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A comparative binding study of BIV Tat peptide against its TAR RNA duplex, RNA–DNA heteroduplex and DNA duplex

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Abstract—Association between RNA and DNA strands to form RNA–DNA heteroduplex is important in many biological processes such as transcription, DNA replication and reverse transcription. Herein, binding affinities of a 17-mer BIV Tat peptide is compared with TAR DNA duplex, TAR RNA–DNA heteroduplex and TAR RNA duplex. It was observed that binding affinities of Tat peptide is comparable against DNA–RNA heteroduplex and RNA duplex, whereas DNA duplex binding is decidedly poor.

Understanding the paradigm of how RNA molecule is able to maintain tight interaction with its binding ligands has been of interest in the last few decades.[1–6] The ability to regulate RNA functions via small molecules or short peptides will have major implications in controlling precise biological processes.[7] For example, RNA–nucleoprotein interaction is involved in ensuring successful replication of the cell cycle, and participates in the expression of genes through catalyzing the maturation of mRNAs via ribozymes. Most notably, RNA is the essential biomolecule responsible for protein synthesis process.[8,9] RNAs generally need to adopt an accurate tertiary fold such that their binders can recognize the binding pocket within the RNA targets. Various classes of RNA-binding small molecules and peptides have been extensively studied;[1–6] however attempts to generate a unifying model for the RNA-recognition process remains elusive. Most probably due to the ease in synthesizing DNA, interaction of DNA molecules is much better understood as compared to RNA. Recently, there is an increasing number of reports of 'cross-over' binding of traditional DNA-binding ligands to RNA targets, and vice versa. For example, it was observed that traditional DNA-binding ligands such as Hoescht 33258 or DAPI are able to bind RNA molecules.[10,11] In addition, RNA binders such as aminoglycoside antibiotics have been observed to enhance efficient triplex DNA formation.[12–14] However, these observations do not necessarily imply that the rules involved in DNA binding could simply be extended for predicting how RNA molecules interact with their binding ligands, and vice versa. In this letter, we report on how conformations of nucleic acid will affect their ligand binding properties.

The tertiary structure and hybridization properties of both DNA and RNA have been well established. Usually, RNA duplex structures adopt the A conformation while DNA duplex adopts the B conformation.[15] Since RNA–DNA heteroduplexes are observed to primarily adopt the A conformation,[15] we aim to investigate if RNA duplex binding peptides are also able to bind to the corresponding DNA duplex or RNA–DNA heteroduplex that contains similar sequences. Our laboratory has been interested in developing novel nucleic acid constructs against naturally-occurring RNA binders.[16] The ability to utilize DNA or DNA–RNA heteroduplex as targets for naturally-occurring RNA binders could offer potential therapeutic approaches in biological pathways involving RNA–protein or RNA–small molecule interactions. Associations between RNA and DNA strands to form RNA–DNA heteroduplexes are implicated in many important biological processes such as transcription, DNA replication and reverse transcription.[17,18] Heteroduplex constructs have drawn special attention because annealing of RNA to DNA is an intermediate state in the conversion of the retroviral RNA genome. In addition, they can be used to selectively block gene

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expression in antisense therapy.[18] Thus, hybridization of RNA with DNA has been actively pursued as potential antisense drugs. There are numerous reports over the last decades in which the furanose ring, the phosphate backbone and the bases of oligonucleotides have been modified in order to generate modified oligonucleotides that will demonstrate higher affinity to the target RNA strand. Instead of modifying the oligonucleotide funtionalities, we hypothesized that novel ligands could be introduced to enhance the stability of the RNA–DNA heteroduplexes. The study of heteroduplexes have been active over the last few years,[19–24] and the generation of tight-binding ligands against heteroduplex may offer a more efficient antisense system without the need to introduce modified oligonucleotide.

In this letter, the BIV Tat peptide–TAR RNA-binding interaction was employed as a model system to investigate the effect of nucleic acid's secondary structure on its Tat peptide binding capability. The BIV Tat–TAR interaction is of particular interest as it represents a new RNA–protein recognition structural motif.[25,26] In specific, the 17-mer BIV Tat peptide has been observed to fold into a β-hairpin structure upon binding to its 26-mer TAR RNA target.[27–29] There are two unpaired RNA nucleotides in the BIV TAR RNA that allows the opening of the major groove of an otherwise regular RNA helix, hence enabling the insertion of the β-hairpin Tat peptide structure. The shape of the two stranded antiparallel β-ribbons closely matches that of a double-stranded nucleic acid, which allows 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.[27–29] Understanding the requirements that enable BIV Tat peptide binding to its TAR RNA had prompted us to address if the corresponding RNA–DNA heteroduplex or DNA duplex of TAR RNA are able to retain Tat peptide binding.

We have recently observed that TAR RNA without its 4 nt hairpin loop drastically reduces its binding affinity to Tat peptide by ~12-fold.[30] However, binding affinity could be fully restored upon replacing the removed 4 nt hairpin loop with two additional G–C base pairs.[30] The ability to convert the 26 nt hairpin TAR RNA to a duplex TAR RNA construct without drastically affecting its Tat peptide binding ability enabled us to investigate the binding effect of a DNA–RNA hybrid TAR construct. In this study, each of the duplex TAR RNA strands has been sequentially replaced with a corresponding DNA strand to afford constructs **B** and **C** (Fig. 1B), whereby construct **B** contains DNA strand 1 (D1, bearing two uridine mismatched nucleotides) annealed to RNA strand 2 (R2). On the other hand, construct **C** contains DNA strand 2 (D2) annealed to RNA strand 1 (R1). The binding affinities of constructs **A**–**D** to the BIV Tat_{65-81} peptide was analyzed by the fluorescence binding method. Pre-purified N-terminus labelled BIV Tat peptide was obtained commercially (Biosynthesis Inc., Lewisville, TX) and its purity was re-analyzed by RP-HPLC when received. Gel-purified RNA and DNA strands utilized to afford constructs **A**–**D** were also obtained commercially (Dharmacon,

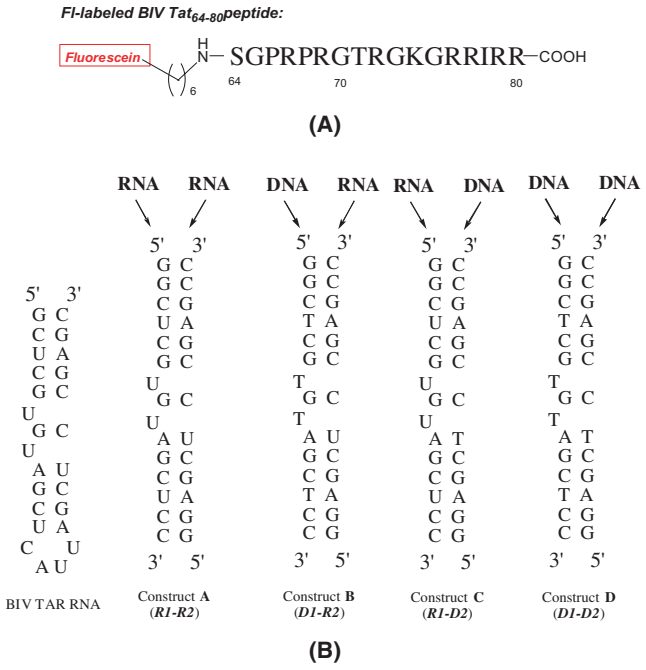

Figure 1. (A) Sequences of the N-terminus fluorescein-labelled BIV Tat_{64-80} peptide, and (B) secondary structures of the wt and various BIV TAR RNA and DNA duplexes employed in this study.

Corvalis, OR) and used as received. The annealing process for various nucleic acid strands were performed at 95 °C for 4 min and cooled to 25 °C over 30 min in an Eppendorf thermal cycler. Upon titration of construct **A** with 10 nM of fluorescein-labelled Tat peptide in a binding buffer consisting of 10 mM Na_2PO_4, 1 mM $MgCl_2$, 100 mM NaCl, pH 7.20, the anisotropy value of the Fl-labelled Tat peptide was observed to increase and reach saturation with ~55 nM of construct **A** (excitation at 490 nm and monitored at 520 nm). The anisotropy measurements were performed at 15 °C using a Yobin–Horiba Fluorolog-3 (Edison, NJ) and a 0.3 cm pathlength quartz curvette. Repeating the same procedures with constructs **B** and **C**, a similar trend in which anisotropy values were observed to increase and achieve saturation with increasing construct concentration. However, a higher concentration of ~65–70 nM is required for both constructs **B** and **C** to achieve saturation in their anisotropy values (Fig. 2C). As for construct **D**, there was a negligible change in the anisotropy value when higher concentration of construct **D** is titrated with Fl-labelled Tat peptide. Upon applying a previously described curve-fitting equation[31] on the anisotropy values obtained for the titration experiment for the three constructs **A**–**C**, dissociation constants values (K_d) of 1.4 ± 0.1, 3.7 ± 0.3, 4.1 ± 0.3 nM were obtained, respectively. As there was no apparent binding interaction between construct **D** and the Tat peptide, no K_d value was obtained. K_d values of all the nucleic acid constructs to the BIV Tat peptide were summarized in Table 1.

Having observed that Tat peptide does indeed bind to constructs **A**–**C**, a thermal denaturation experiment was performed to investigate how the peptide binding

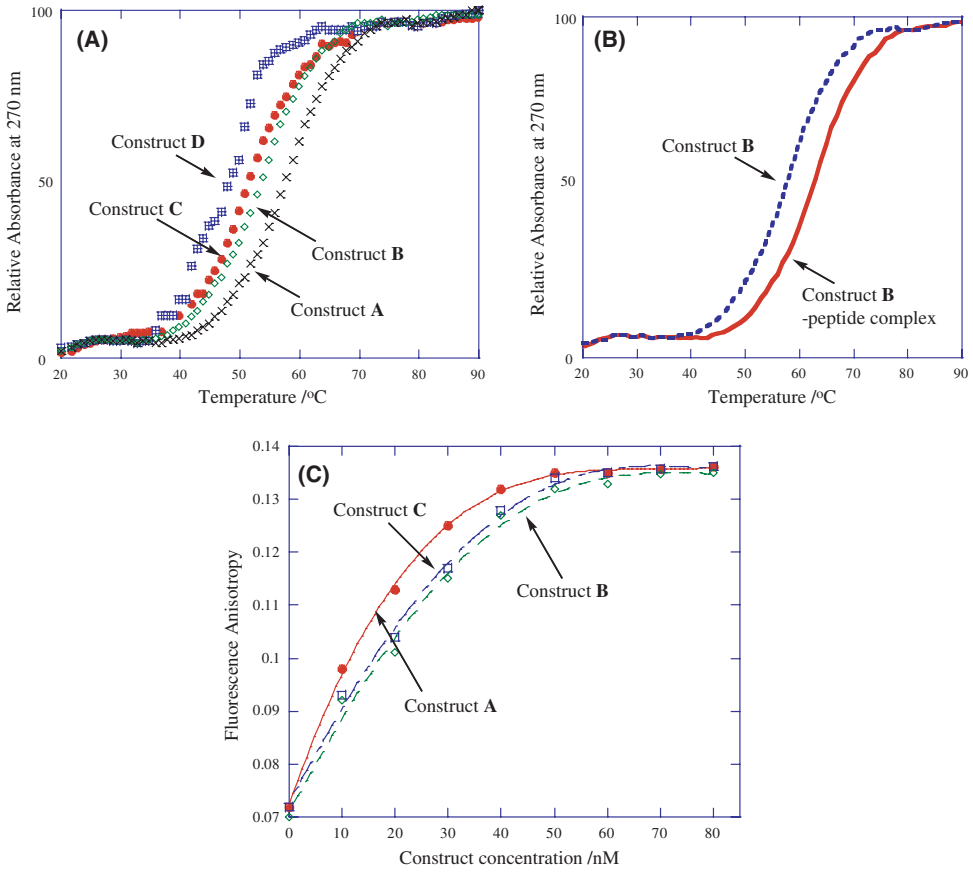

Figure 2. (A) Normalized UV melting curves of constructs **A**—**D**. Through curve-fitting analysis, the melting temperature (T_m) of the four constructs **A**–**D** are 57.1, 54.6, 53.5 and 47.8 °C, respectively, (B) increase in the T_m values of construct **B** (from 54.6 to 61.2 °C; ΔT_m = +6.6 °C) upon the addition of 3 equiv of BIV Tat peptide and (C) fluorescence titration of Fl-BIV Tat peptide (10 nM) as a function of increasing concentration of construct **A** (closed circle), construct **B** (open diamond) and construct **C** (open square).

Table 1. (A) Summary of the dissociation constants (K_d) of the binding of BIV Tat peptide to constructs **A**–**D**, and (B) summary of the melting temperature (T_m) of constructs **A**–**D**, before and after interacting with the Tat peptide and the changes in T_m values

	Construct **A** (R1–R2)	Construct **B** (R1–D2)	Construct **C** (D1–R2)	Construct **D** (D1–D2)
(A)				
Fl-BIV $Tat_{65–81}$ peptide	1.4 ± 0.1 nM	3.7 ± 0.3 nM	4.1 ± 0.3 nM	No measurable binding
(B)				
Without Tat peptide	57.1 °C	54.6 °C	53.5 °C	47.8 °C
With Tat peptide	64.4 °C	61.2 °C	59.9 °C	48.0 °C
ΔT_m	+7.3 °C	+6.6 °C	+6.4 °C	+0.2 °C

event affects the melting behaviour of constructs **A**–**C**. The denaturation UV plots for constructs **A**–**D** was performed with a Perkin–Elmer Lambda 25 equipped with a PTP-1 Peltier control (Norwalk, CT) with its UV measurements taken at 270 nm (binding buffer = 10 mM Na_2PO_4, 1 mM $MgCl_2$, 100 mM NaCl, pH 7.2) and a temperature increase of 1 °C/min. The obtained UV denaturation plot for constructs **A**–**D** is shown in Figure 2A. Through analysis of the obtained denaturation plot with the Perkin–Elmer TempLab software Ver. 2.0, T_m values for constructs **A**–**D** are 57.1, 54.6, 53.5 and 47.8 °C, respectively. Upon the addition of 3 equivalents (equiv) of the Tat peptide to the constructs **A**–**D**, the same denaturation experiment was repeated. A representative plot showing the increase in the T_m upon the addition of the Tat peptide to construct **B** is shown in Figure 2B. Again through analysis of the obtained UV denaturation plot with the Templab software, the T_m value for construct **A**–Tat peptide complex was found to be 64.4 °C. Similarly, the T_m values for construct **B**–Tat peptide and construct **C**–Tat peptide complexes were 61.2 and 59.9 °C, respectively, while the T_m value between construct **D** and Tat peptide is unchanged as there are no measurable interaction. In summary, the obtained data indicates that an increase of 7.3, 6.6 and 6.4 °C were obtained when Tat peptide was added to constructs **A**, **B** and **C**, respectively (data summarized in Table 1B), while no binding to construct **D** is observed.

To investigate if Tat peptide affects the secondary structure of nucleic acid constructs **A**–**D** upon binding, circular dichroism (CD) spectra were recorded for all the

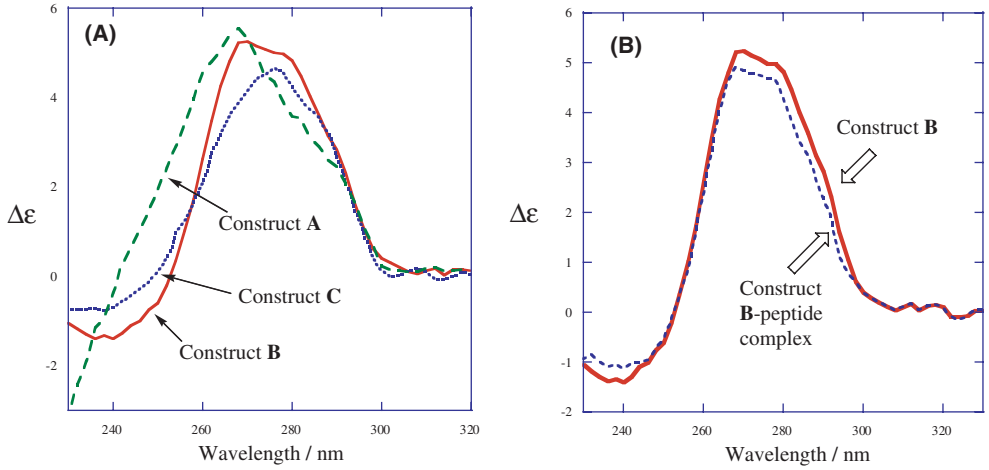

Figure 3. CD spectra of (A) constructs **A** (long-dashed line), **B** (solid line) and **C** (short dashed line), and (B) minor changes in the CD spectrum upon complexation of Tat peptide with construct **B**.

constructs before and after Tat peptide addition. The spectra were recorded at 5 °C with a JASCO J-810 CD spectropolarimeter equipped with a PTC-423S Peltier (Easton, MD) using a 0.1 cm pathlength quartz curvette. The CD spectra obtained for constructs **A**–**C** (20 μM, binding buffer = 10 mM Na_2PO_4, 1 mM $MgCl_2$, 100 mM NaCl, pH 7.2) exhibits a large positive band at 265 nm, which are indicative of a canonical A conformation (Fig. 3A). Of interest is the CD spectrum for construct **C**, which showed a shift in its positive band to ~278 nM. This strongly suggests that the conformation of construct **C** may be in a hybrid state between A- and B-conformation. As shown in Figure 3B, there is a minor decrease in the positive peak (265 nm) for construct **B** when 3 equiv of Tat peptide (60 μM) was added. This minor decrease in the positive peak (~265–270 nm) was also observed upon the complexation of the BIV Tat peptide with constructs **A** and **C**, and is consistent with other RNA–peptide interacting systems as previously reported.[32,33]

It is well established that the Tat protein in lentiviruses such as human immunodeficiency virus (HIV) and BIV enhances the transcription of the viral RNA by binding to the 5′-end of the transcribed mRNA, which is commonly known as TAR RNA.[28,34] Controlling the Tat–TAR binding process may potentially enable the disruption of the viral transcription process. Instead of developing small molecular antagonists against the TAR RNA,[35] we are interested in developing 'decoy' nucleic acid constructs against the Tat peptide. Observations described in this letter demonstrate that TAR RNA–DNA heteroduplex construct is a viable target for the 17-mer BIV Tat peptide. The binding affinity of the RNA–DNA hybrid duplex construct to the Tat peptide is observed to be only ~3-fold lower as compared to the RNA duplex target. Importantly, specificity is also observed as there are no binding between the Tat peptide with the corresponding DNA duplex. These results suggest that modification can be performed on the nucleic acid constructs as long as the overall nucleic acid conformational integrity is not compromised. This is clearly illustrated from the observation that an entire RNA strand within a RNA duplex can be substitute with a DNA strand without drastically changing its binding capabilities. These results have thus provided us with a starting platform to chemically alter the functionalities of the nucleotides to further our effort in developing novel nucleic acid targets for naturally-occurring RNA binding ligands.

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