

Isolation and characterization of an RNA that binds with high affinity to Tat protein of HIV-1 from a completely random pool of RNA.

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Summary

The *trans*-activation (Tat) protein of human immunodeficiency virus type-1 (HIV-1) is vital for the replication of the virus. In a transcription assay *in vitro* in the presence of authentic TAR RNA, we found that authentic TAR RNA inhibits transcription from a template based on the CMV early promoter in a manner that is not related to the Tat/TAR interaction. Using variants of TAR RNA, we identified potential sequences of RNA that seems to be responsible for the inhibition of transcription. In addition, we isolated an RNA aptamer that can bind with high affinity to Tat protein specifically. The isolated aptamer appears to include two TAR-like RNA motifs for higher-affinity binding to Tat peptides. The aptamer's high affinity for Tat peptides, as well as the absence of any inhibitory effects on the transcription of unrelated genes, as opposed to those of TAR RNA, suggests that the novel RNA might be very useful as a Tat-specific decoy.

I. Introduction

The expression of genes encoded by human immunodeficiency virus type-1 (HIV-1) is regulated by the interaction of cellular factors and a viral *trans*-activator protein, Tat, with specific regulatory elements in the long terminal repeat (LTR) of HIV-1 (Gaynor, 1992). The HIV-1 regulatory protein Tat binds to one of the regulatory elements in the LTR region, which is called the *trans*-activating response region, TAR, (Rosen et al., 1985; Dayton et al., 1986; Fisher et al., 1986). This region is located immediately downstream from the site of initiation of transcription at the 5'-end of all the viral transcripts (Berkhout et al., 1989). It is an RNA element consisting of 59 nucleotides (nt), which is the minimal motif that is sufficient for formation of a stable hairpin structure that allows binding of Tat *in vivo* (Rosen et al., 1985; Feng and Holland, 1988; Jakobovits et al., 1988). Tat effectively stimulates transcription after its binding to

TAR RNA (Cullen, 1986; Peterline et al., 1986; Rice and Mathews, 1988). Deletion studies of TAR RNA revealed that so-called bulge residues are obligatory both for the specific binding to Tat and for *trans*-activation, whereas loop sequences are necessary for *trans*-activation but are not essential for the binding of Tat *in vivo* (Feng and Holland, 1988; Berkhout and Jeang, 1989; Dingwell et al., 1989; Cordingly et al., 1990; Roy et al., 1990; Weeks et al., 1990).

Tat is a small cysteine-rich nuclear protein consisting of 86 amino acids. It has two major domains, a cysteine-rich region and highly basic region (Arya et al., 1985; Sodroski et al., 1985). The cysteine-rich region is essential for the function of this protein (Garcia et al., 1988; Kubota et al., 1988) and it has a metal-binding domain that probably mediates the metal-linked dimerization of Tat (Frankel et al., 1988). The basic region is responsible for the specific binding to TAR RNA (Weeks et al., 1990), as well as for nuclear localization (Dang and Lee, 1989;

Endo et al., 1989). Tat belongs to a family of RNA-binding proteins that contain arginine-rich motifs for recognition of the respective cognate RNAs (Lazinski et al., 1989). A short peptide containing an arginine-rich region binds to TAR RNA with specificity similar to that of the intact protein (Weeks et al., 1990; Calnan et al., 1991a). The *tat* gene product not only plays a key role in the *trans*-activation of HIV-1 genes but also has a variety of effects on the growth and metabolism of the host cells (Ensoli et al., 1990, 1993). Moreover, Tat is now known to be important for the efficient reverse transcription of HIV-1 (Harrish et al., 1997).

Despite several studies on the stimulation by Tat of the *trans*-activation of expression of the HIV genome, the precise molecular mechanism by which it operates remains obscure. The rates of viral mRNA and protein synthesis induced by Tat in mammalian cells were estimated to be 100-fold higher than control rates (Hauber and Cullen, 1988). It has been reported that Tat functions as an anti-terminator, an elongation factor in transcription and an enhancer of the initiation of transcription (for reviews, see Vaishav and Wong-Staal, 1991; Cullen, 1992; Jeang et al., 1993). Even though the exact function of Tat remains controversial, the emerging consensus appears to be that Tat functions as a promoter-specific elongation factor that modifies the transcription complex upon binding to TAR RNA. Several early studies showed that, for the stimulation of transcription by Tat, both cellular transcription factors and the integrity of the TAR RNA sequences are essential (for reviews, see Gaynor, 1992; Jones and Peterlin, 1994). However, the order of assembly of components of the transcription complex was not known until recently, it was unknown initially whether Tat binds to the transcription factors first and then binds to the TAR RNA or whether Tat binds initially to the TAR RNA with subsequent binding of transcription factors. Garcia-Martinez et al. (1997) suggested that Tat protein might associate with RNA polymerase II in the preinitiation complex and then the complexed Tat binds to TAR RNA, while stalled RNA polymerase II is passing through the TAR RNA.

A conformational change in TAR RNA upon binding of Tat is among the most intriguing of RNA-protein interactions. The conformational switch was clearly observed by circular dichroism (CD) and NMR studies (Tan and Frankel, 1992; Puglisi et al., 1992; Aboul-ela et al., 1995, 1996). A similar change in conformation of TAR RNA was achieved not only with Tat peptides but also by arginine alone. However, no conformational change was observed either with lysine or with variants of TAR that can no longer bind to Tat. Tat peptides and homopolymers of arginine that bind specifically to TAR RNA, as well as a peptide containing a central single arginine residue, were sufficient to reproduce wild-type *trans*-activation in studies designed to delineate the role of arginine (Calnan et al., 1991a,b). Further NMR studies on

the TAR-argininamide complex suggested that TAR has a specific binding site for arginine (Puglisi et al., 1992). Arginine appears to induce a structure where in a critical residue, U23, in a triple-base bulge makes a Hoogsteen interaction with an A•U base pair in the adjacent stem. This model was supported by replacing the U•A•U bonding in TAR by the isomorphous C•G•C bonding (Puglisi et al., 1993). Recent modification and mutagenesis experiments also support the model in which a Hoogsteen interaction is critical for the structure of TAR (Tao et al., 1997). Despite the discrepancy between results of NMR studies of TAR RNA from two different laboratories with respect to the formation of the base triple (as distinct from base pair), the general consensus on the structure of TAR RNA is in agreement about the approximate location of the bulge U in the major groove and the orientation of the functional groups in the TAR RNA.

Since the Tat protein has various functions in the life cycle of HIV-1, as well as in viral proliferation, it is an important and attractive target in efforts to develop weapons against HIV. Several genetic strategies have been tested, in the past, in attempts to repress the proliferation of HIV. *Trans*-dominant proteins, single-chain antibodies, antisense molecules, ribozymes, decoys (for review, see Yu et al., 1994) and use of the LTR of HIV to produce inducible and toxic gene products have all been tested in cells that were infected by HIV (Harrison et al., 1992). Combinations of these strategies (for example, a ribozyme and a decoy) have also been examined (Yuyama et al., 1994; Yamada et al., 1996). Although the expression and regulation of such therapeutic molecules might be possible *in vivo*, their constitutive expression could lead to cellular toxicity or to an immune response by the host against the engineered cells. This problem is especially significant in the case of toxins and suicide genes. Among various RNA-based strategies against HIV infection, the decoy strategy has a potential advantage over the use of other RNA inhibitors, such as short antisense RNAs and ribozymes, because the generation of escape mutants might be less frequent: alterations in Tat or Rev (HIV-1 protein) that prevent binding to a decoy would also prevent binding to native elements (such as RRE, the Rev-responsive element, and TAR sequences). Both RRE and TAR RNAs have been exploited as decoys and, in cell cultures, these decoys inhibited the replication of HIV by 80% to 97% (Graham and Maio, 1990; Sullenger et al., 1990; Lisiewicz et al., 1993).

Although decoys might act as much more efficient inhibitors (with possible K_i values in the sub-nanomolar range) than other molecules, such as antisense RNAs and ribozymes, decoys might potentially be toxic to cells if they were to sequester cellular factors, in particular when the decoy RNA happens to include regions that can interact with cellular proteins. Several previous studies have shown that cellular factors, such as TRP-185 (Wu-

Bear et al., 1995), Tat-SF1 (Zhou and Sharp, 1996), polymerase II (Wu-Bear et al., 1995, 1996) and others (Sheline et al., 1991; Rounseville and Kumar, 1992; Gatignol et al., 1991) bind efficiently to TAR RNA. Despite these studies, the effects of TAR RNA on the cellular machinery of the host cells have not been analyzed in detail either *in vitro* or *in vivo*.

In the present study, we performed cell-free transcription assays *in vitro* in the presence of TAR RNA and demonstrated that authentic TAR RNA can inhibit transcription in a manner that is independent of the Tat/TAR interaction. Furthermore, we identified important regions of TAR RNA that are responsible for the inhibition of transcription, namely, the loop, residues that surround the loop, the triple-base bulge, and the lower stem region of the TAR RNA. Since authentic TAR RNA of HIV-1 interacts with several cellular factors within the cell, in addition to inhibiting the transcription of unrelated genes, an authentic TAR decoy might not be the most suitable antagonist and specific inhibitor of Tat. In order to isolate more specific high-affinity RNA decoys that would bind to Tat, we used a recently developed technique of genetic selection *in vitro* (Fig. 1, for reviews, see Gold et al., 1995; Osborne and Ellington, 1997) and isolated a high-affinity RNA motif that bound to Tat. The isolated motif, designated 11G-31 RNA, included conserved core elements of TAR RNA (Fig. 2) that have been identified as being necessary for binding to Tat. In addition, it has two TAR-like motifs opposite one another. The binding ability of a truncated mini 11G-31 RNA motif, a 37-mer, and of authentic TAR RNA were analyzed with Tat peptides in competitive binding assays, and the isolated RNA was found to bind with higher affinity. In addition, we performed a transcription assay *in vitro* in the presence of mini 11G-31 RNA to evaluate the effects of this RNA on the transcription of unrelated promoters.

Figure 1. Scheme for genetic selection *in vitro*.

II. Results and Discussion

A. The effects of TAR RNA in a cell-free transcription assay

Studies both *in vitro* and *in vivo* with LTR-based templates and Tat have suggested that addition of exogenous TAR RNA and/or overexpression of TAR RNA can significantly inhibit *trans*-activation. Such significant inhibition might originate from a combination of two effects: a) the expressed or added TAR RNA might act as a decoy by sequestering Tat and interfering directly with the binding of Tat to the TAR in the LTR region after its transcription from LTR templates; and b) the TAR decoy might sequester transcription factors together with other important proteins, such as RNA polymerase II, that are unrelated to the Tat/TAR interaction. Since earlier decoy studies relied on LTR-based vectors, it was not possible to distinguish between the two possible scenarios.

Figure 2. TAR RNA of the core element of HIV-1. TAR motif is indicated by box and the core element that is required for binding to Tat protein is indicated by red.

In order to distinguish between the two possible effects of TAR RNA, we performed transcription assays *in vitro* with an extract of HeLa cell nuclei. This assay has been used routinely to study the Tat-mediated *trans*-activation

of HIV-1 genes (Marciniak et al., 1990). We hoped that this method would provide some insight into Tat-mediated *trans*-activation and would also allow us to screen various inhibitors that might interfere with Tat-TAR interactions. To examine the interaction between TAR RNA and

Figure 3.
Sequences and secondary structures of authentic TAR RNA and mutant TAR RNAs. Bases dissimilar to those in TAR RNA or deleted are indicated by boxes.

Figure 4. Inhibition of transcription from a CMV early promoter-driven template by authentic TAR RNA in an extract of HeLa cell nuclei. A, The template containing the early promoter of CMV was transcribed in the absence (lane 1) and in the presence (lanes 3 and 4) of 100 pmole of TAR RNA, or in the presence of 100 pmole of tRNA (total tRNA from yeast; lane 2). Single-stranded DNA markers were loaded in lane M. The newly synthesized transcript is indicated by the arrow. B, The relative levels of the transcript (364 nt) that was synthesized *in vitro*, as quantitated in four independent experiments (experimental variations are indicated by error bars).

cellular factors, we performed transcription assays using a cytomegalovirus (CMV) early promoter-based template in the presence and in the absence of authentic TAR RNA (**Fig. 3**).

As seen in **Figure 4A**, which shows a representative autoradiogram, the basal level of transcription (lane 1) from the CMV early promoter was greatly reduced by the addition of 100 pmole of authentic TAR RNA (lanes 3 and 4). Quantification of the results of four independent transcription experiments in the presence of TAR RNA revealed that transcription was inhibited by 60-70% (**Fig. 4B**). By contrast, after addition of a similar amount of tRNA (total tRNA from yeast) the basal level of transcription remained either unaffected or was only marginally reduced (about 10-20%; see **Fig. 4**). These results demonstrate that factors that are important in the transcription process can bind to TAR RNA and, therefore, that the TAR decoy can inhibit transcription *in vitro*.

In analogous experiments based on the LTR promoter, we observed similar inhibition of transcription (data not shown). The effects of circular TAR RNA were analyzed recently by Bohjanen et al. (1996), who used an LTR promoter-based template and studied Tat-mediated *trans*-activation under similar conditions to those that we employed here. They found that circular TAR RNA inhibited Tat-mediated *trans*-activation by 77% (Bohjanen et al., 1996). However, the inhibitory effect of circular TAR RNA could not be relieved by the addition of excess Tat (30-fold excess). The observed inhibition of transcription by TAR RNA in the present study, with the CMV promoter-driven template, was 60-70%, a value close to the 77% inhibition of *trans*-activation observed in earlier experiments with the LTR promoter. Therefore, we propose here that the effects of the TAR decoy observed earlier might have been due to inhibition of transcription, at least to some extent, rather than to sequestration of the Tat protein exclusively. In addition, in view of the above results, we 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.

B. Dependence of the inhibition of transcription on the concentration of TAR RNA

Although the concentration of TAR RNA (100 pmole) tested in the above studies *in vitro* might be achievable *in vivo* with various expression vectors, we were interested in determining the minimum concentration of TAR RNA for inhibition. Therefore, we performed transcription reactions *in vitro* in the presence of concentrations of TAR RNA from 0.01-100 pmole. As seen in **Figure 5**, the inhibition of transcription was directly dependent on the concentration of TAR RNA. As expected, lower levels (0.01-1.0 pmole) of TAR RNA resulted in moderate inhibition (about 30%), while at 10 pmole the extent of inhibition by TAR RNA was similar to that (60-70%) that observed in the presence of 100 pmole of TAR RNA (data not shown). Although the levels of TAR RNA that we used appear to be rather high, similar concentrations of TAR RNA were used in the past to examine the decoy effects of TAR in cell-free transcription assays (Bohjanen et al., 1996). As can be seen in **Figure 4**, addition of 100 pmole of tRNA has either no effect or only a marginal effect. These results clearly demonstrated that inhibition of transcription by TAR RNA depended on the concentration of the RNA.

Figure 5. Dependence on the concentration of TAR RNA of the inhibition of transcription. Template containing the early promoter of CMV was transcribed in an extract of HeLa cell nuclei in the absence (lane 1) and in the presence of increasing concentrations of TAR RNA (lane 2, 0.01 pmole; lane 3, 1 pmole; and lane 4, 100 pmole). Newly synthesized transcripts is indicated by arrow.

C. Inhibition of transcription by variants of TAR RNA

In order to identify the regions of TAR RNA that are responsible for interactions with cellular transcription factors *in vitro*, we synthesized and tested four variants. **Figure 3** shows mutant TAR-1 RNA with altered bases in the loop, mutant TAR-2 RNA with a substituted base-pair (mutated bases are boxed), mutant TAR-3 RNA with a deletion of two bulge-bases, and mutant TAR-4 RNA with deletion of the lower stem. These variants were initially tested for their ability to bind to a Tat peptide (CQ peptide; see Experimental Procedures). Only TAR RNA variants with deletion or substitution of conserved bases (such as TAR-2 and TAR-3 RNAs) had significantly reduced affinity for the CQ peptide (**Fig. 6**). Both TAR-1 and TAR-4 RNAs bound efficiently to the CQ peptide (**Fig. 6**). However, the affinity for the CQ peptide of TAR-4 RNA was lower than the affinity of authentic TAR RNA. From these results it appeared, in harmony with previous results (Weeks and Crothers, 1991; Churcher et al., 1993), that the ability to bind to the CQ peptide was abolished only when the conserved residues in TAR RNA were replaced or missing.

Figure 6. Binding of the Tat peptide (CQ) to TAR RNA and its variants. Relative amounts of complex formed (%) were analyzed after titration of increasing concentrations of CQ peptide with labeled RNAs and electrophoresis on non-denaturing gels, as described under Experimental Procedures. TAR RNA-CQ complex (●), TAR-1 RNA-CQ complex (□), TAR-2 RNA-CQ complex (✕), TAR-3-CQ complex (Δ), TAR-4-CQ complex (○).

We next performed cell-free transcription reactions in the presence of authentic TAR RNA or of its variants. Among the parent and variant RNAs tested, authentic TAR RNA had the highest inhibitory effect on transcription from the CMV promoter. Substitution of bases in the loop sequence of TAR RNA (TAR-1), substitution of the conserved base-pair near the triple-base bulge (TAR-2), deletion of the triple-base bulge (TAR-3), and deletion of the lower stem (TAR-4) all halved the inhibitory effect of TAR on transcription. In order to test the validity of the observed inhibition by the TAR RNA variants in nuclear extracts, we performed four independent transcription experiments in the presence of TAR RNA variants and quantitated the amounts of transcript generated (**Fig. 7**). Each variant (TAR-1, TAR-2, TAR-3 and TAR-4) had a clearly reduced inhibitory effect on transcription. These variants of TAR RNA not only were helpful in attempts to identify regions that are important for interactions with cellular factors but also served as good internal controls. In particular, the TAR-3 RNA variant was a good control since this RNA was synthesized and processed under identical conditions to TAR RNA.

Several transcription factors and cellular factors have been isolated from extracts of HeLa cell nuclei that bind to authentic TAR RNA and facilitate *trans*-activation *in vitro*. The importance of specific residues of TAR RNA for specific binding to such cellular factors and to proteins of the host cell has been evaluated in earlier studies and the reported results can be summarized as follow. a) Two distinct nuclear transcription factors, TRP-1 and TRP-2, were isolated (Sheline et al., 1991) that specifically recognize the loop and triple-base bulge residues of TAR RNA, respectively. b) A p140 protein and TRBP (TAR RNA-binding protein) were isolated from HeLa cell nuclei and were shown to interact specifically with the lower stem of authentic TAR RNA (Rounseville et al., 1992; Gagnon et al., 1991). c) For binding to polymerase II, both the loop and the triple-base bulge of TAR RNA were shown to be important (Wu-Baer et al., 1995, 1996). And, finally, d) TRP-185, a cellular factor appeared to bind specifically to the loop of TAR RNA (Wu-Baer et al., 1995, 1996).

Figure 7. Effects of mutant TAR RNAs on the transcription of a CMV early promoter-driven template in extracts of HeLa cell nuclei. The relative level (percentage) of transcript (364 nt) synthesized in four independent experiments was quantitated by reference to the control. Transcription was allowed to proceed in the presence of 100 pmole of TAR RNA or its variants (experimental variations are indicated by error bars).

Substitution or deletion of bases in the loop, of bases near the loop, of the triple-base bulge, and of the lower stem region of TAR RNA abolished the ability of TAR RNA to inhibit transcription. The loss of the ability of TAR RNA to inhibit transcription probably resulted from loss of the ability of cellular factors to bind to TAR RNA. Thus, the variants of TAR RNA used in our study had lost their ability to sequester cellular factors. In several earlier studies, mutations in the loop, bulge, and lower stem of TAR RNA were associated with marked defects in the replication of HIV-1 in human cell lines, as compared with that of wild-type HIV-1 produced under identical conditions, even though some of the variants could have interacted efficiently with Tat *in vivo* (Rounseville et al., 1996; Berkhout and Jeang, 1991; Harrich et al., 1994; Verhoef et al., 1997). Taken together, the results of earlier studies and our present results with the four TAR variants suggest that cellular factors interact efficiently with TAR RNA only when it retains its full integrity. Furthermore, we extended our inhibition studies with TAR RNA to another human cell line, namely, Jurkat cells, and we observed similar inhibition of transcription to that described above in extracts of HeLa cell nuclei (data not shown).

D. Isolation of an RNA aptamer with high affinity for Tat protein

TAR RNA of HIV-1 binds to several cellular factors in the cell. In view of its inhibitory effects on the transcription of unrelated genes, as observed above, authentic TAR RNA might not be the most suitable antagonist and specific inhibitor of Tat. In order to isolate an RNA motif that binds to Tat specifically and with high affinity, we exploited a strategy for genetic selection *in vitro* using a pool of RNAs with a large random core sequence of 120 nt (120 N).

In the first selection cycle, about 10^{13} RNA sequences were allowed to bind to the HIV-1 Tat protein at a molar ratio of protein to RNA of 1:10 in the binding buffer. In subsequent cycles, molar ratios of Tat and RNAs [the 120 N pool, specific competitors (either TAR RNA or selected pool RNA with a random core region of 12-18 nt having about 5% binding ability to Tat) and a non-specific competitor, tRNA] were manipulated in order to increase the stringency of selection (**Table 1**). After each set of two cycles of selection, the RNA pool was analyzed in a filter-binding assay for binding to Tat. As the cycles progressed, levels of specific RNA aptamers that bound to Tat increased in the pool from 1% to 9%. Since the 10^{14} variants in the 120 N pool could not encompass the entire range of possibilities (10^{72}) variants, mutagenic PCR was introduced after the ninth cycle to increase the diversity of functional molecules. However, with the introduction of mutagenic PCR, the number of binding species was reduced in the pool, probably as a number of the mutation of critical residues and, therefore, we cloned products from both the ninth and the eleventh cycle for the analysis of sequences.

In all, we sequenced 64 clones from the ninth and the eleventh cycles and divided the sequences from the eleventh cycle into four classes. Two major classes of sequences (two representative sequences, 11G-22 and 11G-31, are shown in **Fig. 8**) were derived from the eleventh cycle RNA pool, as compared to the ninth cycle, in which many sequences were unrelated. When these RNA sequences were folded by the Mulfold program (Zuker, 1989), 15 clones, representing about 40% of the population in the pool had a TAR-like motif (containing all core elements) in their randomized region. However, some of the clones has two TAR-like motifs, for example, 11G-31 (**Fig. 8**). Combinatorial analysis of TAR core elements predicts that at least one sequence should be found in every 2.16×10^6 nucleotides (Ferbeyre et al., 1997). Despite such a low probability of distribution of TAR core elements, we were able to isolate TAR-like elements from the random pool, and even a double-TAR element, probably because selective pressure was maintained during the entire selection procedure. Although the predominant selected aptamers that belonged to the two major classes contained two bulge residues (UC or UU), as opposed to three in the TAR of HIV-1, mutational analysis has revealed that at least two bulge

residues are necessary for recognition of Tat (Weeks and Crothers, 1991). Moreover, TAR RNA of HIV-2 also contains two (UU or UA in two TAR motifs) bulge residues that allow efficient binding to the HIV-1 Tat peptide (Chang and Jeang, 1992). A similar selection procedure, using an RNA pool with a 30 nt random core, 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).

E. Mini 11G-31 RNA binds efficiently to Tat-derived peptides

Since a short peptide that contains an arginine-rich region binds to TAR RNA with specificity similar to that of the intact protein (Weeks et al., 1990; Calnan et al., 1991a), we used both the CQ (amino acids 37-72) and RE (amino acids 49-86) peptides in further studies. These

peptides were synthesized chemically and purified to homogeneity (>95% purity) by reverse-phase HPLC.

Initially, a representative clone from each class was subjected to a competitive binding assay in the presence of a Tat peptide (CQ or RE; see Experimental Procedures) and authentic TAR RNA. RNA motifs with two TAR-like motifs, such as 11G-31 RNA, appeared to compete with TAR for bind to the Tat peptides (data not shown). In order to locate the binding region in the 11G-31 RNA (one of the RNAs with high affinity for the Tat peptides), we chemically synthesized a minimal RNA (mini 11G-31; 37 mer) that had two TAR-like motifs and analyzed its binding to Tat peptides on non-denaturing gels. Both CQ and RE peptides efficiently bound to mini 11G-31 RNA (**Fig. 9A and B**).

Table 1. Concentrations of RNA and protein used and the ability of the RNA pool to bind to Tat after each selection cycle.

Cycle number	Concentration of				Binding ability ^a	
	Tat	pool RNA	tRNA	RNA [#]	NP	P
	μ M				%	
1	0.50	5.0	-	-	0	2
2	0.50	1.5	3	1	0	4
3	0.50	1.5	5	5	0	5
4	0.50	3.0	10	10	0	4
5	0.50	1.5	40	7	0	6
6	0.50	5.0	50	10	0	5
7	0.50	3.0	50	7	0	6
8	0.50	5.0	50	14	0	7
9	0.50	5.0	50	14	0	9
10	0.33	5.0	50	14	0	7
11	0.17	2.5	25	7	0	9

Figure 8. Representative sequences and secondary structures from each class of RNAs. Regions resembling TAR core elements (for Tat binding) are shaded and

residues are highlighted in either blue or red. Blue and red letters in mini 11G-31 RNA indicate the presence of two TAR core elements.

Figure. 9A. Formation of complex between Tat peptides (CQ and RE) and TAR RNA or mini 11G-31 RNA. Binding reactions contained 5'-end labeled RNA (15,000 cpm) and 5, 10, 20, 40, 60, 80, or 100 nM Tat peptide. Complexes were separated from unbound RNAs by electrophoresis on 20% non-denaturing polyacrylamide gels. (A-1) TAR RNA and CQ peptide; (A-2) TAR RNA and RE peptide; (A-3) mini 11G-31 RNA and CQ peptide; (A-4) mini 11G-31 RNA and RE peptide.

B. Formation of complex between a bulge deletion variant, an RNA lacking the bulge residues in mini 11G-31 RNA, and Tat peptides (CQ and RE). (B-1) The RNA lacking the bulge residues in mini 11G-31 RNA and the CQ peptide; (B-2) the RNA lacking the bulge residues in mini 11G-31 RNA and the RE peptide.

To compare the binding affinities of the aptamer, mini 11G-31, and TAR RNA to the CQ and RE peptides, we

performed binding assay in which labeled RNAs were incubated with various concentrations of CQ or RE peptide, with subsequent separation of complexed and free

RNA on a 20% non-denaturing polyacrylamide gel (**Fig. 9A**). The amount of each complex formed was calculated directly from the intensities of bands on the gel. Authentic TAR RNA formed a complex at a level of about 50% in the presence of 56 nM CQ peptide (**Fig. 9A-1**), whereas mini 11G-31 RNA efficiently formed the same amount of complex even at 14 nM CQ peptide (**Fig. 9A-3**). When we performed a similar analysis using RE peptide, authentic TAR RNA formed a complex at a level of about 50% in the presence of 23 nM RE peptide (**Fig. 9A-2**), whereas mini 11G-31 RNA formed the same amount of complex even at 3 nM RE peptide (**Fig. 9A-4**). These results suggest that the selected aptamer had higher affinity for the Tat peptides.

In order to evaluate the importance of bulge residues, we synthesized an RNA that lacked the bulge residues in mini 11G-31 RNA and analyzed its binding in gel-shift assays with the CQ and RE peptides. No complex was formed even at a high concentration (200 nM) of CQ peptide (**Fig. 9B-1**). However, a small amount of complex was formed at high concentrations (>80 nM) of the RE peptide (**Fig. 9B-2**). A similar experiment was performed with authentic TAR RNA, as well as with the bulge mutant RNA, to examine binding to the RE and CQ peptides and we observed that the CQ peptide efficiently distinguished the bulge variant, as compared to the RE peptide, a result consistent with previous observations (Churcher et al., 1993). From these studies it appears that the bulge residues in mini 11G-31 are important for the recognition of Tat peptides.

Our various studies indicated that core elements of TAR RNA were well conserved in the isolated aptamers that belonged to the two predominant classes, suggesting the importance of the conserved residues. The sequences of the selected RNAs, sequences containing either a single or a double TAR motif, confirm the details of all the core elements that were previously identified as being required for binding of authentic TAR RNA to Tat. Deletion of bulge residues from mini 11G-31 RNA completely abolished the binding of Tat peptides. In addition, the bulge U residue was found in the single and the double TAR motif. This motif probably forms a Hoogsteen base pair with A-U (Watson-Crick paired) residues to form a base-triple U•A•U as proposed for complexes of arginine or the Tat peptide and TAR RNA. Taken together, the recent mutational results of Tao et al. (1997) and our present results suggest that the arginine-binding motif of TAR can be summarized as 5' UX_nGA, where the U residue is predicted to make a Hoogsteen interaction with the A residue, X_n indicates at least one unpaired nucleotide and the G residue forms a pocket for binding of arginine.

F. Comparison of the relative affinities of TAR and mini 11G-31 for the Tat peptide

When authentic TAR RNA and mini 11G-31 RNA were titrated with various amounts of Tat peptides, the aptamer was observed to bind efficiently to the Tat peptides. We next performed competitive binding assays to compare the affinities of authentic TAR RNA and mini 11G-31 RNA directly. The labeled aptamer and TAR RNA were incubated at a ratio of 1 : 1 with unlabeled aptamer and TAR RNA at various molar ratios (ranging from 40-8,000 nM) in the presence of 80 nM RE peptide. The reaction mixture was allowed to equilibrate at 30 °C for 12 hr and resolved on a 20% non-denaturing polyacrylamide gel. The amount of complex formed by the aptamer with the RE peptide was calculated for various ratios and we found that the amount of the aptamer-peptide complex fell by 50% when the molar ratio was one to eighty (mini 11G-31 : TAR = 40 nM : 3,200 nM). These results suggested that the affinity for the RE peptide of the aptamer might be about 80 times higher affinity ($D_{1/2} \approx 3.2 \mu\text{M}$) than that of authentic TAR RNA (**Fig. 10**). By contrast, no TAR-peptide complex was detected when the molar ratio of non-labeled aptamer to non-labeled TAR RNA was 1 : 1.

Figure 10. Competitive binding assay. Formation of a complex when a mixture of 5'-end labeled mini 11G-31 RNA (20,000 cpm), unlabeled mini 11G-31 RNA (40 nM), and unlabeled authentic TAR RNA at various concentrations (40 to 8,000 nM) was allowed to bind with the RE peptide (80 nM) at 30 °C for 12 h. The mixture was fractionated on a non-denaturing gel as mentioned the legend to **Figure 9**.

In earlier studies, peptides derived from the arginine-rich region of HIV-1 appeared to bind to authentic TAR RNA at concentrations between 16 pM to 40 nM

(Churcher et al., 1993; Long and Crothers, 1995). Both results of titration of Tat peptide and of the competitive binding assays suggest that the isolated aptamer not only interfered with Tat/TAR interactions but also efficiently trapped Tat peptides at sub-nanomolar concentrations. This property is clearly desirable for an efficient decoy of viral proteins.

G. The double TAR-like RNA motif has enhanced affinity for the Tat peptide

In order to define clearly the importance of the double TAR-like motif in the efficient binding to Tat peptides, we separated the two strands and deleted the loop sequences (Fig. 11).

Figure 11. Synthetic mini 11G-31 RNA duplexes. Bases deleted from duplex mini 11G-31 RNAs are indicated by boxes. Core elements of TAR RNA that are found in mini 11G-31 RNA are indicated by blue and red letters. Blue and red nucleotides form the two TAR core elements.

Duplex RNA I, that could mimic mini 11G-31 RNA, was prepared by annealing two chemically synthesized 5' and 3' RNA oligomers, (20 mers). Duplex RNA II (with a deletion of the 3'-bulge residues U and C) was prepared by annealing 3' Δ UC and the 5' RNA oligomers. Duplex RNA III (with a deletion of the 5'-bulge residues U and U) was prepared by annealing the 3' and 5' Δ UU RNA oligomers. To prepare duplex RNA IV (with deletion of both pairs of bulge residues) both 3' Δ UC and 5' Δ UU RNA oligomers were annealed. After labeling of the 5'-end of the oligomer in each duplex, we equilibrated the CQ peptide (40 nM) with each duplex in binding buffer at 30 °C for 1 h. The products were resolved on a non-denaturing

polyacrylamide gel (Fig. 12) and the amount of each complex was calculated as mentioned above. The duplex structure that contained both bulges (duplex RNA I) formed a complex at a level of about 80% at 40 nM (Fig. 12, lane 12) and mini 11G-31 RNA formed a similar amount of complex. Deletion of either the 3'-end bulge residues UU (in duplex RNA II); (Fig. 12, lane 8) or the 5'-end bulge residues UC (in duplex RNA III); (Fig. 12, lane 10) reduced by about 50% the amount of complex formed with the CQ peptide. After deletion of both pairs of bulge residues in the duplex (duplex RNA IV) no complex was formed in the presence of the CQ peptide (Fig. 12, lane 4). The results obtained suggest that the ratio of aptamer to peptide in the complex was one to one and that both pairs of bulge residues did indeed play important roles in the efficient binding to the CQ peptide.

Figure 12. Formation of complex between the Tat peptide CQ and duplex RNA I (mini 11G-31), duplex RNA II, duplex RNA III, or duplex RNA IV. Reaction mixtures contained either a duplex strand or RNA (one labeled RNA, usually the 5'-end-labeled oligomer and unlabeled second strand) alone or in the presence of 40 nM CQ peptide. Complexes were separated from unbound RNAs by electrophoresis on a 20% non-denaturing polyacrylamide gel. 3' Δ UC RNA oligo alone (lane 1) or in the presence of CQ peptide (40 nM) (lane 2); duplex RNA IV either alone (lane 3) or in the presence of CQ (lane 4); 3' RNA oligo either alone (lane 5) or in the presence of CQ (lane 6); duplex RNA III either alone (lane 7) or in the presence of CQ (lane 8); duplex RNA II either alone (lane 9) or in the presence of CQ (lane 10); duplex RNA I either alone (lane 11) or in the presence of CQ (lane 12). Large and small arrow heads indicate duplex RNA and CQ peptide complex and duplex RNAs, respectively.

H. The effects of mini 11G-31 RNA in a cell-free transcription assay

As demonstrated above, authentic TAR RNA inhibited the transcription of the CMV template in transcription assay *in vitro*. In order to examine the effect of the

isolated aptamer (mini 11G-31) on transcription of unrelated templates, we performed further transcription assays in extracts of HeLa cell nuclei (**Fig. 13A**). Addition of exogenous authentic TAR RNA (100 pmole) inhibited the transcription of the CMV-derived template by about 50-60%, as mentioned above (**Fig. 13A**, lanes 3 and 4). Transcription from the CMV promoter in the absence (**Fig. 13A**, lane 1) or in the presence of 100 pmole of tRNA (total tRNA from yeast); (**Fig. 13A**, lane 2), and in the presence of 100 pmole of mini 11G-31 RNA (**Fig. 13A**, lane 5) was unaffected or only marginally affected. Quantification of the results of three independent transcription experiments revealed that only TAR RNA inhibited transcription to a significant extent (**Fig. 13B**). Thus, the isolated aptamer bound to Tat peptides with high affinity as compared to authentic TAR RNA and it had no negative effect on the transcription of unrelated genes, as judged from the results of transcription *in vitro*. Therefore, it seems that mini 11G-31 RNA might be very useful as a Tat-specific decoy.

III. Conclusion

The results obtained in this study demonstrate clearly, for the first time, that authentic TAR RNA inhibits transcription from a template under control of the CMV early promoter in a manner that is not related to the Tat/TAR interaction. By analyzing variants of TAR RNA, we identified potential sites in the RNA that were responsible for the inhibition of transcription, in addition to demonstrating the importance of the integrity of authentic TAR RNA for the interactions with several cellular factors in human cell lines. Using a strategy of genetic selection *in vitro*, we isolated an RNA aptamer, 11G-31, with high affinity to the Tat protein of HIV-1. Both full-length 11G-31 RNA and mini 11G-31 appeared to bind to the Tat peptides with a similar efficiency. The isolated aptamer had two TAR-like RNA motifs opposite one another which were found to assist in the high-affinity binding of the aptamer to Tat peptides. The absence of inhibitory effects on transcription by the aptamer, as compared to the inhibition by authentic TAR RNA, makes the mini 11G-31 RNA an attractive molecule for further analysis as a potential Tat decoy in infections by HIV-1. The next challenge is to test how efficiently the selected aptamer traps the Tat protein in a model system in human cell lines before we proceed to studies with live virus.

Figure 13. Inhibition of transcription from a CMV early promoter-driven template by mini 11G-31 and authentic TAR RNA in an extract of HeLa nuclei. A, The template containing the early promoter of CMV was transcribed in the absence (lane 1) and in the presence (lanes 3 and 4) of 100 pmole of TAR RNA, in the presence of 100 pmole of tRNA (total tRNA from yeast; lane 2), or in the presence of 100 pmole of mini 11G-31. Single-stranded DNA markers were loaded in lane M. The newly synthesized transcript is indicated by an arrow. B, The relative level of transcript (364 nt) synthesized *in vitro* was quantitated in three independent experiments (experimental variations are indicated by error bars).

IV. Experimental Procedures

A. Preparation of TAR and mutant TAR RNAs

Oligodeoxyribonucleotide templates containing the T7 promoter and sequences that corresponded to the RNAs shown in **Figure 1** were synthesized with a DNA synthesizer (model 392A; Applied Biosystems, USA). In the presence of the reverse primer 5'-GGGTTCCCTAGTTAGCCAGA-3', single-stranded DNA oligonucleotides were converted to double-stranded DNA (dsDNA) by *Taq* DNA polymerase (Nippon Gene, Japan). Each reaction was carried out in a 100- μ l mixture that contained 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 1.5 mM MgCl₂, 0.1% Triton X-100, 0.2 mM dNTPs, 100 pmole of reverse primer, 78 pmole of DNA oligonucleotide and 2.5 units of *Taq* DNA polymerase (Takara, Japan). The reaction mixture was subjected to cycles of 94 °C for 1 min, 45 °C for 1 min and 68 °C for 2 min, until a product of the desired size was obtained. The resulting dsDNA template was precipitated in ethanol and transcribed by T7 RNA polymerase to generate TAR RNA or a mutant TAR RNA. Transcription *in vitro* was completed during incubations at 37 °C for 2 hours using a T7 Ampliscribe kit (Epicentre Technologies, USA). After the synthesis of RNAs and treatment with DNase I, reaction mixtures were fractionated by electrophoresis on a 10% denaturing polyacrylamide gel (PAGE). RNAs were extracted and recovered from the gel after ethanol precipitation.

TAR-4 RNA was synthesized chemically. The functional groups were deprotected by established protocols (ABI manual) and the RNA was purified on a 15% PAGE.

B. Transcription assays *in vitro* in the presence of TAR RNA and its variants

In order to investigate the effects of TAR RNA on the cellular machinery at the transcriptional level, we used the cytomegalovirus (CMV) immediate early promoter that either contained or lacked enhancer elements. We chose CMV DNA as the template, as an example, for evaluation of the effect of TAR RNA on the LTR-independent transcription of a template. The CMV early-promoter region (from nt -238 to 364) was amplified by *Taq* DNA polymerase with specific primers [5'-TTAGTCATCGCTATTACCATGG-3' and 5'-AGGCTTGGATTACAGGACGGGTG-3'] by PCR [94 °C for 3 min, 50 °C for 1.15 min, and 72 °C for 3 min; 30 cycles]. The resulting product of PCR (602 nt) was recovered by ethanol precipitation and used in the transcription assay. The transcription reaction was carried out with an extract of HeLa cell nuclei (Promega, USA) in the presence of [α -³²P]CTP. Initially, 13 units of the nuclear extract, 3 mM MgCl₂, 0.4 mM each ATP, GTP and UTP and 16 μ M CTP plus 10 μ Ci [α -³²P]CTP (3,000 Ci/mmol; Amersham, U.K.) were combined in buffer [20 mM HEPES (pH 7.9), 100 mM KCl, 0.2 mM EDTA, 0.5 mM DTT and 20% glycerol], mixed with 100 pmole of TAR RNA, of a variant or of total tRNA from yeast (Boehringer Mannheim, Germany) and allowed to equilibrate for 15 min at 30 °C. This reaction mixture was supplemented with 100 ng of template DNA for PCR to give a final reaction volume of 25 μ l and incubation was continued at 30 °C for a further 45 min. The reaction was terminated by addition of 175 μ l of stop solution [0.3 M Tris-HCl (pH 7.4), 0.3 M sodium acetate, 0.5% SDS, 2 mM EDTA and 3 mg/ml of tRNA] and the products were extracted once with phenol and chloroform before precipitation in ethanol. The newly synthesized RNAs were denatured in loading buffer (25 mM

EDTA and 4.5 M urea) at 90 °C for 5 min, loaded on a 6% polyacrylamide gel that contained 7 M urea and fractionated by electrophoresis. Bands on the gel were quantitated with an image analyzer (BAS 2000; Fuji Film, Japan).

C. Synthesis of Tat peptides and gel-shift assays

Two Tat peptides that spanned the arginine-rich region of Tat protein were synthesized chemically: CQ (amino acids 37-72, CFTTKALGISYGRKKRRRQRRPPQGSQTHQVSL SKQ, 36 mer); and RE (amino acids 49-86, RKKRRQRR RPPQGSQTHQVSLSKQPTSQSRGDPTGPKE, 38 mer). These peptides were purified by HPLC and their compositions were confirmed, after hydrolysis, by reverse-phase HPLC.

CQ peptide was titrated against 5'-labeled TAR or a variant in an 8- μ l binding reaction [10 mM Tris-HCl (pH 8.0), 70 mM NaCl, 2 mM EDTA, 40 nM total tRNA from yeast (Boehringer Mannheim) and 0.01% Nonidet P-40 (Shell Chemicals, USA)]. Initially, each labeled TAR RNA was denatured at 94 °C for 2 min and allowed to equilibrate at room temperature for 10 min before mixing with various concentrations of the peptide. The mixtures were incubated at 30 °C for 1 h and the complex and free RNAs were separated on a 15% non-denaturing polyacrylamide gel. The amount of each complex on the gel was quantitated with the image analyzer.

D. Tat protein and the RNA pool

The Tat protein of HIV-1 that we used for selections was purchased from RepliGen (USA). Initially this Tat protein was tested for LTR-dependent *trans*-activation in a cell-free transcription assay with an extract of HeLa nuclei and it was demonstrated that the supplied Tat efficiently supported *trans*-activation. The Tat was also analyzed for efficient binding to TAR. From its properties, we reasoned that the supplied protein contained active Tat protein of high purity (>90%).

The RNA pool (169.1) for use in selections was described initially by Ellington and Szostak (1992). The RNAs in the pool contained 120 nt (N) random core region that was flanked by two constant regions for amplification, as follows: 5'-GGGAGAATTCCGACCAGAAGCTT--120N--CATATGTGCGTCTACATGGATCCTCA-3'. The primers used for amplification of the pool were 5'-AGTAATACGACTCAC TATAGGGAGAATTCCGACCAGAAG-3' (designated 39.169) and 5'-TGAGGATCCATGTAGACGCACATA-3' (designated 24.169). In the selection cycles, yeast tRNA (Boehringer Mannheim) was used as a non-specific competitor.

E. Selection *in vitro*

The protocol that we followed for selection *in vitro* resembled that reported by Urvil et al. (1997). The first cycle of selection was carried out in binding buffer that contained 5.0 μ M (final concentration) RNA (representing approximately 4 x 10¹³ RNA sequences) and 0.5 μ M Tat protein (HIV-1). Before mixing of Tat protein and pool RNAs, initially, the RNAs in the pool were denatured in binding buffer at 90 °C for 2 min and allowed to cool at room temperature for 10 min to facilitate the equilibration of different conformers. The reaction mixture was

incubated for 1 h and filtered as described elsewhere (Urvil et al., 1997). After each of the next five cycles, concentrations of pool RNAs were manipulated and RNAs were allowed to compete for binding to Tat in the presence of increasing concentrations of both non-specific RNA (*E. coli* tRNA) and specific competitor RNA (TAR RNA containing nts +18 through +44) up to the sixth cycle. From the seventh to the eleventh cycle, the pool RNAs were allowed to compete additionally with another specific pool of competitor RNAs [a selected pool (12-18 N pool, with Tat-binding ability of about 5%)]. For the last two cycles, the concentration of Tat protein was reduced significantly. The binding buffer consisted of 50 mM Tris-HCl (pH 7.8) and 50 mM KCl. Pool 0 RNA was pre-filtered through a prewetted nitrocellulose acetate filter (HAWP filter, 0.45 μ m, 13.0 mm diameter; Millipore, USA) in a "Pop-top" filter holder (Nucleopore, USA) to select against RNAs that bound selectively to the filter. This pre-filtering was performed after each additional cycle. The Tat-RNA complexes were collected on a filter after each cycle of selection by washing with 1 ml of binding buffer. Bound RNAs were eluted from filters with 0.4 M sodium acetate, 5 mM EDTA and 7 M urea (pH 5.5) at 90 °C over the course of 5 min. After ethanol precipitation, reverse transcription and amplification by PCR were performed with AMV reverse transcriptase (Seikagaku, Japan) and *Taq* DNA polymerase (Nippon Gene), respectively, as described elsewhere (Urvil et al., 1997).

In addition, a mutagenic PCR protocol (Leung et al., 1989) was also employed during the ninth, tenth and eleventh cycles. In these cycles, half of the cDNA reaction mixture was amplified as described above, while the remaining half was amplified in 100 μ l of a reaction mixture for PCR that contained 67 mM Tris-HCl (pH 8.8), 16.6 mM (NH₄)₂ SO₄, 6.1 mM MgCl₂, 6.7 mM EDTA (pH 8.0), 0.17 mg/ml BSA, 10 mM β -mercaptoethanol, 1% DMSO, 0.2 mM dATP, 1 mM each of dCTP, dGTP and dTTP, 0.5 mM MnCl₂, 5 U of *Taq* DNA polymerase and 0.4 mM of each primer. The reaction mixture was cycled at 94 °C for 1.15 min, at 50 °C for 1.15 min and at 72 °C for 2.15 min for as many cycles as were needed to produce a band of a product of the appropriate size. The product from this PCR (*ca.* 0.25 μ g) was combined with the product of the standard PCR (*ca.* 1.0 μ g) prior to transcription with T7 RNA polymerase.

After the eleventh cycle of selection, the product of PCR was ligated directly into the pCRII vector (Invitrogen, USA) in accordance with the protocol provided by Invitrogen. DNA was isolated from individual clones by the alkaline lysis method and sequenced with a Dye Terminator Sequencing Kit [Applied Biosystems Inc. (ABI)] on a DNA sequencer (model 373A; ABI).

F. Filter Binding Assay

For evaluation of the binding activities of pool RNAs from different selection cycles, as well as those of individual aptamers, internally labeled RNAs were prepared using 0.5 μ Ci/ml [α -³²P]CTP. Conditions for binding and transcription *in vitro* were similar to those used for selection except that the molar ratio of RNA to Tat was 1:1 (330 nM : 330 nM). The filters were washed with 1 ml of binding buffer, air-dried, and radioactivity on filters was quantitated with the image analyzer (BAS2000). To ensure that the binding was specific, we added a ten-fold molar excess of tRNA as a nonspecific competitor to each binding reaction.

G. Gel-shift competitive binding assay

The 5'-end labeled mini 11G-31 RNA and TAR RNA were mixed initially at a molar ratio of one to one. Various ratios of TAR to mini 11G-31 RNA, ranging from 1:1 to 1:200, were prepared after adjustment of concentrations with non-labeled mini 11G-31 and TAR RNA (40 nM mini 11G-31 and increasing concentrations of TAR RNA, from 40-8,000 nM). Both RNAs were denatured at 94 °C for 2 min and then allowed to equilibrate at ambient temperature. The RNA samples were then allowed to bind to 80 nM RE peptide at 30 °C for 12 h, to allow quantitation of the RNA-protein complex at the equilibrium point. The reaction products were separated on a non-denaturing gel and the amounts of complexes formed by the two RNAs were analyzed.

To characterize the individual aptamers from the eleventh selection cycle, we performed similar competitive binding assays, in which individual RNAs (10-100 nM) were allowed to compete with authentic TAR in the presence of 100 nM CQ peptide.

H. Synthesis of mini 11G-31 RNA duplexes

In order to establish the importance of double TAR-like motif of 11G-31 for efficient binding to the Tat peptide (CQ), four strands of oligoribonucleotides were chemically synthesized to prepare four duplex RNAs after deleting the loop sequences from mini 11G-31 RNA and their sequences are as follows: 5' RNA oligo (5'-ACGAAGCUUGAUCCCGAGAC-3'), 3' RNA oligo (5'-GUCUCGGUCGAUCGCUUCGU-3'), 5'AUU RNA oligo (5'-ACGAAGCGAUCCCGAGAC-3'), 3'ΔUC RNA oligo (5'-GUCUCGGGAUCGCUUCGU-3'). These oligo's functional groups were deprotected by established protocols (ABI manual) and purified on a 20% polyacrylamide gel.

I. Transcription assay *in vitro* in the presence of mini 11G-31 RNA

In order to determine whether or not the isolated aptamer mini 11G-31 RNA could interfere with transcription of unrelated templates, we performed transcription assays *in vitro* with extracts of HeLa cell nuclei as described above for studies of TAR.

References

- Aboul-ela, F., Karn, J., and Varani, G. (1995). The structure of the human immunodeficiency virus type-1 TAR RNA reveals principles of RNA recognition by Tat protein. *J. Mol. Biol.* 253, 313-332.
- Aboul-ela, F., Karn, J., and Varani, G. (1996). Structure of HIV-1 TAR RNA in the absence of ligands reveals a novel conformation of the trinucleotide bulge. *Nucleic Acids Res.* 24, 3974-3982.
- Arya, S.K., Guo, C., Joseph, S.F., and Wong-Staal, F. (1985). *Trans*-activator gene of human T-lymphotropic virus type III (HTLV-III). *Science* 229, 69-73.

- Berkhout, B., Silverman, R.H., and Jeang, K.T. (1989). Tat *trans*-activates the human immunodeficiency virus through a nascent RNA target. **Cell** 59, 273-282.
- Berkhout, B., and Jeang, K.T. (1989). *Trans*-activation of human immunodeficiency virus type 1 is sequence-specific for both the single-stranded bulge and loop of the *trans*-acting-responsive hairpin: a quantitative analysis. **J. Virol.** 63, 5501-5504.
- Berkhout, B., and Jeang, K.T. (1991). Detailed mutational analysis of TAR RNA: critical spacing between the bulge and loop recognition domains. **Nucleic Acids Res.** 19, 6169-6176.
- Bohjanen, P.R., Colvin, R.A., Puttaraju, M., Been, M.D., and Garcia-Blanco, M.A. (1996). A small circular TAR RNA decoy specifically inhibits Tat-activated HIV-1 transcription. **Nucleic Acids Res.** 24, 3733-3738.
- Calnan, B.J., Biancalana, S., Hudson, D., and Frankel, A.D. (1991a). Analysis of arginine-rich peptides from the HIV Tat protein reveals unusual features of RNA-protein recognition. **Genes Dev.** 5, 201-210.
- Calnan, B.J., Tidor, B., Biancalana, S., Hudson, D., and Frankel, A.D. (1991b). Arginine-mediated RNA recognition: the arginine fork. **Science** 252, 1167-1171.
- Chang, Y.-N., and Jeang, K.-T. (1992). The basic RNA-binding domain of HIV-2 Tat contributes to preferential *trans*-activation of a TAR-2-containing LTR. **Nucleic Acids Res.** 20, 5465-5472.
- Churcher, M.J., Lmont, C., Hamy, F., Dingwell, C., Greem, S.M., Lowe, A.D., Butler, P.D.G., Gait, M.J., and Karn, J. (1993). High-affinity binding of TAR RNA by the human immunodeficiency virus Tat protein requires amino acid residues flanking the basic domain and base pairs in the RNA stem. **J. Mol. Biol.** 230, 90-110.
- Cordingly, M.G., La Femina, R.L., Callahan, P.L., Condra, J.H., Sardana, V.V., Graham, D.J., Nguyen, T.M., Le Grow, K., Gotlib, L., Schlabach, A.J., and Colonno, R.J. (1990). Sequence-specific interaction of Tat protein and Tat peptides with the transactivation-responsive sequence element of human immunodeficiency virus type 1 *in vitro*. **Proc. Natl. Acad. Sci. U.S.A.** 87, 8985-8989.
- Cullen, B.R. (1986). *Trans*-activation of human immunodeficiency virus occurs via a bimodal mechanism. **Cell** 46, 973-982.
- Cullen, B.R. (1992). Mechanism of action of regulatory proteins encoded by complex retroviruses. **Microbiol. Rev.** 56, 375-394.
- Dang, C.V., and Lee, W.M.F. (1989). Nuclear and nucleolar targeting sequences of c-erb-A, c-myc, N-myc, p53, HSP70, and HIV tat proteins. **J. Biol. Chem.** 264, 18019-18023.
- Dayton, A.I., Sodroski, J.G., Rosen, C.A., Goh, W.C., and Haseltine, W.A. (1986). The *trans*-activator gene of the human T cell lymphotropic virus type III is required for replication. **Cell** 44, 941-947.
- Dingwell, C., Ernberg, I., Gait, M.J., Green, S.M., Heaphy, S., Karn, J., Lowe, A.D., Singh, M., Skinner, M.A., and Valerio, R. (1989). Human immunodeficiency virus 1 Tat protein binds *trans*-activation-response region (TAR) RNA *in vitro*. **Proc. Natl. Acad. Sci. U.S.A.** 87, 8985-8989.
- Ellington, A.D., and Szostak, J. (1992). Selection *in vitro* of single-stranded DNA molecules that fold into specific ligand-binding structures. **Nature** 355, 850-852.
- Endo, S.-I., Kubota, S., Siomi, H., Adachi, A., Oroszlan, S., Maki, M., and Hatanaka, M. (1989). A region of basic amino-acid cluster in HIV-1 Tat protein is essential for *trans*-acting activity and nucleolar localization. **Virus Genes** 3, 99-110.
- Ensoli, B., Barillari, G., Salahuddin, S.Z., Gallo, R.C., and Wong-Staal, F. (1990). Tat protein of HIV-1 stimulates growth of cells derived from Kaposi's sarcoma lesions of AIDS patients. **Nature** 344, 84-86.
- Ensoli, B., Buonaguro, L., Barillari, G., Fiorelli, V., Gendelman, R., Morgan, R.A., Wingfield, P., and Gallo, R.C. (1993). Release, uptake, and effects of extracellular human immunodeficiency virus type 1 Tat protein on cell growth and viral *trans*-activation. **J. Virol.** 67, 277-287.
- Feng, S., and Holland, E.C. (1988). HIV-1 Tat *trans*-activation requires the loop sequence within TAR. **Nature** 334, 165-167.
- Ferbeyre, G., Bourdeau, V., and Cedergren, R. (1997). Does HIV tat protein also regulate genes of other viruses present in HIV infection? **Trends in Biochem. Sci.** 22, 115-116.
- Fisher A.G., Feinberg, M.B., Josephs, S.F., Harper, M.E., Marselle, L.M., Reyes, G., Gonda, M.A., Aldovini, A., Debouk, C., Gallo, R.C., and Wong-Staal, F. (1986). The *trans*-activator gene of HTLV-III is essential for virus replication. **Nature** 320, 367-371.
- Frankel, A.D., Bredt, D.S., and Pabo, C.O. (1988). Tat protein from human immunodeficiency virus forms a metal-linked dimer. **Science** 240, 70-73.
- Garcia, J.A., Harrich, D., Pearson L., Mitsuyasu, R., and Gaynor, R. (1988). Functional domains required for tat-induced transcriptional activation of the HIV-1 long terminal repeat. **EMBO J.** 7, 3143-3147.
- Garcia-Martinez, L.F., Ivanov, D., and Gaynor, B. (1997). Association of Tat with purified HIV-1 and HIV-2 transcription preinitiation complexes. **J. Biol. Chem.** 272, 6951-6958.
- Gatignol, A., Buckler-White, A., Berkhout, B., and Jeang K.-T. (1991). Characterization of a human TAR RNA-binding protein that activates the HIV-1 LTR. **Science** 251, 1597-1600.
- Gaynor, R. (1992). Cellular transcription factors involved in the regulation of HIV-1 gene expression. **AIDS** 6, 347-363.
- Gold, L., Polisky, B., Uhlenbeck, O., and Yarus, M. (1995). Diversity of oligonucleotide functions. **Annu. Rev. Biochem.** 64, 763-797.
- Graham, G.J., and Maio, J.J. (1990). RNA transcription of the human immunodeficiency virus *trans*-activation response element can inhibit action of the viral *trans*-activator. **Proc. Natl. Acad. Sci. U.S.A.** 87, 5817-5821.
- Harrich, D., Hsu, C., Race, E., and Gaynor, R.B. (1994). Differential growth kinetics are exhibited by human

- immunodeficiency virus type 1 TAR mutants. **J. Virol.** 68, 5899-5910.
- Harrich, D., Ulich, C., Garcia-Martinez, L.F., and Gaynor, R.B. (1997). Tat is required for efficient HIV-1 reverse transcription. **EMBO J.** 16, 1224-1235.
- Harrison, G.S., Long, C.J., Curiel, T.J., Maxwell, F., and Maxwell, H. (1992). Inhibition of human immunodeficiency virus-1 production resulting from transduction with a retrovirus containing an HIV-1-regulated diphtheria toxin A chain gene. **Hum. Gen. Ther.** 3, 461-469.
- Hauber, J., and Cullen, B.R. (1988). Mutational analysis of the *trans*-activation-responsive region of the human immunodeficiency virus type I long terminal repeat. **J. Virol.** 62, 673-679.
- Jakovovits, A., Smith, D.H., Jakobovits, E.B., and Capon, D.J. (1988). A discrete element 3' of human immunodeficiency virus 1 (HIV-1) and HIV-2 mRNA initiation sites mediates transcriptional activation by an HIV transactivator. **Mol. Cell. Biol.** 8, 2555-2561.
- Jeang, K.T., Berhout, B., and Dropulic, B. (1993). Effects of integration and replication on transcription of the HIV-1 long terminal repeat. **J. Biol. Chem.** 268, 24940-24949.
- Jones, K.A., and Peterlin, B.M. (1994). Control of RNA initiation and elongation at the HIV-1 promoter. **Annu. Rev. Biochem.** 63, 717-743.
- Kubota, S., Endo, S.-I., Maki, M., and Hatanaka, M. (1988). Role of cysteine-rich region of HIV Tat protein in its *trans*-activation ability. **Virus Genes** 2, 113-118.
- Lazinski, D., Grzadzilska, E., and Das, A. (1989). Sequence-specific recognition of RNA hairpins by bacteriophage antiterminators requires a conserved arginine-rich motif. **Cell** 59, 207-218.
- Leung, D.W., Chen, E., and Goeddel, D.V. (1989). A method for random mutagenesis of a defined DNA segments using a modified polymerase chain reaction. **J. Methods Cell and Mol. Biol.** 1, 11-15.
- Liszewicz, J., Sun D., Smythe, Lusso, P., Lori, F., Louie A., Markham, P., Rossi, J., Reitz, M., and Gallo, R.C. (1993). Inhibition of human immunodeficiency virus type I replication by regulated expression of a polymeric Tat activation response RNA decoy as a strategy for gene therapy in AIDS. **Proc. Natl. Acad. Sci. U.S.A.** 90, 8000-8004.
- Long, K.S., and Crothers, D.M. (1995). Interaction of human immunodeficiency virus type 1 Tat-derived peptides with TAR RNA. **Biochemistry** 34, 8885-8895.
- Marciniak, R.A., Calnan, B.J., Frankel, A.D., and Sharp, P.A. (1990). HIV-1 Tat protein *trans*-activates transcription *in vitro*. **Cell** 63, 791-802.
- Osborne, S.E., and Ellington, A.D. (1997). Nucleic acid selection and the challenge of combinatorial chemistry. **Chem. Rev.** 97, 349-370.
- Peterline, B.M., Luciw, P.A., Barr, P.J., and Walker, M.D. (1986). Elevated levels of mRNA can account for the *trans*-activation of human immunodeficiency virus. **Proc. Natl. Acad. Sci. U.S.A.** 83, 9734-9738.
- Puglisi, J.D., Tan, R., Calnan B.J., Frankel, A.D., and Williamson, J.R. (1992). Conformation of the TAR RNA-arginine complex by NMR spectroscopy. **Science** 257, 76-80.
- Puglisi, J.D., Chen, L., Frankel, A.D., and Williamson, J.R. (1993). The role of RNA structure in arginine recognition of TAR RNA. **Proc. Natl. Acad. Sci. U.S.A.** 90, 3680-3684.
- Rice, A.P., and Mathews, M.B. (1988). Transcriptional but not translational regulation of HIV-1 by the Tat gene product. **Nature** 332, 551-553.
- Rosen, C.A., Sodroski J.G., and Haseltine W.A. (1985). The location of *cis*-acting regulatory sequences in human T cell lymphotropic virus type III. **Cell** 41, 813-823.
- Rounseville, M.P., and Kumar, A. (1992). Binding of a host cell nuclear protein to the stem region of human immunodeficiency virus type 1 *trans*-activation-response RNA. **J. Virol.** 66, 1688-1694.
- Rounseville, M.P., Lin, H.-C., Agbottah E., Shukla, R.R., Rabson, A.B., and Kumar, A. (1996). Inhibition of HIV-1 replication in viral mutants with altered TAR RNA stem structures. **Virology** 216, 411-417.
- Roy, S., Delling, U., Chen, C. H., Rosen, C.A., and Sonenberg, N. (1990). A bulge structure in HIV-1 TAR RNA is required for Tat binding and Tat-mediated *trans*-activation. **Genes Dev.** 4, 1365-1373.
- Sheline, C.T., Milocco, L.H., and Jones, K.A. (1991). Two distinct nuclear transcription factors recognize loop and bulge residues of the HIV-1 TAR RNA hairpin. **Genes Dev.** 5, 2508-2520.
- Sodroski, J., Patarca, R., Rosen, C., Wong-Staal, F., and Haseltine, W.A. (1985). Location of the *trans*-acting region on the genome of human T-cell lymphotropic virus type III. **Science** 229, 74-77.
- Sullenger, B.A., Gallardo, H.F., Ungers, G.E., and Gilboa, E. (1990). Overexpression of TAR sequences renders cells resistant to human immunodeficiency virus replication. **Cell** 63, 601-608.
- Tan, R., and Frankel, A.D. (1992). Circular dichroism studies suggest that TAR RNA changes conformation upon specific binding of arginine or guanidine. **Biochemistry** 31, 10288-10294.
- Tao, J., Chen, L., and Frankel, A.D. (1997). Dissection of the proposed base triple in human immunodeficiency virus TAR RNA indicates the importance of the Hoogsteen interaction. **Biochemistry** 36, 3491-3495.
- Tuerk, C., and MacDougall-Waugh, S. (1993). In vitro evolution of functional nucleic acids: high-affinity RNA ligands of HIV-1 proteins. **Gene** 137, 33-39.
- Urvil, P.T., Kakiuchi, N., Zhou, D.-M., Shimotohno, K., Kumar, P.K.R., and Nishikawa, S. (1997). Selection of RNA aptamers that bind specifically to the NS3 protease of hepatitis C virus. **Eur. J. Biochem.** 248, 130-138.
- Vaishav, Y.N., and Wong-Staal, F. (1991). The biochemistry of AIDS. **Annu. Rev. Biochem.** 60, 577-630.

- Verhoef, K., Tijms., and Berkhout, B. (1997). Optimal Tat-mediated activation of the HIV-1 LTR promoter requires a full-length TAR RNA hairpin. **Nucleic Acids Res.** 19, 6169-6176.
- Weeks, K.M., Ampe, C., Schults, S.C., Steitz, T.A., and Crothers, D.M. (1990). Fragments of the HIV-1 Tat protein specifically bind to TAR RNA. **Science** 249, 1281-1285.
- Weeks, K.M., and Crothers, D.M. (1991). RNA recognition by Tat-derived peptides: interaction in the major groove? **Cell** 66, 577-588.
- Wu-Baer, F., Lane, W.S., and Gaynor R.B. (1995). The cellular factor TRP-185 regulates RNA polymerase II binding to HIV-1 TAR RNA. **EMBO J.** 14, 5995-6009.
- Wu-Baer, F., Lane, W.S., and Gaynor, R.B. (1996). Identification of a group of cellular factors that stimulates the binding of RNA polymerase II and TRP-185 to human immunodeficiency virus 1 TAR RNA. **J. Biol. Chem.** 271, 4201-4208.
- Yamada, O., Kraus, G., Luznik, L., Yu, M., and Wong-Staal, F. (1996). A chimeric human immunodeficiency virus type 1 minimal rev responsive element-ribozyme molecules exhibit dual antiviral function and inhibits cell-cell transmission. **J. Virol.** 70, 1596-1601.
- Yu, M., Poeschla, E., and Wong-Staal, F. (1994). Progress towards gene therapy for HIV infection. **Gene Ther.** 1, 13-26.
- Yuyama, N., Ohkawa, J., Koguma, T., Shirai, M., and Taira, K. (1994). A multifunctional expression vector for an anti-HIV-1 ribozyme that produces a 5'- and 3'- trimmed *trans*-acting ribozyme, targeted against HIV-1 RNA, and *cis*-acting ribozymes that are designed to bind to and thereby sequester *trans*-activator proteins such as Tat and Rev. **Nucleic Acids Res.** 22, 5060-5067.
- Zhou, Q., and Sharp, P.A. (1996). Tat-SF-1: Cofactor for stimulation of transcriptional elongation by HIV-1 Tat. **Science** 274, 605-610.
- Zuker, M. (1989). On finding all suboptimal foldings of an RNA molecule. **Science** 244, 48-52.

Belongs to Table I

a. The binding assays were performed either in the presence (P) or absence (NP) of Tat protein. Both Tat and individually labeled RNA pools from different cycles were incubated together and then filtered under similar conditions to those used during selection *in vitro* in the presence of a 10-fold excess a non-specific competitor (tRNA) in 100- μ l binding buffer [50 mM Tris-HCl (pH 7.5), 50 mM KCl]. # Specific RNA competitor (see Experimental Procedures)