

Molecular beacon aptamer fluoresces in the presence of Tat protein of HIV-1

Rika Yamamoto^{1–3} and Penmetcha K. R. Kumar^{1,*}

¹National Institute of Bioscience and Human Technology, and ²National Institute for Advanced Interdisciplinary Research, AIST Center, MITI, Tsukuba 305-8566, Japan, ³Institute of Applied Biochemistry, University of Tsukuba, Tsukuba 305-8572, Japan

Abstract

Background: We reported an aptamer, RNA^{Tat} that binds to the Tat protein of HIV with two orders of magnitude greater (133-fold) affinity over the TAR RNA of HIV-1 and specifically inhibits the Tat-dependent *trans*-activation of transcription, both *in vitro* and *in vivo* (demonstrated in the accompanying article, Yamamoto *et al.*, this issue pp. 371–388). We now report the use of aptamer-derived oligomers to analyze the Tat of HIV and the possible applications of such constructs in the field of biosensors.

Results: To make new molecular beacon, we constructed two RNA oligomers that derived from RNA^{Tat}. To one of the split RNA oligomers that forms a hairpin structure, the fluorophore and quencher were attached at the 5'- and 3'-ends, respectively. Specifically in the presence of Tat or its

peptides, but not in the presence of other RNA binding proteins, the two oligomers undergo a conformational change to form a duplex that leads to relieving of fluorophore from the quencher, and thus a significant enhancement of the fluorescence of fluorescein was observed.

Conclusion: A novel strategy for exploiting aptamers in the analysis of Tat (analyte) has been described. A similar strategy could be used to study other analytes such as proteins and small molecules. In addition, the molecular beacon aptamer requires half the length of target sequence (eight nucleotides) in comparison with molecular beacons. Thus, it is conceivable that we could insert an analyte-binding site into molecular beacons to convert them to signalling beacons.

Introduction

Molecular beacons, a nucleic acid motif that possesses a stem-loop structure, have been exploited to find a complementary target sequence (Tyagi & Kramer 1996). Molecular beacons essentially contain two structural components, a loop and a stem. The loop sequence serves as a probe, which is complementary to the target sequence, and the annealing of two complementary arm sequences that are flanked by the probe sequence forms the stem. One fluorescence and one non-fluorescent quencher are linked covalently at each end of the arm. The stem of the beacons brings the fluorophore and quencher into close proximity to each other, resulting in zero fluorescence. When the molecular beacon encounters a target molecule, it forms a probe–target hybrid that is stronger and more

stable than the stem in the hairpin. The resulting conformational change in the stem-loop oligomer forces the sequence of the two arms apart, thus permitting the fluorophore to fluoresce. Consequently, molecular beacons allow a real-time detection of specific nucleic acids without interrupting their reactions which is also applicable to living cells (Piatek *et al.* 1998; Matsuno 1998; Sokol *et al.* 1998). However, the most significant parameters affecting the conformational switch in the molecular beacon (Tyagi & Kramer 1996), appear to be the length of the arm and probe sequences. In addition, the probe sequence (target sequence) should be at least twice the length of each arm sequence in order to render the conformational change (Tyagi & Kramer 1996). Thus, the specificity of the molecular beacons depends entirely on the target sequence (loop of the molecular beacon).

In our previous studies, we isolated a novel RNA-binding motif (aptamer RNA^{Tat}; Fig. 1, right) that bound to the Tat protein of HIV with two orders of

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*Correspondence: E-mail: pkrkumar@nibh.go.jp

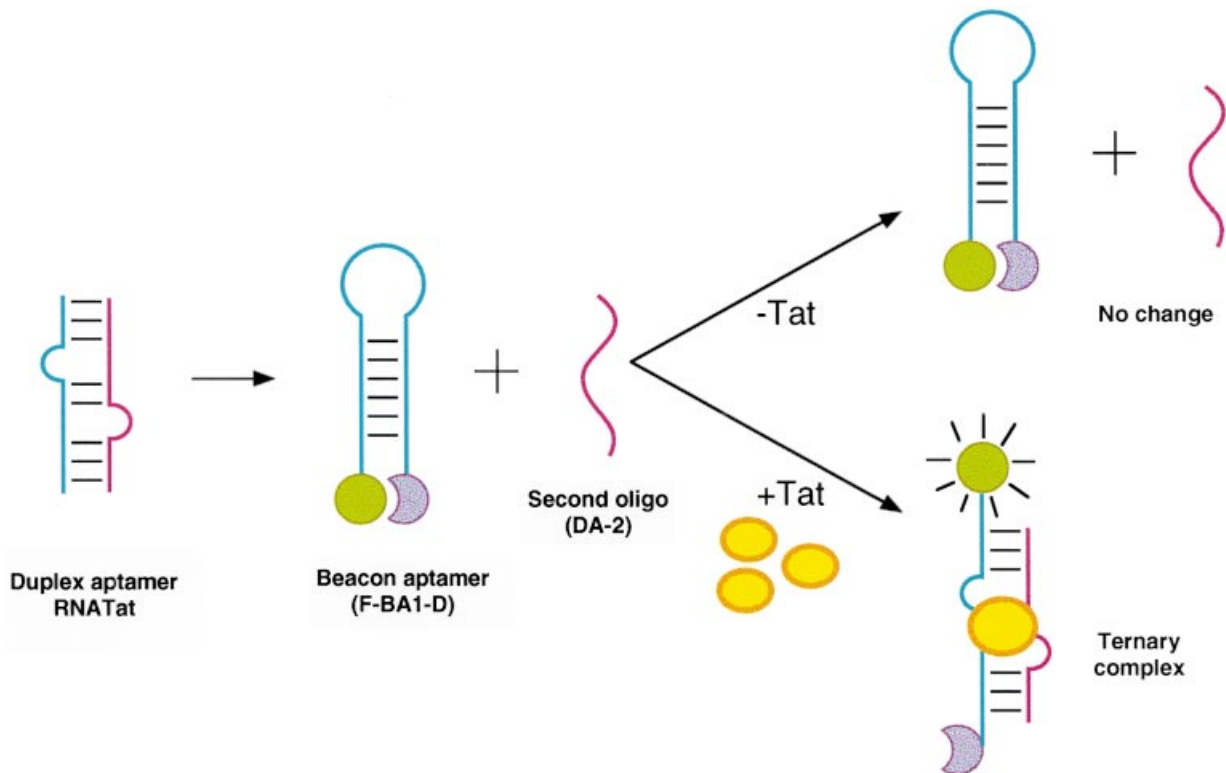


Figure 2 Schematic representation of molecular beacon aptamer strategy for analyzing the viral protein (Tat).

Results

Biochemical studies previously identified a minimal recognition region of Tat-1 that is sufficient for TAR-1 RNA binding. A peptide, CQ (37–72), and Tat-1 protein are reported to have similar binding affinity to the TAR-1 RNA (Feng & Holland 1988; Roy *et al.* 1990; Weeks *et al.* 1990) and therefore, we used sometimes CQ peptide in our binding studies. In order to screen for efficient molecular beacon aptamer, we designed and synthesized several aptamer RNA^{Tat}-derived RNA oligomers.

Gel-shift analysis

We carried out gel-shift assay to monitor efficient RNA oligos (derived from aptamer RNA^{Tat}) that undergo conformational change in the presence of Tat-1-derived peptide, CQ. Initially, both split the oligomers (DA-1 and DA-2) were denatured in Tat-binding buffer independently. Binding reactions were carried out with split oligomers either in the absence or presence of an increasing concentration of CQ (1–100 nM). Samples containing either a single oligomer (DA-2;

Fig. 3B, lane C) or both oligomers (DA-1 and DA-2; Fig. 3B, lane 0) migrated at the same position on the non-denaturing polyacrylamide gel. On the other hand, a band of ternary complex was visible in the presence of two aptamer-derived oligomers (DA-1 and DA-2) with increasing concentrations of Tat-derived peptide, CQ (Fig. 3B, lanes 1–100). The amount of ternary complex increased progressively with increasing the amount of CQ from 1 to 100 nM (Fig. 3B, lanes 1, 5, 10, 50 and 100). In the absence of Tat protein, two oligomers did not form a duplex (Fig. 3B, lane 0), even in the presence of a high concentration of a second oligomer (DA-2) suggesting that DA-1 undergoes a conformational change from hairpin to duplex in the presence of a second strand and CQ peptide (100 nM). As little as 10 nM of CQ peptide was sufficient to form about 50% of the ternary complex, suggesting that the conformational switch for the beacon aptamer is an efficient process (Fig. 3B, lane 10). However, when the target length was shortened by one base-pair at each end of the duplex, the amount of ternary complex formed was significantly affected (data not shown). These results suggest that the Tat-derived peptide mediates the annealing of the two oligomers and the resulting structure is stabilized.

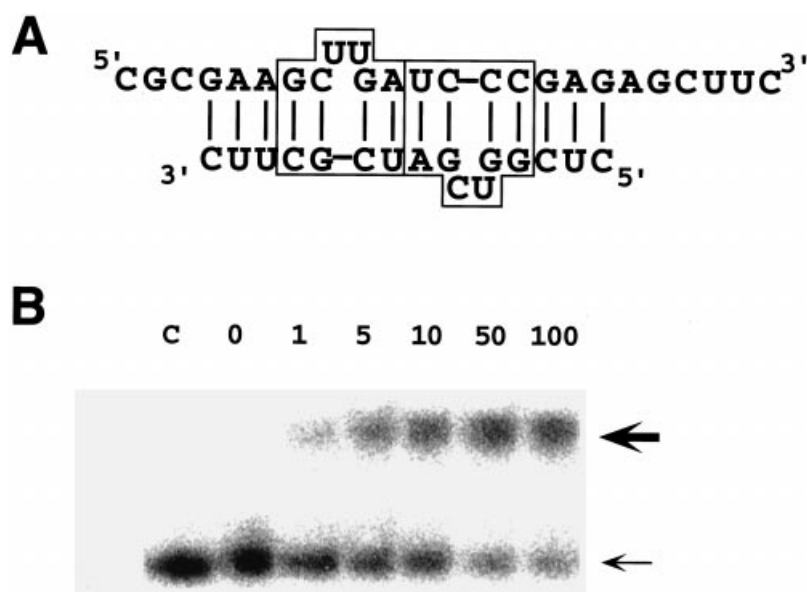


Figure 3 Potential of DA-1 and DA-2 oligomers to form complex that are derived from aptamer RNA^{Tat}. (A) DA-1 and DA-2 are oligomers ability to form duplex in the presence of Tat or its peptides. TAR-like core elements are boxed. (B) Representative autoradiogram obtained from gel-shift assays for the analysis of DA-1 and DA-2 oligomers to form duplex in the presence of CQ. All assays were carried out in 10 μ L of Tat-binding buffer containing DA-1 (\approx 2 nM, 500 c.p.m.) and 40 nM of tRNA. Lane C, 5'-end labeled DA-1 oligomer, lane 0, labeled DA-1 and unlabeled DA-2 (200 nM), lanes 1–100, labeled DA-1 and unlabeled DA-2 (200 nM) oligomers mixed with varying concentrations of CQ peptide (1, 5, 10, 50 and 100 nM). The samples were loaded on 15% non-denaturing polyacrylamide gel. The bold and thin arrows indicate the ternary complex and DA-1 oligomer positions, respectively.

Molecular beacon aptamer studies

Since the DA-1 RNA oligomer undergoes a conformational switch to form the duplex with second oligomer, DA-2, in the presence of the Tat-derived peptide, we synthesized a beacon aptamer (F-BA1-D). The synthesized oligomer contains a fluorescein and a DABSYL that are linked at 5'- and 3'-ends, respectively. The purified oligomer was denatured independently in Tat-binding buffer before analyzing the formation of the duplex (Fig. 3A). In order to estimate the maximum intensity of fluorescence that the F-BA1-D oligomer achieves when it hybridizes to perfect complementary sequence oligomer, we synthesized an additional DA-2 oligomer. To prepare the self-complementary RNA duplex (25 base pairs), both F-BA1-D and DA-3 oligomers were combined in Tat-binding buffer and denatured, followed by annealing at ambient temperature.

When we analyzed the concentration ratio of F-BA1-D/DA-2 between 2 and 50-fold, the resulting fluorescein intensity did not change at the same concentration of CQ (data not shown). Figure 4A shows that in the presence of beacon aptamer (10 nM, F-BA1-D) second oligomer (100 nM, DA-2) and CQ peptide (100 nM) the fluorescein intensity increased, target-to-background (T/B) ratio, 4.9 ± 0.7 (red bar). In the absence of CQ, but in the presence of two oligomers (F-BA1-D and DA-2), fluorescein intensity was considered as background (Figs 4A and 4B, black

bar). These results suggest that, as reported for molecular beacons (Tyagi & Kramer 1996; Piatek *et al.* 1998; Matsuno 1998; Sokol *et al.* 1998), F-BA1-D oligomer undergoes a conformational change only in the presence of CQ and therefore, the fluorescein relieved from the quencher (DABSYL) in F-BA1-D oligomer. When a similar assay was carried out in the presence of other Tat-1-derived peptide, RE (containing the same basic amino acid region), an enhancement of fluorescein intensity was observed comparable to that observed with CQ (Fig. 4A, orange bar).

The binding core elements of aptamer RNA^{Tat} for Tat binding consist of a central four-base pair helix flanked by two residues each (Yamamoto *et al.* 2000). In addition, site-specific mutagenesis studies suggested that the U residues at both bulges are very important for binding to the Tat protein (data not shown). Our studies on modulating aptamer RNA^{Tat} show that the residues forming the two bulges upon binding to the second strand of the aptamer-derived oligomer are also important. To test whether these residues are important for the conformational change that is required for Tat binding, we substituted the U of bulge for C in the target sequence (DA-6i) as well as in F-BA1-D. The resulting oligonucleotide pair (F-BA1A-D/DA-6i) failed to form a ternary complex (data not shown). This result suggests that the functional groups responsible for the binding of Tat-1 to the hairpin aptamer (for example N-3 of U residues) are also important for Tat-1 binding to the

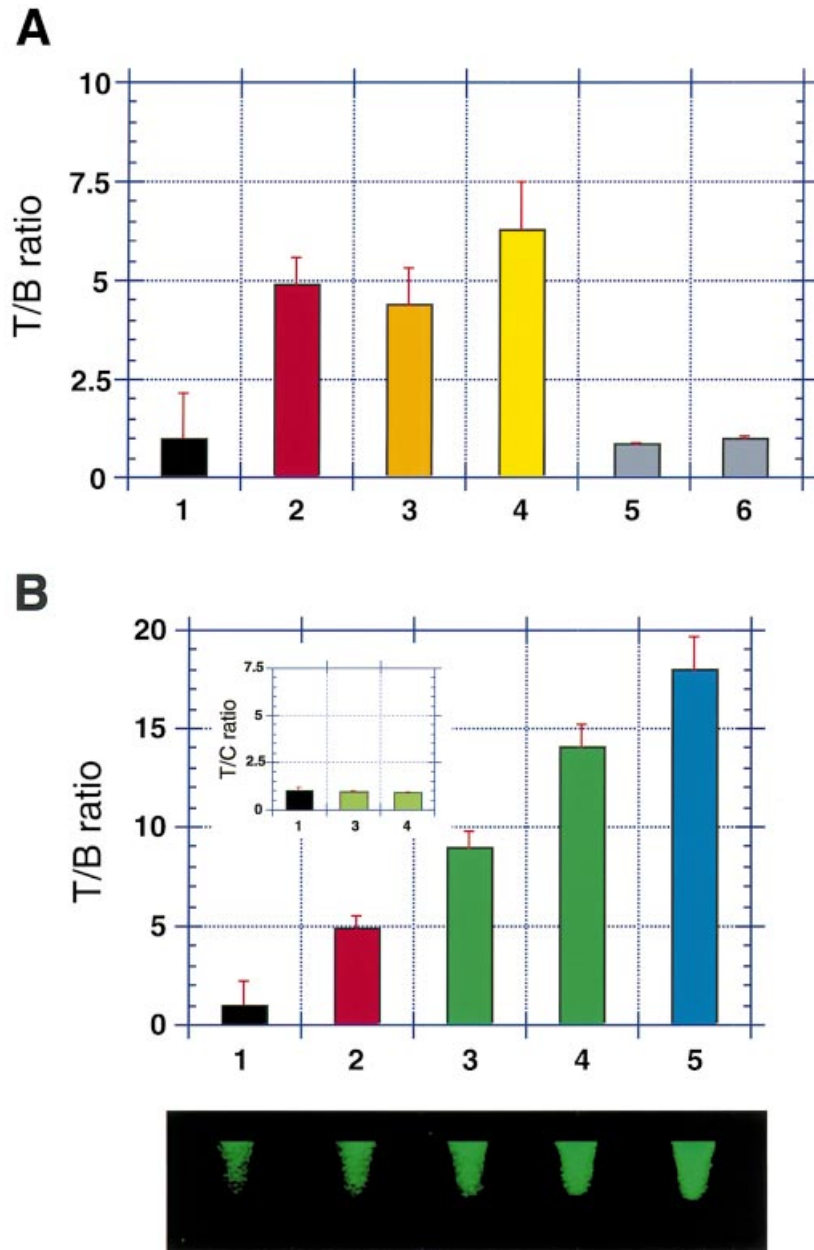


Figure 4 Fluorescence intensity of molecular beacon aptamer in the presence or absence of Tat protein and its peptides. (A) Ratio of enhancement of fluorescence of oligomer F-BA1-D by the Tat-derived peptides. All assays were performed at 30 °C in 50 μ L of Tat-binding buffer containing 40 nM of tRNA. The assays were performed with DA-2 (100 nM) and F-BA1-D (10 nM) in the absence of Tat-peptides (black bar) or in the presence of 100 nM of CQ (red bar), 100 nM of RE (orange bar), 100 nM of CP (Tat-2-derived peptide, yellow bar) 200 nM of Rev (HIV-1 protein, gray bar, 5) or 200 nM of NS3 (HCV protein, gray bar, 6). (B) Ratio of enhancement of fluorescence of oligomer F-BA1-D by the CQ and Tat-1 protein. The assays were performed with DA-2 (100 nM) and F-BA1-D (10 nM) in the absence of Tat-peptides (black bar) or in the presence of 100 nM of CQ (red bar), 100 nM of Tat-1 protein (green bar, 3) or 200 nM of Tat-1 protein (green bar, 4). The blue bar indicates the complementary duplex. The target-to-control ratio (T/C ratio) was obtained without DA-2 in the presence of F-BA1-D (10 nM) in the absence of Tat-peptides (black bar) or in the presence of 100 nM of Tat-1 protein (right green bar, 3) or 200 nM of Tat-1 protein (right green bar, 4). For the visual presentation of the assay, we downloaded the image file from the FluorImager to a Photoshop file (Adobe Inc., version 5.0) for the respective samples and changed the colour to green (to image the fluorescein colour). The target-to-background ratio (T/B ratio) was calculated by taking the fluorescence intensity in the absence of Tat-peptides as the background value. The T/C ratio was calculated by taking the fluorescence intensity only in the presence of F-BA1-D (10 nM) as the control value. The error bars indicate experimental variations between three independent assays.

beacon aptamer; however, other functional groups may be also involved in binding to the modulating aptamer.

In order to examine whether the conformational change observed for beacon aptamers is specific for Tat protein, we carried out an analysis using two RNA binding proteins (NS3 of HCV and Rev of HIV-1). Neither of these two proteins enhanced the fluorescein intensity of beacon aptamer (F-BA1-D; Fig. 4A, grey bars). Previous studies indicate that the Tat proteins from HIV-1 and HIV-2 have about a 65% homology in their core regions (residues cystein36 to proline57 of Tat-2; Guyader *et al.* 1987; Chang & Jeang 1992). In addition, our studies have shown that a Tat-2 peptide (CP, amino acids 66–97) binds to the aptamer RNA^{Tat} with a greater affinity than to TAR-2 RNA (see Yamamoto *et al.* 2000), suggesting similar RNA binding for the two proteins. Figure 4A shows that the Tat-2-derived peptide CP also efficiently promoted duplex formation with the F-BA1-D and DA-2 (yellow bar), indicating that the beacon aptamer responds specifically to the Tat protein or its peptides.

We next compared the efficiency of the conformational change between the Tat-derived peptide CQ and the full-length Tat-1 protein. Figure 4B shows that the full-length Tat-1 protein (100 nM) favoured a conformational change more efficiently than CQ (Fig. 4B-3; green bar), the T/B ratio was 8.9 ± 0.9 . Further increases in the concentration of the Tat-1 protein (200 nM) increased the T/B ratio to 14.0 ± 1.2 (Fig. 4B-4; green bar), which is close to the T/B value (18.0 ± 1.7) of that preformed with the complete duplex (Fig. 4B; blue bar).

Although Tat-derived peptide and Tat-1 protein enhanced the intensity of fluorescein of F-BA1-D with DA-2, no enhancement occurred in the absence of DA-2 (Fig. 4B, inset). This result suggests that the conformation of F-BA1-D was changed by hybridizing with DA-2 in the presence of Tat-derived peptide or Tat protein.

Our previous studies have shown that affinities for CQ peptide-duplex RNA^{Tat} and CQ-RNA^{Tat} complexes were found to be similar (see accompanying article). However, when oligomers form a shorter stem structure (less than four base pairs) at either side of the bulges of the duplex, the affinity for such duplexes to CQ was found to be affected by as much as three orders of magnitude (data not shown). These results suggest that splitting of RNA^{Tat} into two oligomers does not affect the affinity of the oligomers to the Tat, as long as they possess the core binding sequence and at least a four base pair stem on either side of the bulges.

Discussion

We designed a molecular beacon aptamer that fluoresces

only in the presence of the Tat protein of HIV-1. Contrary to earlier reported molecular beacons for hybridizing nucleic acids, the molecular beacon aptamer described here does not depend on the target sequence (nucleic acid) alone, but does depend on the Tat protein of HIV.

In this study, the full-length Tat-1 protein (100 nM) was found to more favourably enhance the conformational change of the aptamer: efficiency was significantly greater with the full-length Tat-1 protein than with CQ. Further increases in the concentration of the Tat-1 protein (200 nM) increased the T/B, closer to the T/B value of the preformed complete duplex. The reason for the higher fluorescence (of fluorescein) from the samples containing Tat protein compared to the CQ peptide at similar concentrations (100 nM) could not be explained. It is tempting to speculate that the stability of the Tat-F-BA1-D-DA-2 (ternary complex) is higher than the CQ-F-BA1-D-DA2 complex, particularly when one oligomer forms a hairpin following dissociation of the ternary complex.

The gel-shift study shows (Fig. 3B) that 10 nM of CQ is sufficient for forming an efficient ternary complex. Although the molecular beacon aptamer appears to require higher amount of Tat protein or its peptide, compared to the gel-shift assay, the present fluorescence assay could be converted into a more sensitive assay by altering the design of the molecular beacons. For example, when the fluorophore EDANS [5'-(2' aminoethyl) aminonaphthalene-1-sulfonic acid] was covalently linked to the DABSYL, the efficiency of quenching was reported to increase 200-fold (Wang *et al.* 1990). Moreover, as suggested by Tyagi & Kramer (1996), it is worthwhile to link the fluorophore and quencher to nucleotides, which are located in the stem, rather than the ends of the RNA. When these modifications are considered in the present studies, we believe that the sensitivity of the molecular beacon aptamer could improve several-fold for the detection of Tat of HIV. A perfect duplex (F-BA1-D and DA-3) was formed to enhance the fluorescein intensity by as much as 18.0 ± 1.7 . Previously, about a 25-fold enhancement of fluorescein (T/B ratio) in the presence of target oligomer has been reported for similar length of DNA (Tyagi & Kramer 1996). One of the reasons for this discrepancy in T/B ratios for a similar length of oligomers between the two studies, could be due to the presence of three extra unpaired nucleotides in our construct. Furthermore, the beacon aptamer can be improved by optimizing the stem and target sequence, which may result in a lower background.

On the other hand, in the presence of other RNA binding proteins, an enhancement of the fluorescein intensity from the beacon aptamer (F-BA1-D) was not

observed. In addition, the Tat-2-derived peptide (CP, amino acids 66–97) also efficiently promoted duplex formation with the F-BA1-D and DA-2. The beacon aptamer responds specifically to the Tat protein or its peptides. These results suggest that the beacon aptamer is indeed suitable for a specific detection of Tat protein, either derived from HIV-1 or HIV-2.

Furthermore, several modifications are now considered for stabilizing the F-BA1-D against the nucleases. Such a stabilized molecular beacon aptamer RNA^{Tat} could find applications in detecting the Tat protein in living cells (such as HIV infected cells). Although only a preliminary study, the present results open the possibility of the use of molecular beacon aptamers as an analytical tool for monitoring viral protein levels, both *in vitro* and in infected cells.

Experimental procedures

Tat-peptides, Tat protein and RNA

Tat-1 derived peptides, CQ (amino acids 37–72), RE (amino acids 49–86) and Tat-2 derived peptide, CP (amino acids 66–97), were chemically synthesized, deprotected and purified by HPLC. The amino acid composition of synthetic peptides was confirmed after cleaving the peptide with 6 N HCl containing 0.1% phenol at 10 °C for 1 h followed by HPLC analysis.

RNA sequences used in the present study were DA-1, 5'-CGCGAAGCUUGAUCCCGAGAGCUUC-3'; DA-2, 5'-CUCGGUCGAUCGCUUC-3'; DA-3, 5'-UAAGCUCUCGGGAUCAAGCUUCGCG-3', where underlined residues form bulges in the duplex. The above RNA oligomers were synthesized chemically on an RNA/DNA synthesizer (Applied Biosystems Model 394) using a Phosphoramidites from Glen research (Glen Corporation, USA). All oligoribonucleotides were deprotected and purified as described previously (Yamamoto *et al.* 1998), whereas fluorescence and DABSYL [4-(4'-dimethylamino-phenylazo) benzoic acid] labeled RNA oligomer was deprotected by following instructions of the manufacturers.

Gel-shift assay

Duplex formation by the RNA oligomers was assayed using a previously reported protocol (Yamamoto *et al.* 1998) in the presence of CQ peptide. Two RNA oligonucleotides with the potential to form a duplex were characterized (Fig. 3A). The DA-1 oligomer of the duplex aptamer RNA^{Tat} was labeled with [γ -³²P] ATP. Initially, both RNA oligomers (DA-1 and DA-2) were denatured independently in Tat-binding buffer (10 mM Tris-HCl, pH 7.8, 70 mM, NaCl, 2 mM EDTA and 0.01% Nonidet P-40) and equilibrated at ambient temperature. 5'-End labeled RNA (~2 nM, 500 c.p.m.) was mixed with 200 nM of unlabeled DA-2 oligomer in the presence of 40 nM of unlabeled *E. coli* tRNA (total tRNA) in 10 μ L of Tat-binding buffer. CQ peptide (1–100 nM) was added and the reaction mixture was incubated at 30 °C for 1 h. The

reaction products were separated on a non-denaturing polyacrylamide gel (15%) and the amount of complex formed in the presence and absence of protein or peptides was quantified by an image analyzer (BAS2000, Fuji Film, Japan).

Molecular beacon aptamer studies

A molecular beacon was used for most of the experimental work described here. The sequence of molecular beacon aptamer, F-BA1-D, fluorescein-5'-CGCGaagcuuGAUCCCGAgagcuua-3'-DABSYL was synthesized chemically as described above, where the underlined nucleotide constitute the probe sequence and small letters form the stem structure. Initially, beacon aptamer oligomer and target oligomer (F-BA1-D and DA-2) were denatured independently at 90 °C for 3 min in Tat-binding buffer. Two oligomers, F-BA1-D and DA-2, were mixed and incubated with Tat-derived peptides (CQ, RE or CP) or Tat-1 protein with specified amount in each case in 50 μ L Tat-binding buffer containing 40 nM of tRNA. The reaction mixture was incubated at 30 °C for 30 min. Fluorescein intensity was quantified in the samples using a FluorImager (Molecular Dynamics, USA) with excitation at 488 nm and detection at 530 nm. Simultaneously, controls are also placed with all other components of test samples except the Tat-1 protein or its peptides. In order to establish the maximum fluorescence intensity of fluorescein that the F-BA1-D oligomer achieves when it hybridizes to the perfect complementary sequence oligomer, the DA-3, we combined two oligomers in Tat-binding buffer followed by denaturing at 90 °C for 3 min and annealing at ambient temperature for 15 min.

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