Chapter 5

General discussion

TAR-1 RNA inhibited a transcription of CMV-based template that is irrelevant to Tat/TAR interaction. Therefore, I propose here that a part of the effects of the TAR decoy observed previously might have been due to inhibition of general transcription. Some variants of TAR-1 RNA lost the ability to inhibit the transcription of CMV-based template although TAR-1 RNA inhibited the transcription. This was probably resulted from loss of the ability of TAR-1 RNA to bind to cellular factors. In view of the above results, I cannot exclude the possibility that, upon expression of a TAR decoy in the host cell, transcription might be inhibited not only on templates that are dependent on Tat-mediated *trans*-activation but also on other templates, including those that encode housekeeping genes. The TAR-1 variants also lost the ability to bind to Tat peptide. Since TAR RNA appeared to be unsuitable for inhibitor of Tat-dependent *trans*-activation, new RNA was required to use as a decoy for inhibition of the *trans*-activation without inhibition of other transcription.

I was able to isolate new RNA motif containing two TAR core elements from the random RNA pool (120 random nucleotides; 120N) because selective pressure was maintained probably during the entire selection procedure. A similar selection procedure, using an RNA pool with a random core (30N), resulted in isolation of other structural forms (Tuerk and MacDougal-Waugh, 1993). In this case, selection might have been hampered by the short random core region over the fixed sequences for amplifications. The selected RNA^{Tat} showed lower K_d value than TAR-1 RNA and the difference in the K_d values (TAR-1 RNA *versus* selected aptamer RNA^{Tat}) was about 130-fold (16 nM *versus* 120 pM). The selected RNA^{Tat} also showed low K_d value to Tat-2 peptide, CP (7.0 nM). Since RNA^{Tat} can bind specifficaly to Tat peptides of HIV-1 and HIV-2 and the interaction between RNA^{Tat} and Tat peptides is very

strong, RNA^{Tat} may be suitable for inhibition of viral replication and use in biosensor as a molecular recognition element.

At present, the exact mechanism for higher affinity to Tat by the RNA^{Tat} is not completely understood. However, atomic mutation analysis and kinetics studies suggested, at least in part, that the aptamer achieved higher affinity to Tat protein by twining the core residues of TAR RNA as have been observed in other naturally occurring RNA, DNA, and proteins.

The isolated aptamer RNA^{Tat} has strong affinity to the Tat even in the presence of a large excess of TAR RNAs of HIV-1 and HIV-2. It is suggested that the selected RNA^{Tat} has no effect on the general transcription when RNA^{Tat} was tested in cell free transcription assay. Importantly, the RNA^{Tat} showed inhibition as effectively as TAR-1 RNA in analyses *in vitro* and *in vivo* even in the absence of interaction sites with cellular factors, such as cyclin T1. These results suggest that RNA^{Tat} has the higher affinity to Tat without any aids from the cellular protein, whereas, TAR-1 RNA achieved similar affinity to Tat only in the presence of cellular factors, for example, cyclin T1. In addition, the present use of TAR RNA as decoy may limit its application because they might exhibit cellular toxicity. Thus, the aptamer RNA^{Tat} not only serves as high affinity receptor to specifically sequester the Tat protein efficiently without cellular factors (in contrast to TAR RNA) but also is useful as efficient decoy even under mixed infections caused by HIV-1 and HIV-2.

Since the binding affinity of RNA^{Tat} for Tat is comparable to the affinities of antibodies for antigens, I designed a novel binding assay (ADHONA) for Tat detection 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 Tat

protein of HIV. RNA^{Tat} is suitable to use for this assay because the RNA^{Tat} has a higher affinity to Tat protein, i.e. 130-fold higher K_d than the TAR-1 for Tat-1 and 40-fold higher K_d than the TAR-2 for Tat-2. In addition, the loop sequences of RNA^{Tat} are not part of the binding site for binding to Tat protein, and RNA^{Tat} could be split into two oligonucleotides. 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 a modulating aptamer. At this stage, ADHONA may not be sensitive enough to measure the amount of Tat released from infected cells or found in the serum of infected patients because of the low intensity of fluorescence. This problem should be improved by using a reagent which enhances the intensity of fluorescence, for example, alkaline phosphatase anti-fluorescein and its substrate producing a color. Moreover, an improvement will be obtained when modulating RNA aptamers can be 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 toward their application in proteomics.

In conclusion, to the best of my knowledge, an interaction between novel aptamer RNA^{Tat} and Tat is one of the tightest interaction in RNA-protein interactions. Thus, RNA^{Tat} is very useful not only for gene therapy of HIV infection but also as a tool for novel detection assay of Tat, such as ADHONA.