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The ability of tRNA-embedded ribozymes to prevent replication of HIV-1 in cell culture

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Summary

A *trans*-acting tRNA-embedded ribozyme targeted to HIV-1 RNA is flanked by *cis*-acting ribozymes at both its 5' and its 3' end so that, upon transcription, the *trans*-acting ribozyme is trimmed at both its 5' and its 3' end, with resultant liberation of a small tRNA-embedded ribozyme. The *trans*-acting ribozymes targeted to a different site in HIV-1 RNA were expressed under the control of the SRa promoter and were examined in cell culture in a co-transfection transient assay with an HIV-1 infectious molecular clone, pNL4-3, for their ability to suppress HIV-1 replication. Although the extent of inhibition depended on the target site, all constructs caused significant inhibition of the HIV-1 p24 antigen production in culture supernatant. The ribozyme targeted to either 5' SS (5' major splicing site) or *tat*1 site within *tat*-coding region had the highest inhibitory effect (>80%) when the molar ratio of template DNA for the target HIV-1 RNA to that for the ribozyme was 1:8.

I. Introduction

Gene therapy is thought to be the ultimate and radical treatment for several diseases, attributed to genetic disorders. It is reasonable to cure these genetic disorders with genetic approaches, whether it is acquired or congenital. In those cases, AIDS (acquired immunodeficiency syndrome) is one of the most suitable candidates for gene therapy using ribozyme techniques because HIV belongs to the retrovirus, its viral genome consists of RNA, while a ribozyme catalyzes the cleavage of substrate RNA without an aid of proteinaceous apparatus (Altman, 1989; Symons, 1989; Cech, 1990; Dropulic et al., 1992; Scanlon, 1997; Turner, 1997). Therefore, the strategy to overcome the HIV infection using ribozymes as specific restriction enzymes is a hopeful idea to gene therapy for AIDS patients. Indeed, the first demonstration that a hammerhead ribozyme can disarm HIV-1, at least in cells in culture, without any associated detrimental effects (Sarver et al., 1990), has accelerated attempts to use it as an anti-HIV agent (Ojwang, 1992; Altman, 1993; Yu et al., 1993; Yamada et al., 1994a, 1994b; Bertrand and Rossi, 1996, 1997; Scanlon, 1997; Turner, 1997).

Since their first discovery, the list of ribozymes with new functions is increasing (Noller et al., 1992; Piccirilli et al., 1992; Bartel and Szostak, 1993; Lorsch and Szostak, 1994; Dai et al., 1995; Wilson and Szostak, 1995; Zhang and Cech, 1997; Vaish et al., 1998). Although there are many kinds of ribozymes with respect to their

catalytic mechanisms, hammerhead-type ribozymes (Buzayan et al., 1986; Hutchins et al., 1986; Prody et al., 1986; Foster and Symons, 1987a, 1987b; Symons, 1989) offer particularly attractive possibilities (Taira and Nishikawa, 1992; Eckstein and Lilley, 1996; Scanlon, 1997; Turner, 1997; Kawasaki et al., 1998). These ribozymes work in *cis* (intramolecularly). However, they have been engineered to work in *trans* (intermolecularly) both by Uhlenbeck (1987) and by Haseloff and Gerlach (1988). Target RNAs can have any sequence as long as the cleavage site contains NUX, with N being any nucleotide and X being any nucleotide except G (Koizumi et al., 1988; Ruffner et al., 1990; Perriman et al., 1992), although the cleavage efficiency changes dramatically depending on the combination of N and X: in general, GUC offers the best cleavage site (Shimayama et al., 1995). Because of the ease with which the hammerhead ribozyme can be manipulated and of its high activity *in vitro*, it has been tested and successfully exploited as a regulator of gene expression (Erickson and Izant, 1992; Murray, 1992; Eckstein and Lilly, 1996; Kawasaki et al., 1996, 1998).

Since HIV is notorious for its high mutation rate (Preston et al., 1988; Roberts et al., 1988; Peliska and Benkovic, 1992), it is not only difficult to develop vaccines against HIVs but also this genetic variability limits the application of ribozymes for cleavage of HIV RNAs. This is because, once a target site has undergone mutation, the ribozyme targeted to that specific site obviously loses its effectiveness. One way to overcome the problems associated with the mutability of HIV is to use ribozymes that target several conserved sites simultaneously (Taira et al., 1991; Ohkawa et al., 1993). Then, even if one or more sites were to undergo mutation and become resistant to cleavage by a ribozyme, the other conserved sites could still potentially be cleaved by additional ribozymes targeted specifically to those sites. In fact, the use of antisense DNAs targeted simultaneously to different sites has been shown to prevent the development of escape mutants (Lisziewicz et al., 1992) and the effectiveness of a multi-targeting strategy has also been demonstrated in the case of ribozyme-catalyzed reactions (Chen et al., 1992; Weizacker et al., 1992; Ohkawa et al., 1993).

We previously proposed the usefulness of self-trimming vectors (Taira et al., 1990) for liberation of multiple ribozymes, each with a different target site (Taira et al., 1991; Ohkawa et al., 1993). Our strategy involves the combination of *cis*-acting ribozymes with *trans*-acting ribozymes that have been embedded in tRNA (Cotten and Birnstiel, 1989; Yuyama et al., 1992) so that several *trans*-acting ribozymes, targeted to HIV (or any other sequence), are trimmed at both their 5' and 3' ends by the actions of the *cis*-acting ribozymes, with resultant liberation of several tRNA-embedded *trans*-acting ribozymes that should function independently of one another (shotgun type; Ohkawa et al., 1993).

These shotgun type ribozyme-activities *in vitro* should hold promise for similarly efficient ribozyme function in cell culture, provided that each tRNA-embedded 5'- and 3'-trimmed ribozyme is stable and sufficiently active *in vivo*. However, some earlier reports indicated that, in order to detect ribozyme activities *in vivo*, a huge excess of ribozymes over the target sequence had to be used (Cameron and Jennings, 1989; Cotten and Birnstiel, 1989). Moreover, when ribozymes were stabilized by being inserted into a long RNA transcript, the ribozyme activity decreased dramatically (Bertrand et al., 1994). Kinetic analysis also indicated that the longer the binding sequence, the lower the activity of the ribozyme, most probably due to formation of an inactive complex (Heidenreich and Eckstein, 1992; Zhou and Taira, 1998). Ribozyme activity in cell culture must be maximized considering the stability of the ribozyme (for this purpose, a longer binding sequence may be preferable) and its kinetic activity (for this purpose, a shorter binding sequence is preferable).

Cotten and Birnstiel (1989), in their pioneering ribozyme design for *in vivo* usage, have embedded a ribozyme into tRNA anticodon region in order to increase the stability of the ribozyme and to utilize the tRNA's internal promoter. In our present study, as mentioned above, we also designed the ribozyme expression vector in the tRNA embedded form, that did not completely depend on the pol III promoter but it was under the control of SRa promoter. We also introduced our self-trimming strategy into this system (Taira et al., 1991; Ohkawa et al., 1993). We expect not only additive but also synergistic effect *in vivo* after using this shotgun type of ribozyme, targeting different sites simultaneously. Prior to the employment of targets for shotgun type ribozyme, it is very important to evaluate the efficiency of each ribozyme for individual targets in cell culture in order to explore the more effective combination of target sites.

We report here the anti HIV-1 activities of five different tRNA-embedded ribozymes, targeting five different sites in HIV-1 RNA, in a transient assay. We could detect significant ribozyme activity in cell culture when the molar ratio of template DNA coding for the target HIV-1 RNA to that for the ribozyme was 1:8.

II. Results

A. Ribozyme-expression plasmids and transcription in cell culture

In order to examine the anti-HIV activity of tRNA-embedded 5'- and 3-' trimmed ribozymes in cell culture,

each ribozyme unit (**Fig. 1**) was connected to a mammalian expression vector under the control of SRa promoter (Takebe et al., 1988). **Figure 2** shows the scheme for the construction of ribozyme expression plasmid. Five relatively conserved regions on HIV-1 RNAs were chosen as the target sites of anti HIV-1 ribozymes (**Fig. 3**).

We previously demonstrated the usefulness of 5'- and 3'-trimmed ribozymes, based on transcription and kinetic studies *in vitro* (Ohkawa et al., 1993). Since there has been no proof of *cis*-trimming activities in cell culture, we first examined the self-trimming activities by detecting 5'- and 3'-trimmed ribozymes in total RNA from COS7 cells that had been transfected with the ribozyme expression plasmid, pME18-LTR 1. Trimmed-tRNA-embedded ribozyme was detected directly by Northern hybridization (**Fig. 4**).

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Figure 1. The 5'- and 3'-trimming plasmid vector, pV3TA-A2, derived from pV3T-A2 (Ohkawa et al., 1993). The tRNA-embedded *trans*-acting ribozyme is trimmed at both the 5' and the 3' end during the transcription reaction.

Figure 2. Scheme for construction of a mammalian ribozyme-expression vector.

Figure 3. Locations and sequences of HIV RNA that can be targeted by ribozymes. Positions of nucleotides relative to the initiation site are indicated by numbers. Cleavage occurs on the 3' side of the outlined triplets (GUC or GUA).

Figure 4. Detection of tRNA-embedded ribozymes. tRNA-embedded ribozymes trimmed *in vivo* in COS7 cells were detected by Northern hybridization with 5' end-labeled synthetic oligonucleotide that was complementary to the *Apa*I-*Eco*RV sense strand of the ribozyme targeted at LTR 1. Lane 1 contains total RNA prepared by T7 transcription from the pV3TA-LTR 1 plasmid (**Fig. 1**; Ohkawa et al., 1993). Overnight transcription produced only fully-trimmed ribozymes. Lane 2 contains total RNA transcribed *in vivo* in COS7 cells from the pME18-LTR 1 plasmid (**Fig. 2**) under the control of SRa promoter. The total RNA was isolated 24 hours after transfection.

Overnight transcription reaction (<24h) with concomitant self-trimming *in vitro,* from the pV3TA-LTR 1 plasmid (**Fig. 1**; under the control of T7 promoter), produced only fully-trimmed ribozymes (lane 1). Judging from the Northern analysis on the total RNAs isolated 24 hours after transfection of COS7 cells with the pME18-LTR 1 plasmid (**Fig. 2**; under the control of SRa promoter), the trimming reaction in cell culture appears much slower (lane 2): the main component was the unprocessed ribozyme. Nevertheless, the tRNA-embedded 5'- and 3'-trimmed ribozymes were at least stable enough in cell culture to be detectable by Northern hybridization, in accord with the previous studies (Cotten and Birnstiel, 1989; Yuyama et al., 1992).

The reason for the slower processibility of the transcribed RNA in COS7 cells remains obscure. Large quantities of ribozyme transcripts require higher magnesium ions since ribozymes are metalloenzymes (Yarus, 1993; Uchimaru et al., 1993; Piccirilli et al., 1993; Steitz and Steitz, 1993; Pyle, 1993; Pontius et al., 1997; Lott et al., 1998; Zhou et al., 1996, 1997; Zhou and Taira, 1998). The transcribed RNA *in vitro* in nuclear extracts from HeLa cells could be processed almost completely upon addition of magnesium ions (5 mM) (data not shown).

B. Inhibition studies on replication of HIV-1

We have confirmed that all five ribozymes transcribed from pV3TA-HIV were active as catalysts since all of them successfully cleaved the HIV-1 RNA *in vitro* (**Fig. 5**). No attempts were made to obtain quantitative kinetic parameters because the substrate RNAs used were not the same, as a result of the different target sites (**Fig. 3** and **Fig. 5**). Ribozyme-mediated inhibition of the replication of HIV-1 was then examined in cell culture as described in Materials and Methods according to the procedure outlined in **Figure 6** (Sczakiel et al., 1992).

In **Figure 7A**, the relative inhibitory effects of the ribozymes are compared to the results for the control, into which an equal amount of CAT RNA had been introduced in place of the ribozyme plasmid (Homann et al., 1993). We can clearly conclude from **Figure 7A** that each ribozyme could successfully inhibit the replication of HIV-1 in cell culture. The extent of inhibition was, however, dependent on the target sequence. Among the four target sites chosen, the *tat*1 site was the most susceptible, with 94-98% inhibition of replication of HIV-1 in 24 experiments.

In order to exclude the possibility that a significant portion of the inhibitory effect shown in **Figure 7A** originated from the titration of transcription factors by the strong SRa promoter, we then constructed a new control plasmid. In case of the newly constructed control plasmid pME18-*lac* Z, the target sequence of the ribozyme (**Fig. 2**) was set at a 5' region of *lac* Z. In parallel, pME18-5' SS was also constructed. In **Figure 7B**, the relative inhibitory effects of the five kinds of ribozymes are evaluated with respect to the new control, pME18-*lac* Z. The relative inhibitory potentials shown in **Figure 7B** are pertinent to those shown in **Figure 7A** and the SRa promoter had a little inhibitory effect. Among the five target sites chosen (**Fig. 3**), the 5' SS site was the most susceptible one, with 88-94% inhibition of replication of HIV-1. This 5' SS site turned out to be more susceptible than the *tat*1 site.

Through out these experiments, the molar ratio of the template DNA coding for the target HIV-1 RNA to that for the ribozyme was kept at 1:8. It is to be noted that greater inhibition could be achieved by choosing a higher molar excess of ribozyme template. These results clearly demonstrate that our tRNA-embedded ribozyme-expression system under the control of the SRa promoter inhibited HIV-1 replication in cell culture.

Figure 5. Ribozyme activities *in vitro*. Co-transcriptional cleavage of two types of HIV-1 RNA (*tat* RNA or LTR-*gag* RNA; see details in **Figure 1** of Ohkawa et al., 1993) by five kinds of ribozyme-expression plasmids: each target site is shown in **Figure 3**. Substrate RNAs are indicated by arrows.

III. Discussion

A. Ribozyme activities

In our previous reports, the ribozyme activity associated with the catalytic sequence against RNA coliphages has been intensively investigated *in vitro* and *in vivo* (Yuyama et al., 1992; Inokuchi et al., 1994). RNA coliphages provide systems in which ribozyme activity can be rapidly evaluated in cell culture. We found that (i) a ribozyme designed to cleave the A2 gene (coding the maturation enzyme) of RNA coliphage SP, when transcribed from a plasmid in *Escherichia coli,* caused failure of the proliferation of progeny phage, and (ii) inactive ribozymes with altered catalytic sequences, which might be expected to form more stable RNA duplex than the active ribozyme, did not have significant inhibitory effects on phage growth. These results indicated that it is mainly the catalytic activity of the ribozyme and not its function as an antisense molecule that

Figure 6. Co-transfection transient assay used for testing the antiviral activity of the ribozyme-expression plasmids. human $CD4^-$ epithelioid colon carcinoma cells (SW480) were co-transfected with infectious proviral DNA (pNL4-3) and ribozyme-coding test plasmids (pME18-HIV). The HIV-1 virus initially produced in SW480 cells was amplified in co-cultivated human $CD4^+$ T-lymphoid MT-4 cells that replicate HIV-1 efficiently. The concentrations of HIV-1 antigen p24 were measured 4 days after transfection in cell-free culture supernatants by a polyspecific ELISA.

is responsible for suppressing the proliferation of the RNA phage (Inokuchi et al., 1994).

The antisense effect of ribozyme starts to play a role when the binding site becomes longer. In our previous study, the total length of the binding site was set at 20 nucleotides and no antisense effect was recognized (Inokuchi et al., 1994). Having the information that the inhibitory effect of a ribozyme should originate mainly from its cleavage activity (Kawasaki et al., 1998), in the present investigation, we constructed five active ribozymes with the binding sites of 16 nucleotides targeted at five different sites in HIV-1 RNA and their activities in cell culture were compared.

B. Ribozyme trimming system

Although all five ribozymes were active *in vitro* against synthetic substrate RNAs as shown in **Figure 5**, no attempts were made to obtain quantitative kinetic parameters because (i) the substrate RNAs used were not identical, as a result of the different target sites (**Fig. 3** and **Fig. 5**) and (ii) in general, the kinetic parameters obtained *in vitro* depended heavily on the length and higher order structure of the substrate RNA used (Heidenreich and Eckstein, 1992; Bertrand et al., 1994). In fact, the highest activity in cell culture exerted by the pME18-5' SS (**Fig. 7B**) could not be predicted from the *in vitro* kinetic data of **Figure 5**. Similarly, the activity also depends on the length of the ribozyme transcript (Yuyama et al., 1992; Bertrand et al.,

Figure 7. Replication of HIV-1 in the presence of various ribozyme-expression plasmids. The co-transfection transient assay was carried out as described previously (Homann et al., 1993). All constructs (120 ng) were tested by co-transfection in human SW480 cells with infectious proviral HIV-1 DNA (pNL4-3, 40 ng). Either (**A**) CAT RNA (120 ng) or (**B**) plasmid that codes for a ribozyme targeted to *lac*Z (pME18-*lac*Z, 120 ng) served as the control 100% replication. The bars represent averages of 24 measurements: the average

values in
% replication are given in numbers above the bars.

When we originally examined the activities *in vitro* of tRNA-embedded 5'- and 3'-trimmed ribozymes against synthetic HIV-1 RNA, five target sites were chosen (**Fig. 3**). Then all five ribozyme units were separately ligated into the pME18-226HygB vector by the procedure shown in **Figure 2**. We later realized, upon restriction analysis, that a part of the ribozyme region of 5' SS was missing from the final pME18-HIV that had been isolated. We therefore used four different kinds of ribozyme constructs in the study shown in (**A**). The control plasmid pME18-*lac*Z and the missing pME18-5' SS were later constructed and the results are shown in (**B**).

1994). Our previous kinetic analysis *in vitro* indicated that the shorter ribozyme had the higher cleavage activity (Yuyama et al., 1992), although the effect in cell culture is a combination of the kinetic efficiency (the shorter the better) and the lifetime of the ribozyme (a naked ribozyme will be degraded quickly). It should be noted that when a hammerhead ribozyme (called Rz6) was expressed as a large 3 kb transcript, no cleavage products were observed after incubation with the 1335 nt substrate RNA *in vitro*, whereas the same Rz6 ribozyme, when expressed as a small 60 nt ribozyme, efficiently cleaved the same substrate RNA (Bertrand et al., 1994). Moreover, in accord with the results *in vitro*, when the Rz6 ribozyme was expressed in cell culture as a large 2.4 kb transcript from the HIV-1 LTR promoter, there was no inhibition of HIV-1 replication (Bertrand et al., 1994).

Since we had a belief long ago that ribozyme transcripts should be as compact as possible, we have been developing a trimming system (Taira et al., 1991; Taira and Nishikawa, 1992; Ohkawa et al., 1993). The accumulating evidence that increasing the size of the flanking regions of the ribozyme would result in a significant reduction of its cleavage efficiency encouraged us to use trimmed ribozymes at their 5' and 3' ends. The trimming in cell culture was first demonstrated in COS7 cells (**Fig. 4**). The trimmed ribozymes are expected to show a significantly higher cleavage efficiency (Bertrand et al., 1994). In fact, our tRNA-embedded ribozyme-expression system under the control of the SRa promoter inhibited HIV-1 replication in cell culture (**Fig. 7**).

Cameron and Jennings (1989) and Cotten and Birnstiel (1989) demonstrated activities in cell culture of hammerhead ribozymes by transient co-transfection or micro-injection assays. Though both groups successfully demonstrated the usefulness of ribozymes, the extent of inhibition was limited. A huge excess of template DNA coding for the ribozyme over the DNA coding for the target site had to be used for detection of meaningful inhibition. Cotten et al. also showed that short ribozymes were not stable and, therefore, they stabilized the ribozymes by embedding them into tRNA (Cotten and Birnstiel, 1989; Cotten et al., 1989). As a result, RNA polymerase III rather than RNA polymerase II was expected to generate short, correctly folded ribozymes in cells, with stable tertiary structures (Jennings and Molloy, 1987; Cotten and Birnstiel, 1989). However, the pre-tRNA form containing the embedded ribozyme was poorly processed, possibly because the precursor tRNA/ribozyme interacted poorly with the nuclear factors required for both 5' and 3' processing and for addition of the 3' terminal CCA (Cotten and Birnstiel, 1989). When either the target or the ribozyme sequence is inserted into a long RNA, the catalytic activity is expected to be decreased as discussed above, because unfolding of the interacting site may become the rate-limiting step. Therefore, although the activity of a tRNA-

embedded ribozyme itself could have been high enough (Cotten and Birnstiel, 1989; Yuyama et al., 1992), that of the precursor tRNA/ribozyme might have been lower.

In order not to depend on nuclear factors for both 5' and 3' processing, we combined *cis*-acting ribozymes with each *trans*-acting ribozyme (Taira et al., 1990) such that the *trans*-acting ribozyme is trimmed at both its 5' and its 3' end by the actions of the *cis*-acting ribozymes (Taira et al., 1991; Yuyama et al., 1992). The usefulness of such a strategy has been demonstrated *in vitro* (Ohkawa et al., 1993). Moreover, the results in cell culture, shown in **Figure 7**, indicate that such a strategy might also be useful *in vivo* since all five *trans*-acting ribozymes against HIV-1 RNA inhibited the replication of HIV-1 when the molar ratio of template DNAs for target and ribozyme, respectively, was 1:8. It is to be emphasized that the intention of these assays was not to obtain a large reduction in HIV-1 replication, but to compare the inhibitory potential of each ribozyme construct targeted at different sites. A much greater inhibition could be achieved in our system by choosing a higher molar excess of a ribozyme template.

C. Possible strategies for avoiding "escape" phenomena

There are several reports that demonstrate the potential usefulness of ribozymes in the suppression of the proliferation of HIV-1 in cell culture (Sarver et al., 1990;

Chen et al., 1992; Dropulic et al., 1992; Ojwang et al., 1992; Yu et al., 1993; Yamada et al., 1994a, 1994b; Bertrand and Rossi, 1996; Zhou et al, 1996; Bertrand et al., 1997; Smith et al., 1997; Li et al., 1998). However, in many cases, the effectiveness of ribozymes was temporary because cells infected with HIV-1 gradually became resistant to the ribozymes (Dropulic et al., 1992). Antisense DNA can also effectively suppress the proliferation of HIV-1 at the early stages of treatment with antisense DNA. However, this suppressive effect is also temporary and, at a later stage, treatment with antisense DNA becomes ineffective (Lisziewcz et al., 1992). These "escape" phenomena may possibly be the result of the high rates of mutation of HIV-1 (Preston et al., 1988; Roberts et al., 1988; Peliska and Benkovic, 1992) or of incomplete shut-down of the expression of HIV, with resultant subsequent breakthrough (Sarver and Rossi, 1993). Since hammerhead ribozymes have high substrate-specificity, mutations in HIV-1 RNA can abolish the effectiveness of the ribozymes (Scanlon, 1997; Turner, 1997; Kawasaki et al., 1998; Zhou and Taira, 1998). Moreover, some conserved sites are less accessible than others because of the complicated higher-order structure of HIV-1 RNA (**Fig. 7**). In order to overcome the escape phenomenon associated with HIV-1, several conserved sequences of HIV-1 RNA need to be targeted by multiple ribozymes. Our strategy involves the combination of *cis*-acting ribozymes with *trans*-acting ribozymes so that several *trans*-acting ribozymes, targeted to HIV (or any other sequence), are trimmed at both their 5' and 3' ends by the actions of the *cis*-acting ribozymes, with resultant liberation of several *trans*-acting ribozymes that should function independently of one another (shotgun type; Ohkawa et al., 1993). Chen et al. (1992) have already demonstrated in cell culture the usefulness of multitarget ribozymes in a system in which several ribozymes, each with a different target site, are connected in tandem.

Our present finding that tRNA-embedded ribozymes can act in cell culture to inhibit the replication of HIV-1 and our previous finding of the superiority *in vitro*, in terms of kinetic effectiveness, of shotgun-type multitarget ribozymes relative to the simply connected type of multitarget ribozymes (Ohkawa et al., 1993) encourage us to try to use this system to overcome the "escape" phenomenon. To this end, stable transformants that generate shotgun-type multitarget tRNA/ribozymes are being isolated in our laboratory.

IV. Experimental Procedures

A. Construction of Ribozyme-Expression Plasmids

The ribozyme-expression vectors were constructed based on the plasmid, pV3TA-A2, as previously reported (**Fig. 1**, Yuyama et al., 1992), in which Cotten and Birnstiel's tRNA-embedded ribozyme (1989) was combined with self-trimming vector (Taira et al., 1990, 1991) that encodes a *trans*-acting ribozyme targeted to the A2 gene of RNA phage SP (maturation protein; Inokuchi et al., 1994). The tRNA-embedded ribozyme portion of pV3TA-A2 (**Fig. 1**; *Apa*I-*Eco*RV fragment) was replaced by various ribozyme sequences with 8 bases on both substrate-binding arms targeted to relatively well conserved sequences in HIV-1 RNA (**Fig. 3**). The plasmids generated were designated pV3TA-HIV. During this process, a *Pst*I site was inserted into the stem II loop, replacing the *Bst*PI site of pV3TA-A2, and the newly introduced unique *Pst*I site was used for the confirmation of successful introduction of each *trans*-acting ribozyme. As described previously (Yuyama et al., 1992; Shimayama et al., 1993; Sawata et al., 1993; Amontov and Taira, 1996; Kuwabara et al., 1996; Zhou and Taira, 1998), the stem II/loop region is inert in terms of catalysis and, therefore, it can be used for manipulations [According to the report of Tuschl and Eckstein (1993), stem II with 2 base pairs rather than the conventional 4 base pairs has essentially unaltered catalytic activity, independent of the composition of the tetraloop.]. The

whole unit within the *Eco*RI-*Hind*III fragment in **Figure 1** was then inserted into the *Eco*RI-*Not*I region of pME18-226HygB (**Fig. 2**). The resulting ribozyme expression plasmids under the control of the SRa promoter (Takebe et al., 1988) were designated pME18-HIV, that includes pME18-LTR 1, pME18-5'SS, pME18-*gag*1, pME18-*tat*1, and pME18-*tat*3 (**Fig. 3**).

B. Detection of trimmed-tRNA-embedded ribozymes in cell culture

tRNA-embedded ribozymes trimmed in cell culture were detected by Northern hybridization procedures. COS7 cells in a 10 cm dish-plate (80% confluent) were transfected with 10 mg of the plasmid that encoded a ribozyme targeted to LTR 1, according to the lipofectin reagent protocol (GIBCO BRL). Total RNA were harvested 24 hours after transfection as described previously (Inokuchi et al., 1994). Total 20 mg of isolated RNA was denatured by glyoxal method and electrophoresed onto 1% agarose gel, then transferred to a nylon membrane (Hybond N, Amersham Inc.). Trimmed-tRNA-embedded ribozyme was detected by Northern hybridization with 5'-end-labeled synthetic oligonucleotide that was complementary to the *Apa*I-*Eco*RV sense strand of the ribozyme targeted at LTR 1.

C. Co-transcriptional cleavage reactions

A mixture for co-transcriptional cleavage reactions, in a total volume of 50 μl, contained 1 pmol of *Hind* III-linearized pME18-HIVs. In all cases examined, the amount of [^{32}P]-labeled HIV RNA substrate was kept at 1 pmol. The co-transcriptional cleavage reactions were carried out at 37°C in 40 mM Tris-HCl (pH 8.0), 20 mM $MgCl_2$, 2 mM spermidine, 1 mM DTT, 1 mM NTP (Amersham, UK), 100 units of human placental ribonuclease inhibitor (Takara Shuzo, Kyoto), and 100 units of T7 RNA polymerase (Takara Shuzo).

D. Co-transfection transient assay

Human $CD4^-$ epithelioid colon carcinoma cells (SW480) (Leibovitz et al., 1976) in 48-well plates (grown to semi-confluence) were co-transfected with test plasmids (pME18-HIV; 120 ng) and infectious proviral HIV-1-DNA pNL4-3 (Adachi et al., 1986) (40 ng) by the protocol of Chen and Okayama (1987), which is a modified Ca^{2+}-co-precipitation protocol. Subsequently, transfected SW480 cells were co-cultivated with $CD4^+$ T-lymphoid MT-4 cells, that replicate HIV-1 efficiently (Harada et al., 1985) (2 x 10^5 per well), and the p24 HIV-1 antigen was quantified in supernatants from co-cultures 4 days after transfection with a commercially available polyspecific ELISA (Organon). Measured absorbance was transformed into a relative concentration of the antigen. As a control, the identical amount (120 ng) of either CAT RNA (Homann et al., 1993) or a plasmid that encoded a ribozyme targeted to unrelated RNA of *lac Z* was used in place of the HIV-1 targeted ribozyme expression plasmid. In total, three series of experiments were performed with eight determinations (8 samples were prepared separately so that they represented 8 different experiments) for each construct. Thus, the data shown in **Figure 7** are the means of 24 values.

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