Chapter 4

Modulating aptamer RNA for HIV-1 Tat: potential use in diagnostics and biosensors

Introduction

Several high affinity RNA or DNA aptamers have been isolated from combinatorial libraries of nucleic acids that bind to targets such as RNA binding proteins and other small molecules (Osborne and Ellington, 1997). The affinities of some aptamers (nucleic acids) for their target proteins are comparable to the affinities of antibodies for antigens. Therefore, they have possibility as a fundamental molecular recognition element in biosensors (Osborne and Ellington, 1997). Previously, a high affinity aptamer RNA (K_d~140 pM) was exploited in developing an enzyme-linked oligonucleotide assay (ELONA) for the detection of human vascular endothelial growth factor (hVEGF) levels in sera (Drolet et al., 1996). The ELONA showed equivalent to enzyme-linked immunosorbent assay (ELISA) with similar accuracy, specificity, and interference. Therefore, it appears that the *in vitro* evolved or selected aptamers could be potential source to substitute the antibody use in clinical research and diagnostics. Although initial studies carried out with full-length aptamers have been encouraging and promising (Drolet et al., 1996; Potyrailo et al., 1998), this approach has several limitations: for example, the efficiency of chemical synthesis reduces with increasing length of the aptamer; modification of the full-length aptamer is mandatory for protection against nucleases; and in the case of multiple analysis, the relative efficiency of folding and refolding is poor for longer aptamers.

In order to allow their effective use in biosensors, a novel strategy was developed in which the aptamer reassembles only in the presence of analyte; this design is termed a modulating aptamer. This assay, called the analyte-dependent hybridizing oligonucleotide assay (ADHONA), detects HIV-1 Tat protein efficiently and rapidly without significant interference

from nuclear extract components. Thus, it appears that ADHONA may have potential uses similar to those of DNA arrays, and could be readily adopted and expanded to detect viral proteins or other small molecules.

Materials and Methods

RNA, Tat-peptide and Tat protein

Tat-1 derived peptides, CQ (aa 37-72) RE (aa 49-86) and Tat-2 derived peptide, CP (aa 66-97), were chemically synthesized, deprotected and purified by HPLC. The amino acid composition of synthetic peptides was confirmed by reverse HPLC analysis. Modulating aptamer RNAs^{Tat} oligonucleotides shown in Figure 25 were synthesized on an RNA/DNA synthesizer (Applied Biosystems Model 394) using Phosphoroamidites from Glen research (Glen Corporation, USA). The RNAs were deprotected and purified as described previously (Yamamoto et al., 1997). The 5'-strands of the modulating RNAs were labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (Yamamoto et al., 1998). Similarly, 3'-fluoresceinated oligomer RNA (3'-fluorescein DA-9) and 3'-biotin labeled DA-10 RNA were chemically synthesized and deprotected using established protocols. DT-1 and DT-2 RNAs (duplex derived from TAR-1 RNA) were chemically synthesized and deprotected as described above.

Gel-shift assay

Duplex formation by the RNA oligonucleotides was assayed using a previously reported protocol (Yamamoto et al., 1998) in the presence of Tat or Tat peptides CQ, RE and CP. Eight RNA oligonucleotides with potential to form five duplexes were characterized (Figure 25). In all cases, the 5'-strand (DA-1, DA-3, DA-5, DA-7, DA-5i, and DT-1) of the duplex aptamer RNA was labeled with [γ-^{32}P]ATP. 5'-End labeled RNA (2,000 cpm) was mixed with 200 nM of unlabeled complementary RNA (DA-2 for DA-1, DA-4 for DA-3, DA-1 for DA-4, DA-6 for DA-5, DA-8 for DA-7, DA-6i for DA-5i, and DT-2 for DT-1 oligo) in the presence

of 40 nM of unlabeled *E. coli* tRNA in 10 µl Tat-binding buffer [10 mM Tris-HCl (pH 7.8), 70 mM NaCl, 2 mM EDTA and 0.01% Nonidet P-40]. CQ peptide (20 nM) or Tat-1 protein (200 nM) was added and the assay 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 the image analyzer BAS2000.

Equilibrium dissociation constants were determined for modulating aptamer RNAs DA-1/DA-2 and DA-5/DA-6 with CQ peptide. 5'-End labeled RNA (50 pM) DA-1 or DA-5 was mixed with the appropriate complementary RNA in 10 µl of Tat-binding buffer and 40 nM of tRNA was added as a non-specific competitor. CQ peptide (0.5 nM to 64 nM) was added and incubation proceeded at 30 °C for 1 h. Products were separated on a non-denaturing polyacrylamide gel (15%) and quantified. B_{max} and K_d were determined as mentioned above.

Analyte- (Tat-) Dependent Hybridizing Oligonucleotide Assay

Streptavidin-coated microtiter plates were purchased from Labsystems, Finland. The 3'-biotinylated oligonucleotide DA-10 (5 pmol) was added to the well and allowed to bind with streptavidin in 50 µl Tat-binding buffer at ambient temperature for 10 min followed by washing with 200 µl binding buffer. 50 µl Tat-binding buffer containing 10 pmol 3'-fluoresceinated DA-9 and 200 pmol Tat or Tat peptide (CQ or CP) was added. The microtiter plate was incubated at 30 °C for 30 min and then washed with 200 µl Tat-binding buffer. Intensity of fluorescence was quantified using a FluorImager (Molecular Dynamics, USA) with excitation at 488 nm and detection at 530 nm. Controls were carried out in the absence of Tat-1 protein or Tat-derived peptides. Assays were also

carried out using 8 units of HeLa nuclear extract (Promega) in the presence of 40 units of RNase inhibitor (Toyobo, Japan).

Results

Screening for efficient modulating aptamers

Five modulating aptamers were tested for complex formation using a gel shift assay (Figs. 25A and 25B, i to v). Four of these, DA-1/DA-2, DA-3/DA-4, DA-5/DA-6, and DA-1/DA-4 (i, ii, iii and v, respectively) formed a high affinity recognition complex (Fig. 25B) in the presence of Tat protein (200 nM, lanes 4) or Tat-derived peptide CQ (20 nM, lanes 3) but not in the absence of Tat protein (lanes 2). The DA-5/DA-6 oligonucleotides formed a ternary complex more efficiently than the other modulating aptamers in the presence of Tat protein (50%) or the CQ peptide (84%) (Fig. 25B, iii *vs* i, ii and v).

In order to compare the efficiency of the two good modulating aptamers, K_d values for DA-5/DA-6-CQ and DA-1/DA-2-CQ ternary complexes were analyzed by gel-shift assays in the presence of varying concentrations of CQ (0.1 to 12.8 nM and 2 to 64 nM, respectively). The K_d values for the DA-5/DA-6-CQ and DA-1/DA-2-CQ complexes were 0.5 nM and 400 nM, respectively (Figs. 26A and 26B). The K_d for the DA-1/DA-2-CQ complex was 800-fold higher than that for the DA-5/DA-6-CQ complex. These results suggest that the modulating aptamer reconstitutes the duplex in the presence of Tat protein or CQ peptide, and has comparable specificity and affinity for Tat protein as the hairpin aptamer RNA^{Tat} (Fig. 12B).

Importance of sequence and functional groups of modulating aptamer

Since the K_d for the DA-5/DA-6-CQ complex was estimated to be close to the value for hairpin aptamer RNA^{Tat} (Fig. 12B), it is likely that the modulating aptamer, especially DA-5/DA-6, reconstitutes the binding

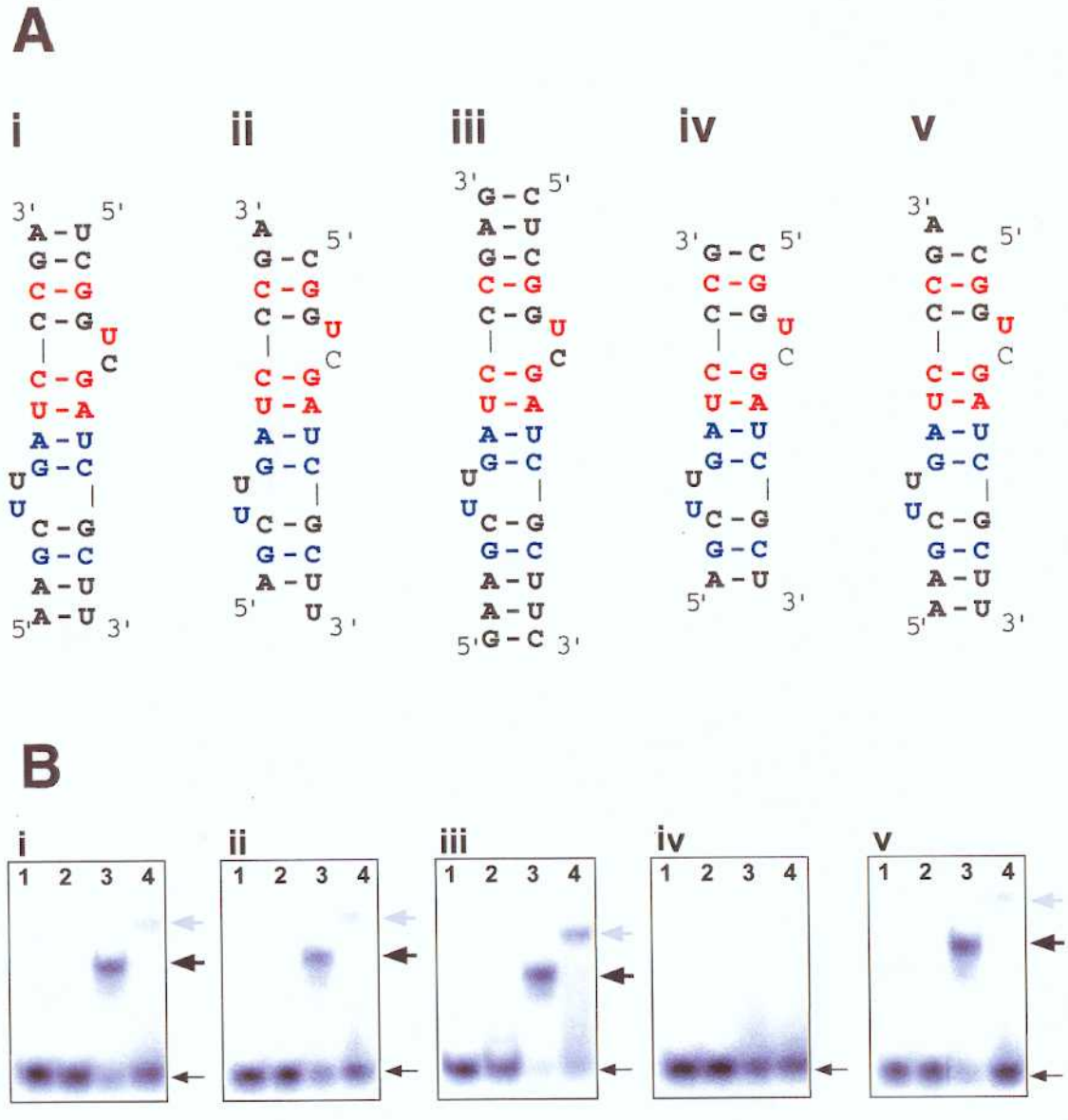

Figure 25. A, Modulating aptamer RNAs (i, DA-1/DA-2; ii, DA-3/DA-4; iii, DA-5/DA-6; iv, DA-7/DA-8; v, DA-1/DA-4). Red and blue letters indicate TAR core elements. B, Representative autoradiograms from gel-shift assays for modulating aptamers: lane 1, radiolabeled 5'-end oligo (10 nM); lane 2, radiolabeled 5'-end oligo (10 nM) and unlabeled 3'-end oligo (200 nM); lane 3, radiolabeled 5'-end oligo (10 nM) and unlabeled 3'-end oligo (200 nM) in the presence of CQ (20 nM); lane 4, radiolabeled 5'-end oligo (10 nM) and unlabeled 3'-end oligo (200 nM) in the presence of 200 nM of Tat-1. Shadow and bold arrows indicate the positions of modulating RNA-Tat and modulating RNA-CQ complexes, respectively. Thin arrow indicates free 5'-oligonucleotide.

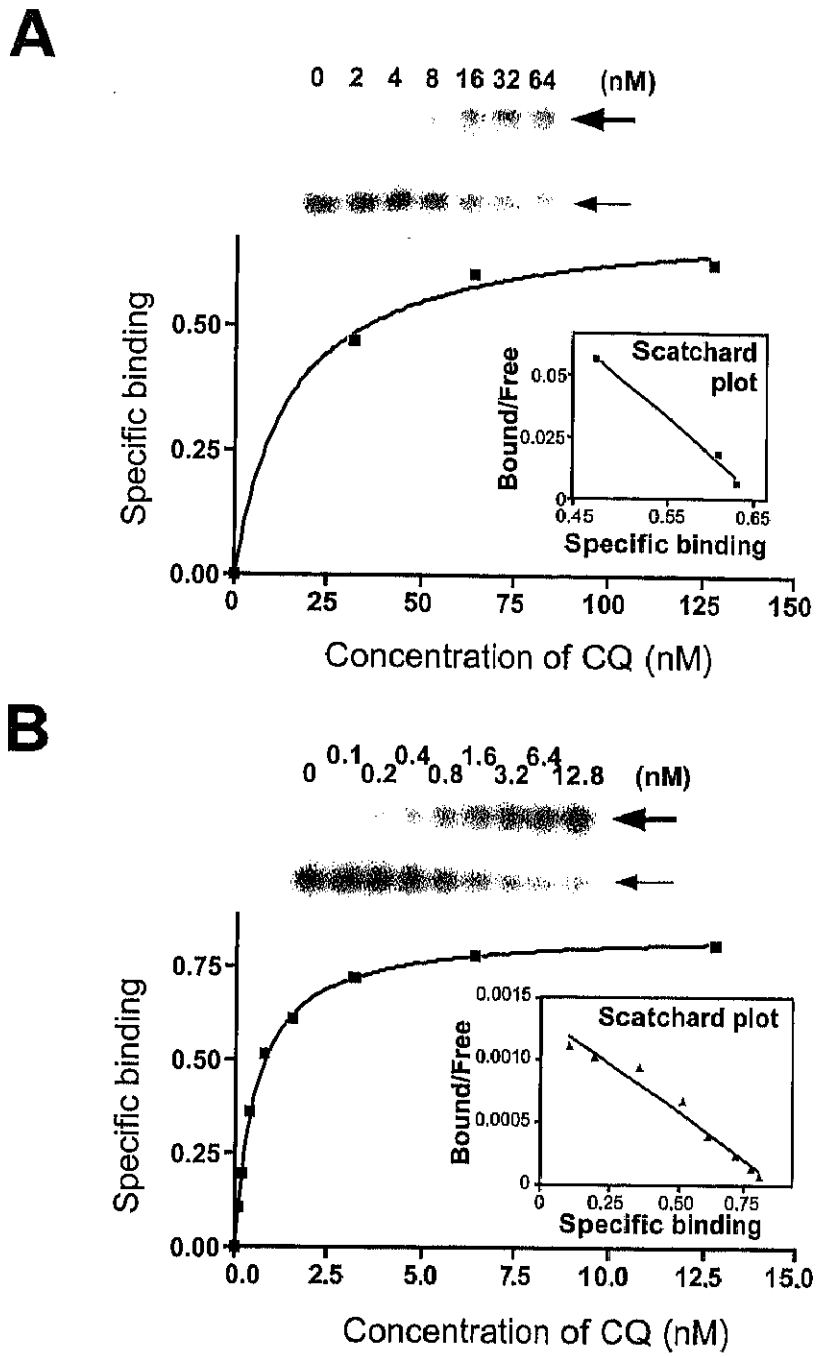

Figure 26. Determination of equilibrium dissociation constant (K_d) for binding of modulating RNAs, DA-1/DA-2 and DA-5/DA-6, to CQ. Representative autoradiograms from gel-shift assays for analysis of binding of DA-1/DA-2 and DA-5/DA-6, to CQ (A and B, respectively). Bold and thin arrows indicate the positions of modulating RNA-CQ complex and free 5'-oligonucleotide, respectively. Saturation curves and Scatchard plots are shown for DA-1/DA-2-CQ and DA-5/DA-6-CQ (A and B, respectively).

motif in the presence of Tat protein or CQ peptide. The binding core elements of RNA^{Tat} for binding to Tat protein consist of a central 4-bp helix flanked by two bulges containing two residues each (see Chapter 2). In addition, site-specific mutagenesis suggested that the U residues at both bulges are very important for binding to Tat protein (see Chapter 2). Based on kinetic studies of hairpin RNA^{Tat} and the modulating aptamer oligonucleotides DA-5/DA-6, it appears that both RNA components, DA-5 and DA-6, interact to Tat protein. To confirm this notion, two derivatives of DA-5 and DA-6 were synthesized with C to U substitutions. The pair of these oligonucleotides (DA-5i/DA-6i) failed to form a ternary complex (Figs. 27A i and 27B i). This result suggests that the functional groups of the hairpin aptamer which are responsible for the binding to Tat-1 (for example, N-3 of U residues) are also important for the modulating aptamer DA-5/DA-6; however, other functional groups of the modulating aptamer may also be involved in the binding to Tat. Nevertheless, these results indicate the importance of the core binding sequence to the interaction between Tat-1 and the modulating aptamer DA-5/DA-6.

The binding of CQ to DA-5/DA-6 was also compared to another set of RNA oligonucleotides from TAR-1 RNA, DT-1/DT-2. Figures 27A, ii and 27B, ii show that DT-1/DT-2 did not form a ternary complex even in the presence of a large excess of oligonucleotide (DT-2) and CQ. Taken together, the results suggest that the modulating aptamer displays a high affinity, sequence specific binding to the Tat protein or to the CQ peptide by inverted repeats of the core elements of the TAR-1 RNA.

In order to examine whether the modulation is specific to Tat, modulating aptamer oligonucleotides were also subjected to a gel-shift binding assay in the presence of other RNA binding proteins such as HCV NS3 protein (which has a protease domain and an RNA helicase domain).

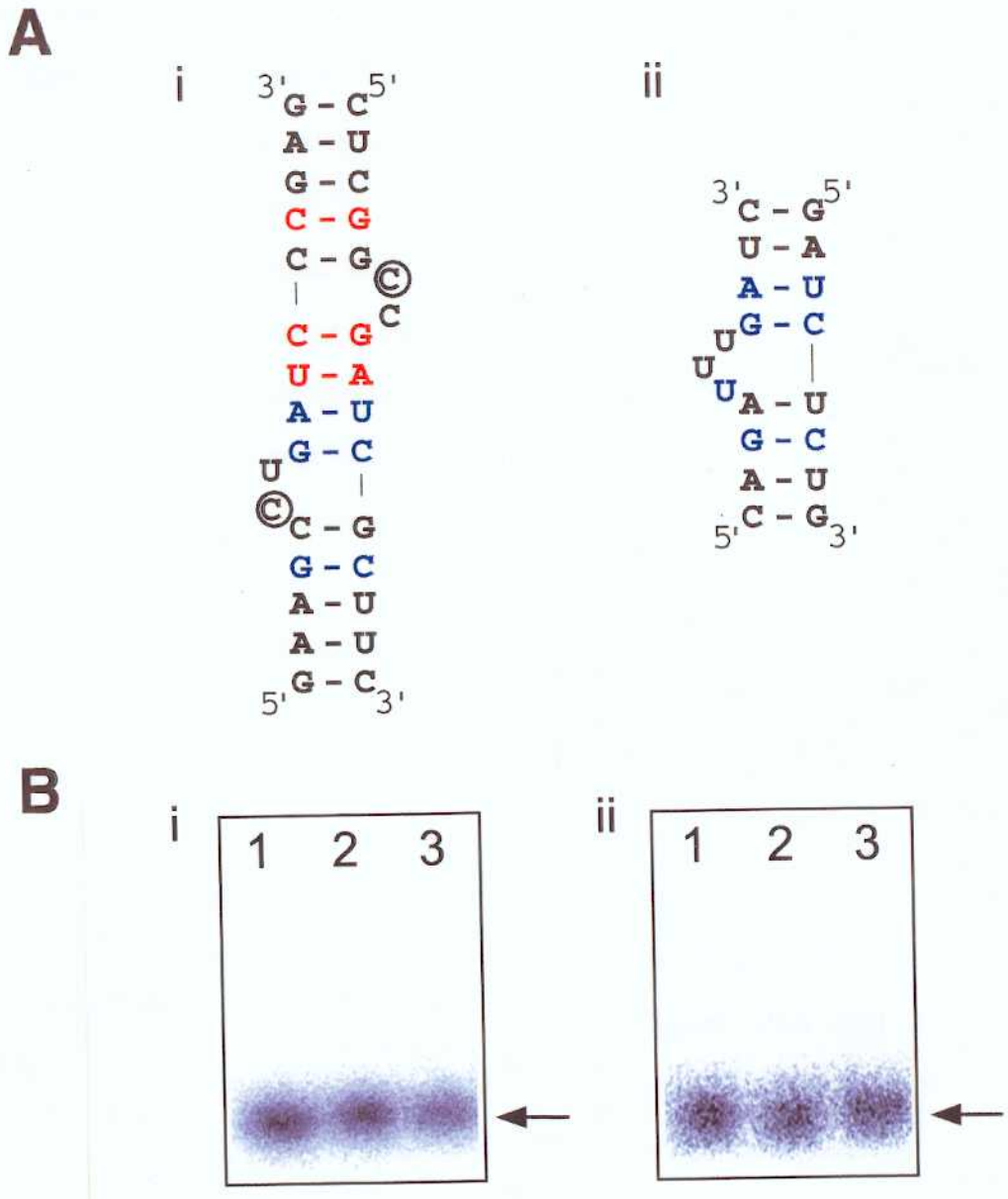

Figure 27. Analysis of binding of inactive modulating variants and TAR-1-derived oligonucleotides to Tat-derived peptide by gel-shift assay. A, Secondary structures of inactive modulating aptamer variants (i, DA-5i/DA-6i) and duplex TAR-1 RNA (ii, DT-1/DT-2). Red and blue letters indicate TAR core elements. B, Representative autoradiograms from gel-shift assays for binding of inactive modulating aptamer variants and TAR-1-derived duplex to CQ (A and B, respectively): lane 1, radiolabeled 5'-oligo (10 nM); lane 2, radiolabeled 5'-oligo and unlabeled 3'-oligo (200 nM); lane 3, radiolabeled 5'-oligo and unlabeled 3'-oligo (200 nM) in the presence of CQ (20 nM).

The NS3 protein failed to form a complex with the modulating aptamer oligonucleotides as revealed by gel-shift analysis (data not shown). My studies have shown that a Tat-2 peptide (CP, aa 66-97) binds to RNA^{Tat} with higher affinity than to TAR-2 RNA (in Chapters 2 and 3), suggesting similar RNA binding properties for the two proteins. Figure 28 shows that the Tat-2 peptide (CP) also efficiently promotes duplex formation with the modulating aptamer DA-5/DA-6, although at reduced efficiency compared to the Tat-1 peptides CQ or RE (Fig. 28). Thus, it's clearly suggested that the conformational change of the modulating aptamer specifically depend upon Tat proteins and its peptides either derived from HIV-1 or HIV-2 but not upon other RNA-binding proteins.

Analyte- (Tat-) Dependent Hybridizing Oligonucleotide Assay

The modulating aptamer described here has potential as a diagnostic tool for the detection of Tat protein. One such diagnostic assay was developed and tested and is presented in Figure 29. This assay, called analyte-dependent hybridizing oligonucleotide assay (ADHONA), uses a 3'-fluorescein oligonucleotide (DA-9), a 3'-biotin oligonucleotide (DA-10) and streptavidin-coated microtiter plates (Figure 29A and 29B). The biotinylated DA-10 oligonucleotide (5 pmol) was added to wells of the streptavidin plates, the unbound oligonucleotide removed by washing, followed by addition of the fluorescein-modified DA-9 oligonucleotide (10 pmol) with Tat or CQ peptide and a final washing step to remove unbound material. Appropriate controls were carried out in the absence of Tat or CQ peptide. Fluorescence intensity was quantified using a fluoroimage analyzer (FluorImager) and the results are summarized in Figure 30A. In the presence of increasing amounts of CQ peptide (10 to 100 pmol) the fluorescence increased 500- to 2500-fold compared to the control without CQ. A higher concentration of Tat-1 protein (200 pmol)

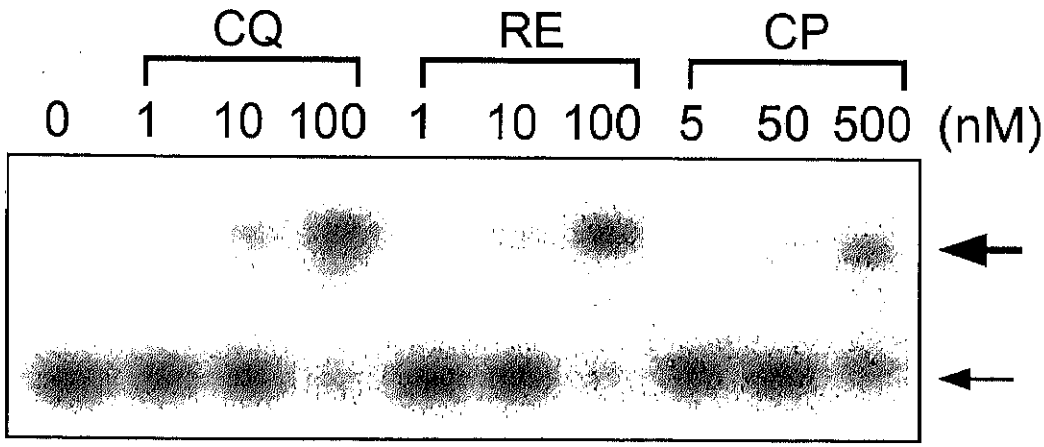

Figure 28. Analysis of binding of modulating RNAs, DA-5/DA-6, to Tat-1 peptides (CQ and RE) and Tat-2 peptide (CP). Representative autoradiograms from gel-shift assays for binding of modulating RNAs DA-5/DA-6 to Tat-derived peptides (CQ, RE and CP). Bold and thin arrows indicate the positions of modulating RNA-peptide complex and free 5'-oligonucleotide, respectively.

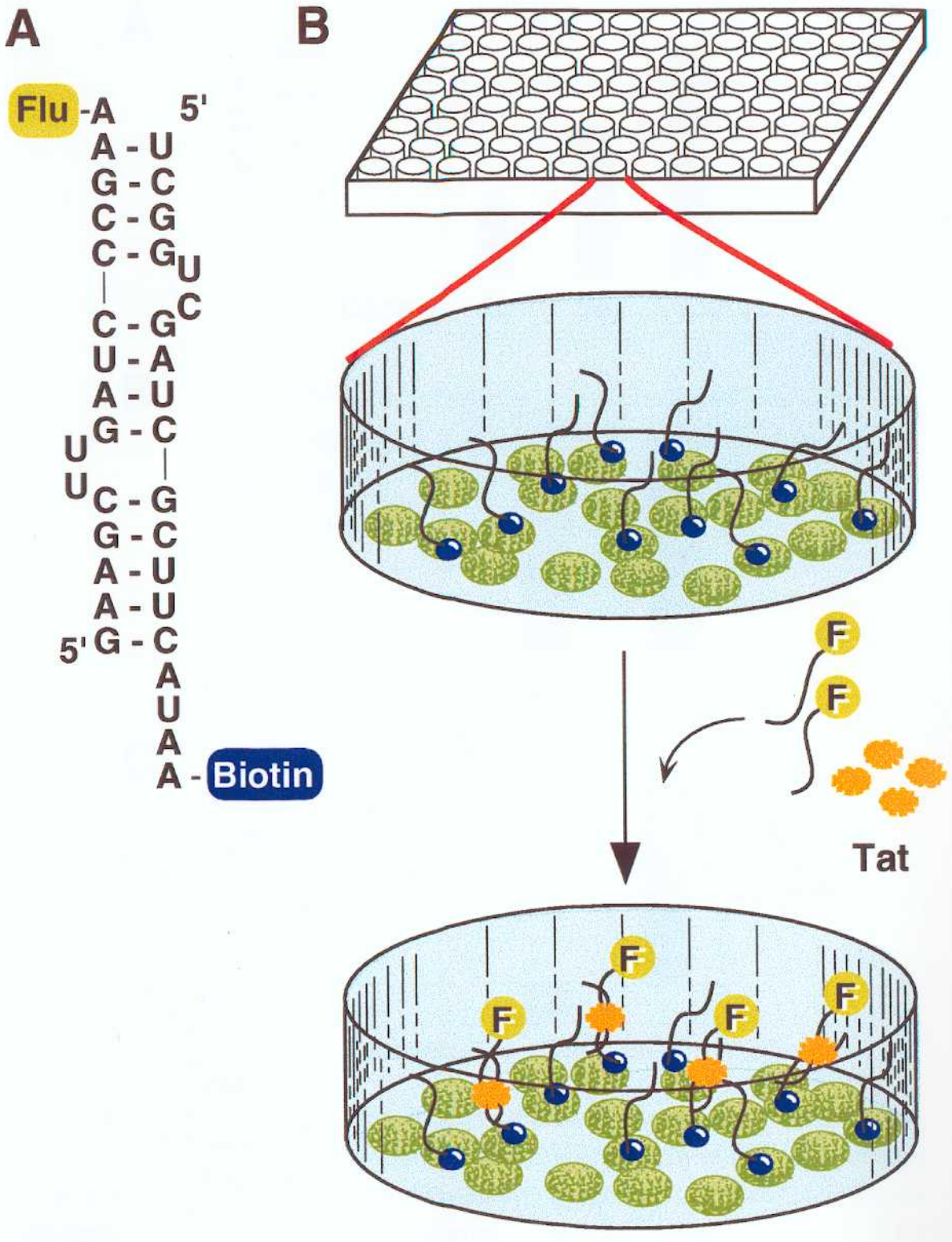

Figure 29. Analyte-dependent Hybridizing Oligonucleotide Assay (ADHONA). A, Modulating RNAs DA-9 and DA-10 used in ADHONA. B, Schematic representation of ADHONA. Flu and F indicate fluorescein.

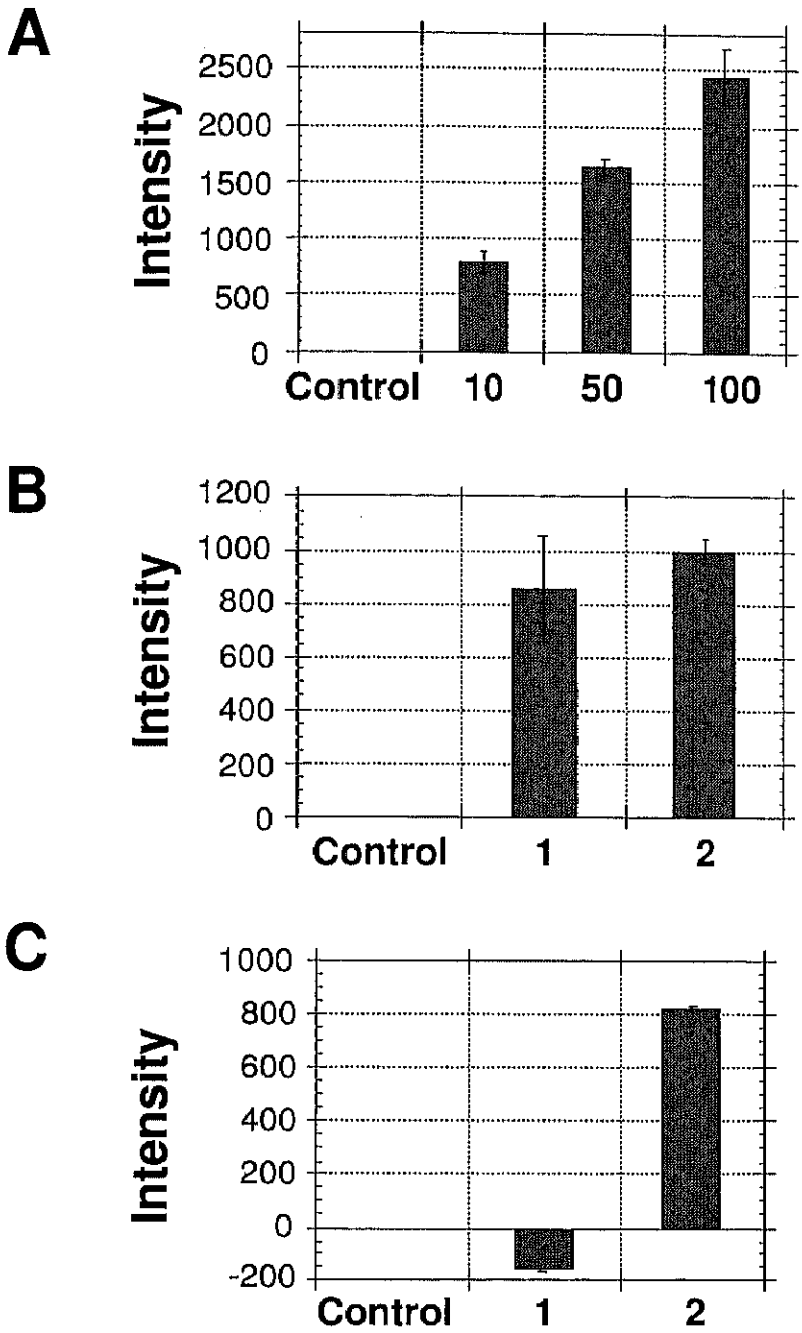

Figure 30. Analyte-Dependent Hybridizing Oligonucleotide Assay (ADHONA). A, Concentration dependence of ADHONA on CQ peptide: intensity of fluorescence signal in the absence of Tat-derived peptide, CQ (control) or in the presence of CQ (10, 50, and 100 pmol). B, ADHONA using Tat-1: control, in the absence of Tat-1 or CQ; 1, in the presence of CQ (10 pmol); 2, in the presence of Tat-1 (200 pmol). C, The effect of HeLa nuclear extract on the ADHONA: intensity of fluorescence signal in the absence of CQ (control); or in the presence of 8 units of HeLa nuclear extract, 1 ; or in the presence of CQ (10 pmol), 2. Relative fluorescence intensities shown in panels A, B, and C are from three independent experiments (S.E.M. indicated by error bars).

was required to make a fluorescence signal comparable to that of CQ peptide (10 pmol; Fig. 30B).

To be an effective diagnostic tool, the assay shown in Figure 29B should provide quantitative results in the presence of crude samples such as a mammalian cell nuclear extract. Crude samples could contain proteins and compounds that interfere with the assay, as well as proteins that promote annealing of complementary sequences (Weeks et al., 1990). The assay described above was carried out with DA-9/DA-10, HeLa nuclear extract and an RNase inhibitor. Figure 30C shows that the modulating aptamer oligonucleotides do not assemble into duplex and are not retained in the microtiter well in the presence of HeLa extract alone. (It should be noted here that a small amount of RNase activity was detected despite in the presence of 40 units of RNase inhibitor). This result suggests that this assay may be suitable for detection of Tat protein in a mammalian nuclear extract, i.e. from an infected cell.

Discussion

The studies presented here demonstrate a novel binding assay using a modulating aptamer RNA that reconstitutes a duplex aptamer only in the presence of analyte. The method has been characterized using oligonucleotides that target HIV-1 Tat protein. There are at least two features in favor of using RNA^{Tat}, especially a modulating aptamer, instead of TAR RNA as a molecular recognition element for Tat protein: 1) the aptamer has a higher affinity to Tat protein, i.e. 130-fold higher K_d than the TAR-1 (59 mer) for Tat-1 and 40-fold higher K_d than the TAR-2 (123 mer) for Tat-2; 2) the loop sequences of hairpin RNA^{Tat} are not a part of the binding site for the Tat protein, hence RNA^{Tat} can be divided into two oligoribonucleotide strands. The modulating aptamer forms a ternary complex efficiently in the presence of Tat protein but not in the presence of other RNA binding proteins. Thus, the Tat protein can be quantified using the modulating aptamer. The specific modulating aptamer sequence and design that is based on the Gibbs free energy (i.e., DA-5/DA-6; energy= -15.2) determines its ability to assemble into a ternary complex. The bulge residues appear to be very important for interaction with Tat proteins from HIV-1 or HIV-2. The hairpin RNA^{Tat} and the modulating aptamer DA-5/DA-6 displayed high affinity for Tat protein. Therefore, modulating aptamers may be sensitive enough to measure the amount of Tat released from infected cells or found in the serum of infected patients. Importantly, the specificity of the modulating aptamer DA-5/DA-6 was retained in a crude sample such as a HeLa nuclear extract.

The modulating aptamer method has many advantages over a method that uses a longer non-modulating aptamer sequence, including the following: 1) shorter RNA oligonucleotides can be synthesized with

higher efficiency than longer molecules; 2) modifications to stabilize the nucleic acid are necessary for only a portion of the aptamer; 3) proper folding of the modulating aptamer is facilitated by the analyte; 4) lower cost. Moreover, the results presented above suggest that the modulating aptamer method is sensitive and specific and can be readily adapted to nucleic acid array technology for multiple-routine analysis. However, further improvement will be obtained when RNA aptamers are more fully protected from ribonucleases. This approach can be generalized for detection of proteins other than HIV Tat by selecting appropriate non-modulating and/or modulating species from combinatorial libraries. This approach is currently underway for several target HIV proteins.