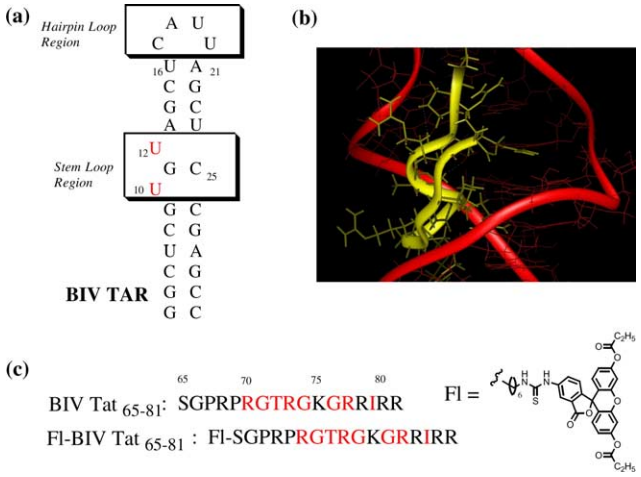

Figure 1. (a) Secondary structure of the wt BIV TAR construct used in this study, (b) a close-up three dimensional NMR structure of the BIV TAR RNA–Tat peptide complex,[11] and (c) sequence of the 17-mer BIV Tat peptide and the fluorescein-labeled (N-terminus) 17-mer Tat peptide used in this study.

easy fit into a wide variety of nucleic acid structures, such as the minor grooves of both DNA and RNA, DNA major grooves, and distorted RNA major grooves.[15] It was also observed that the 2′-OH groups in the RNA minor grooves are also regularly spaced so as to facilitate hydrogen-bonding contacts between the backbone carbonyl groups from the anti-parallel sheets of the peptide and its RNA target.

Regarding the precise atomic requirements for the Tat peptide recognition process, biochemical data have indicated that eight amino acids (three arginines, three glycines, one threonine, one isoleucine; highlighted in Fig. 1c) in the arginine-rich domain of the peptide is integral in maintaining specific contacts with the internal bulge of the TAR RNA.[12] Furthermore, the recognition of the BIV Tat peptide–TAR RNA does not require the pre-binding of accessory proteins, for example, cyclin T1, as is required for the HIV system.[16,17] We thus hypothesized that the hairpin loop region of the BIV TAR RNA construct can be derivatized to afford higher peptide-binding affinities. Additionally, we are also interested in examining how flexible the stem loop is, that is, the two single-uridine bulged stem loop, during its binding interaction with the BIV Tat peptide. In this communication, the synthesis and binding studies of a series of mutant BIV TAR RNA constructs against its Tat peptide is reported. The mutant TAR RNA constructs were broadly divided into two categories: (i) constructs without hairpin loop or with modified RNA loop, and (ii) constructs with modified stem loop region. Understanding the requirement that confers RNA-binding properties (especially at the hairpin loop or stem loop) would enable better design of RNA constructs against the BIV Tat peptide.

2. Materials and methods

With RNA constructs having modified hairpin loops, we include a previously studied semi-synthetic RNA construct, in which a stilbenedicarboxamide moiety was employed to replace natural-nucleotide RNA loop.[18] In addition, we had also incorporated TAR RNA mutant constructs previously studied by Frankel and co-workers.[23] To determine the dissociation constants of all the RNA constructs (secondary structures are shown in Figs. 2 and 3) with BIV Tat peptide, a previously described fluorescence binding method was employed.[24–27] Fluorescence binding method has been previously demonstrated to afford direct and quantitative measurements of peptide–RNA, small molecule–RNA and RNA–RNA interactions.[24–27] Briefly, the fluorescence methodology involves studying the changes in anisotropy values of the fluorescently labeled peptide with

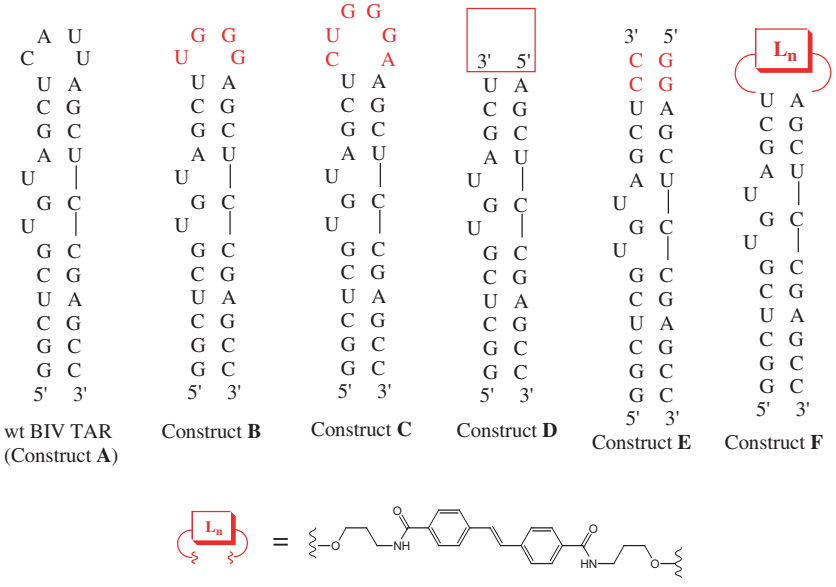

Figure 2. Secondary structures of the mutant BIV TAR RNA constructs with modification at its hairpin loop.

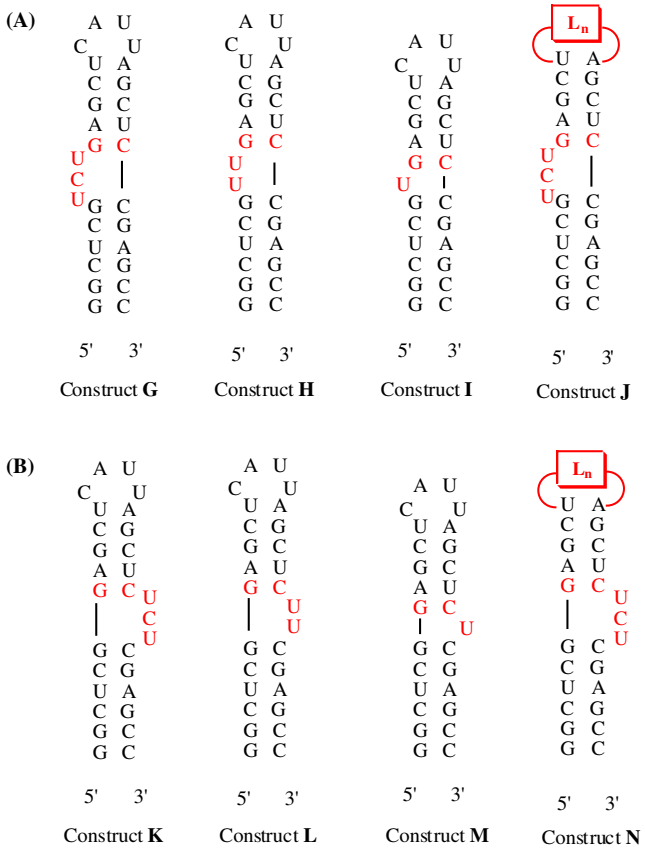

Figure 3. Secondary structures of the mutant BIV TAR RNA constructs with its stem loop modified either at the (A) left strand or the (B) right strand.

increasing concentration of the target RNA molecules. Gel-purified RNA constructs **A**–**F** were obtained commercially (Dharmacon Research; Lafayette, CO) and used directly in the fluorescence study. Exceptions are RNA constructs **A** and **J**, in which the stilbenedicarboxamide moiety was incorporated according to previously published procedures.[19–22] The fluorescently labeled BIV Tat peptide, in which its N-terminal was labeled with the fluorescein fluorophore, was also obtained commercially (Biosynthesis; Lewisville, TX). Upon receiving the purified peptide, its purity was re-analyzed by reverse-phase HPLC and mass spectrometry. The peptides were subsequently suspended in 10 mM Tris–HCl (pH 7.0) and stored frozen in a −20 °C freezer until use.

All fluorescence binding studies were performed in an incubation buffer containing 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 20 mM HEPES (pH 7.40). In each binding studies, 10 nM of the fluorescently labeled BIV Tat peptide was gradually titrated with an increasing concentration of the RNA constructs. In a typical experiment, when increasing concentration of the wt TAR RNA construct **A** was titrated with 10 nM of the fluorescein labeled BIV Tat peptide in a quartz curvette (1 cm pathlength), the anisotropy fluorescence curve (525 nm) was observed to increase correspondingly and saturate around 60 nM of TAR RNA

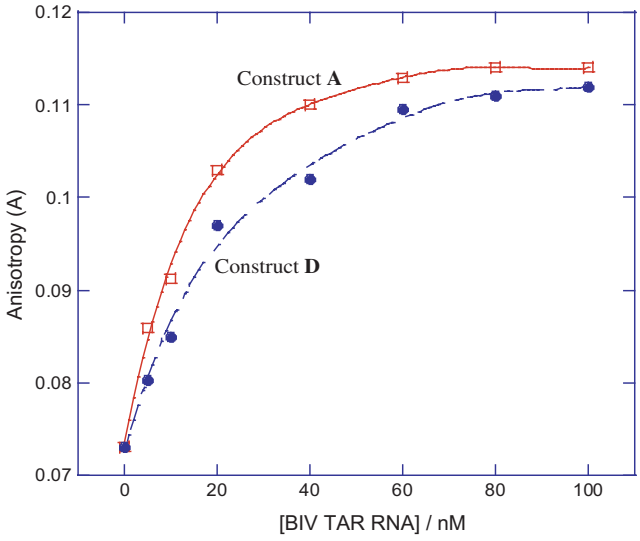

Figure 4. Representative fluorescence anisotropy plots of fluorescein-labeled BIV Tat peptide (10 nM) as a function of increasing concentration of wt BIV TAR RNA construct **A** and mutant TAR RNA construct **D**. Curve fitting for the two obtained plots gave 1.5 nM as the K_d for construct **A** and 53.5 nM as the K_d for construct **D**.

(Fig. 4). Using a previously described curve fitting equation,[24] the dissociation constant (K_d) between the BIV Tat peptide and TAR RNA was calculated to be 1.5 ± 0.1 nM. This value is in good agreement with our previously obtained data using similar fluorescence method,[18,25] and also through gel shift assay results obtained by Frankel and co-workers.[23] This indicates that the fluorescence method is sufficiently robust, and the same experimental approach was subsequently repeated with the remaining RNA constructs **B**–**J**. The fluorescence curve obtained for all RNA construct studied are all observed to achieve saturation upon the addition of all the TAR RNA studied, with the weaker binding RNA constructs requiring higher concentration to achieve saturation (for comparison purposes, constructs **A** and **D** are represented in Fig. 4). Assuming the binding stoichiometry for all the RNA constructs studied is 1:1, the same curve fitting equation[24] was applied for all the mutant BIV TAR RNAs to obtain their respective K_d values (all the K_ds are summarized in Table 1).

3. Results and observations

First, for the RNA mutants with modified hairpin loop (Fig. 2), it was observed that both constructs **B** and **C** behaved similar to the previous observation by Frankel and co-workers,[23] in which construct **B** demonstrated similar K_d to construct **A**, and construct **C** having a ~7-fold decrease in its Tat peptide binding capability. The removal of the loop region (construct **D**) resulted in a 25-fold decrease in its K_d value, however the binding affinity can be fully restored when the duplex end was stabilized with two additional G–C base pairs (construct **E**).

Second, for the RNA mutants with the stem loop modified (Fig. 3), it was observed that RNA constructs **G**

Table 1. Summary of the K_ds of the modified BIV TAR RNA constructs with the fluorescein-labeled BIV Tat peptide: [A] hairpin loop and [B] stem loop

TAR RNA constructs[a]	K_d (nM)
[A] Hairpin loop mutants	
A	1.5 ± 0.1
B	1.9 ± 0.1
C	13.4 ± 1.9
D	53.5 ± 6.8
E	2.2 ± 0.2
F	43.4 ± 4.8
[B] Stem loop mutants	
A	1.5 ± 0.1
G	1.2 ± 0.2
H	1.1 ± 0.1
I	11.0 ± 0.9
J	38.9 ± 3.4
K	44.6 ± 5.7
L	64.3 ± 7.1
M	58.5 ± 5.5
N	64.9 ± 8.3

[a] See Figures 2 and 3 for the secondary structures of the RNA constructs **A**–**N**.

and **H**, in which both contained only a single enlarged stem loop (3 nt and 2 nt, respectively), demonstrated comparable binding affinities when compared to the wt BIV TAR RNA (construct **A**) for Tat peptide binding. This observation slightly deviates from the previously reported 3-fold improvement in binding affinity observed for RNA mutant constructs **G** and **H** by Frankel and co-workers.[23] The slight discrepancy in the K_d values may be attributed to the different method and conditions utilized in these binding studies. Of particular interest is that when the bulge is being located on a different strand (constructs **K**–**N**), their Tat peptide binding abilities are drastically reduced. These results indicate that there is a certain degree of stereospecificity to the Tat peptide–RNA interaction process. This observation is in agreement with previous observations by Rando and co-workers.[28]

In summary, binding affinities were nearly abolished when the hairpin loop region of the BIV TAR construct is either being replaced or removed (constructs **B**, **C**, and **D**). We hypothesized that upon the removal of the hairpin loop (construct **D**), the resulting duplex strand is unable to maintain an 'optimized conformation' at its Tat peptide binding RNA stem loop. However the inclusion of the two G–C base pairs at the end of the RNA duplex is able to stabilize the overall duplex structure, restoring the 'optimized conformation' of Tat peptide binding site. This observation suggests that G–C base pair at the duplex terminal is a viable alternative approach to the commonly utilize UUCG nucleotides to stabilize RNA duplex strands. As for constructs **F** and **J**, in which both constructs contain the stilbenedicarboxamide linker as replacement for the loop region, demonstrate drastic decrease in binding affinities of ~19–24-folds toward the BIV Tat peptide. Again, we hypothesized that the inclusion of the stilbenedicarboxamide linker as replacement for the hairpin loop may induce further destabilization of the duplex strands, thus affecting the conformation of the binding site for binding to the Tat peptide. Furthermore, the enlargement of the two single-nt bulges (construct **A**) into a single two- or three-nt stem loop (constructs **G** and **H**) has been observed to lead to an improvement in Tat peptide binding affinity. This observation suggests that modifications can be tolerated at the stem loop, but not at the hairpin loop. However, results from the 'mirror-image' stem-loop constructs **K**–**N** suggest that modifications be performed with stepwise increments as stereospecificity is an important factor in the binding event.

4. Conclusion

The design of high binding semi-synthetic RNA constructs that could potentially be applied to control the translation process in vivo have been investigated. The data presented in this letter reinforces the notion that appropriate conformation of the RNA-binding site is extremely important to its ligand binding ability. Changes such as removing or replacing the loop structure of BIV TAR RNA led to drastic decrease in their Tat peptide binding abilities. These modifications are thought to alter the conformation of the RNA stem loop, which had previously been established through NMR experiments to be critical in binding the Tat peptide. However, direct modifications to the stem loop such as expanding the size of the internal bulge improved the binding affinity for Tat peptide. Taken together, these results indicate that it is possible, through appropriate placement of nucleotides at the stem loop, could proof beneficial toward our effort in designing and synthesizing RNA decoys that could better regulating gene expression at the translation level.

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