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Importance of independence in ribozyme reactions: Kinetic behavior of trimmed and of simply connected multiple ribozymes with potential activity against human immunodeficiency virus

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ABSTRACT **The kinetic behavior of ribozymes derived from two types of multiple-ribozyme expression vector were examined. In some cases, multiple ribozymes were expressed as a single RNA molecule and all the ribozymes were simply connected in tandem (connected type). In other cases, multiple ribozymes were flanked by cis-acting ribozymes at both their 5′ and 3′ ends so that, upon transcription, multiple ribozymes were trimmed at both their 5′ and 3′ ends, with resultant liberation of multiple independent ribozymes (shotgun type). When levels of ribozyme expression were examined for the shotgun-type vector, the level of the ribozyme transcript was found to be proportional to the number of units (*n*) connected in tandem. Accordingly, the activities of the shotgun-type ribozymes, in terms of the cleavage of HIV-1 RNA *in vitro*, were also found to be proportional to the number of units connected in tandem (*n*). By contrast, the activities of the connected-type ribozymes reached plateau values at around *n* = 3. These results indicate that, when the shotgun-type expression system is used, it is theoretically possible to generate various independent ribozymes, each specific for a different target site, without sacrificing the activity of any individual ribozyme.**

RNA enzymes (ribozymes) catalyze the cleavage and formation of phosphodiester bonds (1–3) and also appear able to catalyze many other types of reaction (4, 5). From the point of view of application, hammerhead-type ribozymes (3, 6–10) are particularly attractive. Hammerhead-type ribozymes work in cis (intramolecularly) in nature, but they have been engineered to work in trans (intermolecularly) both by Uhlenbeck (11) and by Haseloff and Gerlach (12). Because of the portability of the hammerhead ribozyme, it has been used as a regulator of gene expression (13, 14). Moreover, its application to the control of expression of human immunodeficiency virus type 1 (HIV-1) and oncogenes has been emphasized. The finding that a hammerhead ribozyme can disarm HIV-1, at least in cells in culture, without any associated detrimental effects (15), has accelerated attempts at its application as an anti-HIV agent.

HIV is infamous for its high mutation rate, which is caused by the low fidelity of its reverse transcriptase, an enzyme that lacks the proofreading function (16, 17) and has a tendency to add an extra nucleotide when moving from one DNA template to another (18). This mutability of HIV not only makes it difficult to prepare vaccines against HIV but also hinders the application of ribozymes to cleavage of HIV RNA, because once the target site has undergone mutation, the ribozyme targeted to that specific site loses its effectiveness. One way to overcome this mutability of HIV would be to use ribozymes that target several conserved sites simultaneously. Then, even if one or more sites were to undergo mutation and avoid cleavage by the ribozyme, the other conserved sites could still potentially be cleaved by additional ribozymes targeted specifically to those sites. In fact, targeting several sites simultaneously by antisense DNAs prevented the development of escape mutants (19). We previously proposed the use of trimming vectors for liberation of multiple ribozymes, each with a different target site (20). Since then, several groups have constructed expression vectors for multitargeted ribozymes and demonstrated the importance of the multitargeting strategy (21, 22). There are at least two methods for expression of multitargeted ribozymes. The simpler way involves joining several sequences of ribozymes that are specific for different target sites in tandem, such that all the transcribed multitargeted ribozymes are connected in tandem as a single RNA (connected type). The second strategy involves combining cis-acting ribozymes (CARs) with trans-acting ribozymes so that several trans-acting ribozymes, targeted to HIV (or any other sequences), are trimmed at both their 5′ and 3′ ends by the actions of the CARs, with resultant liberation of several trans-acting ribozymes that should function independently of one another (shotgun type). In this paper we compare the activities of both types of expression system and demonstrate the superiority, in terms of kinetic effectiveness, of shotgun-type ribozymes to connected-type ribozymes.

MATERIALS AND METHODS

Construction of Shotgun-Type and Connected-Type Plasmids. Construction of the shotgun-type expression vectors was based on plasmid pV3T-A2 (23), in which Cotten and Birnstiel's tRNA-embedded ribozyme (24) was combined with our trimming vector (20). A poly(A) tail (A_{30} tract) was added at 3′ end of tRNA structure of pV3T-A2 (between GAT of aminoacylation end and GUC of 3′-processing ribozyme cleavage site) to increase the stability of tRNA-embedded ribozyme *in vivo*. The newly constructed plasmid was designated pV3TA-A2. The *Apa* I–*Eco*RV fragment of a trans-acting ribozyme, having an A2 binding site, was replaced by various fragments with different binding sites for HIV (Fig. 1). Then, *Sma* I–*Hin*dIII fragments were connected in tandem in a defined orientation to generate shotgun-type ribozymes. For the construction of the connected-type plasmids, *Xho* I–*Xba* I fragments were similarly connected in tandem. In all cases, oligonucleotides, including linkers for connection of different restriction sites, were synthesized on a DNA synthesizer (model 392; Applied Biosystems).

Synthesis of HIV RNA Substrates. Templates for HIV RNA substrates were prepared by PCR from a template plasmid, pNL4-3 (25). Each PCR primer for the sense strand contained

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Abbreviations: HIV, human immunodeficiency virus; CAR, cis-acting ribozyme; LTR, long terminal repeat.

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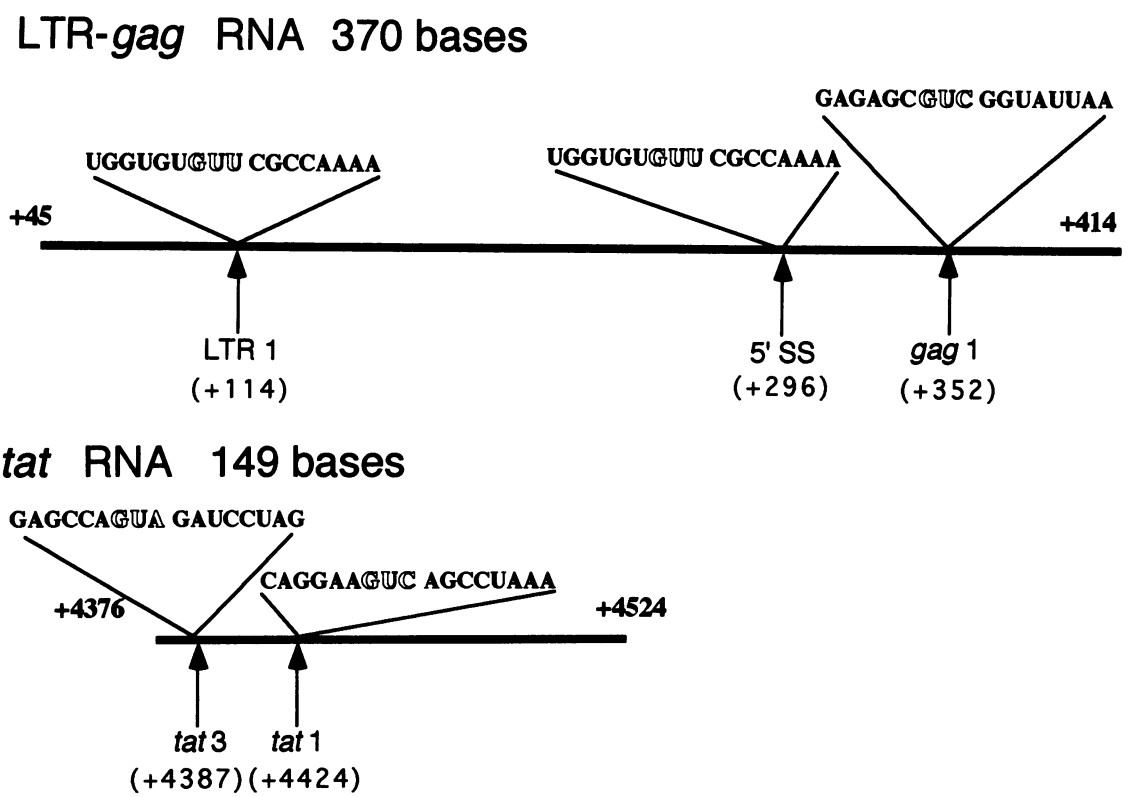

FIG. 1. HIV-1 RNA substrates used for cotranscriptional cleavage reactions and the sequence that can be targeted by ribozymes. Target sites are indicated by arrows. Positions of nucleotides from the initiation site are indicated by numbers. LTR, long terminal repeat.

a T7 promoter. Transcription and gel purification of HIV RNA substrates were carried out as described (20).

Transcription and Cotranscriptional Cleavage Reactions. Self-trimming reactions during transcriptions, as shown in Fig. 3, were carried out as described (20). Cotranscriptional cleavage reaction mixtures (50 μl) either contained 0.1 pmol of circular template DNA that encoded the shotgun-type *tat*-1-specific ribozymes or connected-type *tat*1-specific ribozymes (see Fig. 4) or contained 1 pmol of *Hin*dIII-linearized template DNA that encoded multitargetted ribozymes (Fig. 5). In all cases examined, the amount of ^{32}P-labeled HIV RNA substrate was kept at 1 pmol. The cotranscriptional 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 dithiothreitol/1 mM NTPs (Amersham) with 100 units of human placental ribonuclease inhibitor (Takara Shuzo, Kyoto) and 100 units of T7 RNA polymerase (Takara Shuzo).

RESULTS

Construction of Ribozyme Expression Plasmids. The tRNA-embedded ribozyme portion of pV3TA-A2 [*Apa* I–*Eco*RV fragment; see figure 3(a) of ref. 23] was replaced by various ribozyme sequences with eight bases on both substrate-binding arms targeted to the relatively well-conserved sequences of HIV-1 RNA (Fig. 1). The plasmids generated were each called pV3TA-HIV. As mentioned previously (23, 26), stem II and its loop region are inert in terms of catalysis and, therefore, were used in recombinant manipulations (23).

To compare the *in vitro* ribozyme activities of simply connected multiple ribozymes (connected type; Fig. 2*A*) with the activities of independent multiple ribozymes (shotgun type; Fig. 2*B*), either the tRNA-embedded trans-acting ribozymes alone or the whole unit of pV3TA-HIV, consisting of 5′ and 3′ processing ribozymes and the tRNA-embedded trans-acting ribozyme, were connected in tandem. In both cases, the number of repeats ranged from 1 to 10. We initially chose a ribozyme sequence that is specific for the *tat*1 site (Fig. 1) and connected various numbers of copies of this sequence in the form shown in Fig. 2 (n = 1–10).

Levels of Ribozyme Expression from Shotgun-Type Constructs. Levels of expression of RNAs were examined for six shotgun-type constructs, which differed in the number of ribozyme units connected in tandem (n = 1–5, and 10 in Fig. 2*B*). Under our conditions, the rate of transcription was linear for up to 6 hr (data not shown). Fig. 3 shows the products of transcription, examined after transcription for 6 hr. In all cases, the identical molarity of template DNA was used for each transcription reaction. Therefore, the amount of 5′ CAR (a unique fragment, produced only once per transcription cycle, which is initiated at the promoter and terminated at the cleavage site by the action of the first 5′-processing ribozyme; band 5) was identical in each case, confirming the uniform molarity and purity of each template. Similarly, the intensity of band 2, which consisted of 5′ CAR and the trans-acting ribozyme specific for *tat*1, was equal in all cases and independent of the unit number (n) because this segment is also produced only once per template per individual transcription reaction. This fragment is discernible since, in our construct, the cleavage activity of the 5′ CAR is estimated to be <30% of the 3′ CAR due to the presence of a second binding site for 5′ CAR (J.O. and K.T., unpublished work).

In contrast to the above mentioned bands 2 and 5, the amount of trans-acting ribozymes targeted to *tat*1 (band 3) increased linearly in direct proportion to the number of connected units (n) because the band 3 product is reiterated n times per transcript and a cleavage releases each reiterated unit. Similarly, intensities of band 4, which consisted of 5′ CAR and 3′ CAR, and band 1, the partially processed (3′-processed) fragment, increased with increases in n. Therefore, by keeping the concentration of template DNA constant, it was still possible to increase the amount of trans-acting ribozymes merely by repeating the same units in the form shown in Fig. 2*B*. Moreover, the proportionality was independent of the time at which samplings were made

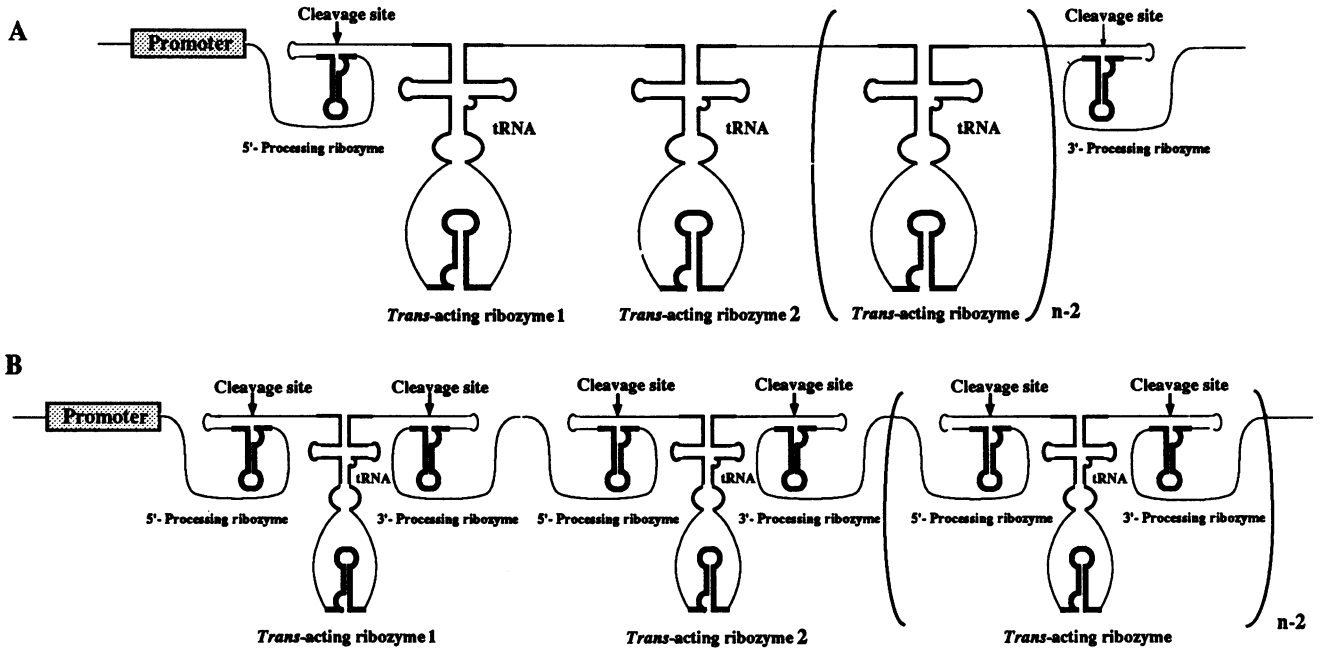

FIG. 2. (*A*) Simple connected-type ribozyme expression vector. Several tRNA-embedded trans-acting ribozymes are connected in tandem. Therefore, trans-acting ribozymes are transcribed as a single RNA molecule. To facilitate the analysis of the products of transcription, CAR sequences are added at each extreme 5′ and 3′ end. (*B*) Shotgun-type ribozyme expression vector. Several units of the entire 5′- and 3′-trimming vector are connected in tandem. Upon transcription, several independent trans-acting ribozymes are liberated.

(data not shown). In other words, the shotgun-type plasmid with $n = 10$ always produced roughly 10-fold higher amounts of trans-acting ribozymes than the plasmid with $n = 1$.

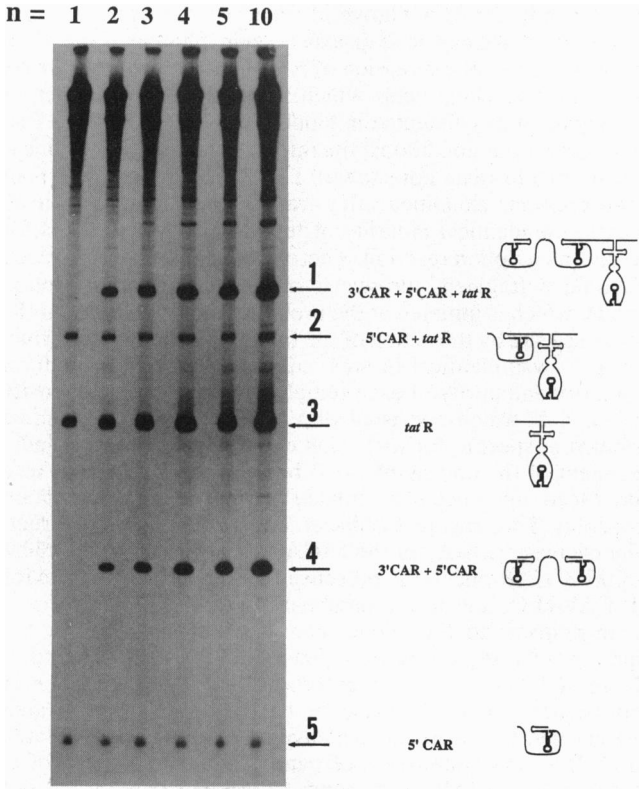

FIG. 3. Effects of the number of ribozyme units connected in tandem in the shotgun-type vector shown in Fig. 2*B*. Transcription and trimming reactions were followed by electrophoresis, 6 hr after initiation of the transcription. The number of connected units (n) is indicated at the top. In all cases, an identical molarity of DNA template was used.

Comparison of Cleavage Activities Between the Connected-Type and Shotgun-Type Ribozymes. Since the amount of trans-acting ribozymes produced from the shotgun-type plasmids was proportional to n (Fig. 3), the cleavage activity was also expected to be proportional to n. In order (*i*) to verify this expectation and (*ii*) to compare the activities with those of the connected-type transcripts (transcripts from Fig 2*A*-type template), cleavage reactions were carried out *in vitro* (Fig. 4). In these experiments, transcription reactions were carried out under the same conditions as discussed in the previous section, with the exception of the presence of the gel-purified fragment of HIV-1 *tat* RNA (Fig. 1).

Degradation of the *tat* RNA fragment from HIV-1 was followed (Fig. 4*A*), and the results revealed that, as expected, the cleavage activities of the shotgun-type plasmids (with $n = 1$–10), measured after a 6-hr incubation, were proportional to n (Fig. 4*B*). By contrast, the activity of the connected-type ribozyme (Fig. 2*A*) increased linearly up to $n = 3$, but the activity failed to increase thereafter, even with larger values of n (see the saturation curve in Fig. 4*B*). To facilitate the analysis of the transcription products, one 3′-processing ribozyme was inserted at the extreme 3′-terminal region as shown in Fig. 2*A*, even in the case of the connected-type construct. Since the binding-site sequence of the 3′-processing ribozyme was also present in each internal region between the tRNA-embedded ribozymes, our connected-type plasmid (e.g., $n = 10$) also produced some ribozymes smaller than those of the expected sizes ($n = 1$–9). Since the activities of the connected-type ribozymes, as shown in Fig. 4*B*, represent the overall activities (including the activities of the smaller independent ribozymes of $n = 1, 2, \ldots$), the actual activities of the full-length connected-type ribozymes are lower than those expected from the saturation curve in Fig. 4*B*.

These results clearly indicate that, to maintain the activity of ribozymes at a level that is directly proportion to the number of connected units (n), it is necessary to produce independent ribozymes. This conclusion should also be applicable to multitargeted ribozymes when four or more ribozymes, each with a different target site, are to be used *in vivo* in order to overcome the mutability of HIV.

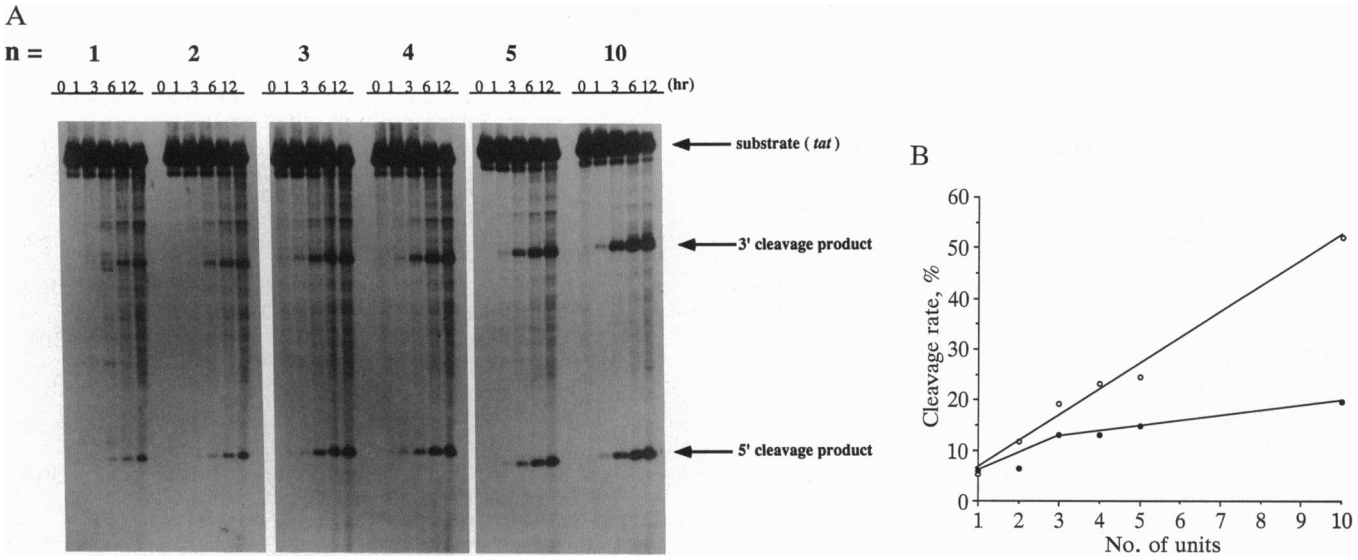

FIG. 4. Cotranscriptional cleavage of HIV-1 RNA with a *tat* sequence (shown as *tat* RNA in Fig. 1*B*). (*A*) Gel electrophoretic analysis of the cotranscriptional cleavages by shotgun-type ribozymes. (*B*) Comparison of the cleavage activities between the connected-type ribozyme-expression system (●) and the shotgun-type ribozyme-expression system (○), measured 6 hr after the initiation of transcription.

Expression Plasmids for Multitargeted Ribozymes. With the idea of using shotgun-type ribozymes *in vivo*, we constructed several multitargeted ribozyme expression vectors. Such plasmids contained one to five different binding sites (shown in Fig. 1). Transcription and cleavage assays were carried out under nearly the same conditions as those described in the previous section. Results of three sets of experiments using a multitargeted ribozymes expression-vector with five different target sites are shown in Fig. 5. In set A, reaction mixtures contained all the reagents, including the two kinds of substrate RNA necessary for examination of the cleavage reactions, with the exception of the ribozyme-expression plasmid (control A). In set B, the two substrate RNAs in reaction mixtures were replaced by an inappropriate substrate without the cleavage site, but the ribozyme-expression plasmid was included (control B). In set C, reaction mixtures contained correct substrates and the ribozyme expression plasmid. Products of the reactions were examined after incubations for 0, 2, 4, and 8 hr. Since only set C produced the expected cleavage products (indicated by thinner arrows), the high substrate specificity was confirmed. Since each ribozyme with its specific target site functioned independently of the others, this kind of multitargeted ribozymes seems to have strong potential as a therapeutic agent in the treatment of HIV.

DISCUSSION

There are several reports that demonstrate the potential usefulness of ribozymes in suppression of the proliferation of HIV-1 *in vivo* (15, 21, 27). However, because hammerhead ribozymes have high substrate specificity, mutations in HIV-1 RNA can abolish the effectiveness of the ribozymes (27). To overcome the mutability of HIV-1, several conserved sequences of HIV-1 RNA need to be targeted by multiple ribozymes. Moreover, some conserved sites are less accessible than others because of the complicated higher-order structure of HIV RNA, and a search for the best target sites for ribozymes would be tedious. Therefore, we developed a strategy to target several conserved sequences of HIV RNA simultaneously (20). Thus, cis-acting ribozymes are combined with trans-acting ribozymes such that each trans-acting ribozyme is trimmed at both its 5′ and its 3′ end by the actions of the cis-acting ribozymes (shotgun-type ribozymes of Fig. 2*B*). Furthermore, Chen *et al.* (21) demonstrated, *in vivo*, the usefulness of multitarget ribozymes in a system in which several ribozymes, each with a different target site, were connected in tandem.

Since there have been no reports of the kinetic behavior of the two kinds of multitarget ribozyme—namely, connected-type ribozymes (Fig. 2*A*) and shotgun-type ribozymes (Fig. 2*B*)—we constructed expression plasmids for both types of ribozyme with repeating units ranging in number (n) from 1 to 10. Analysis of cleavage efficiencies *in vitro* revealed that the activity of the shotgun-type expression system was directly proportional to the number of ribozyme units connected in tandem (n), whereas that of the connected-type system reached a plateau value when n was >3 (Fig. 4*B*). These results clearly indicate that, when three or more ribozymes are to be connected in tandem, the shotgun-type expression system (Fig. 2*B*) is kinetically superior to the simple connected-type expression system (Fig. 2*A*). In the former case, each trans-acting ribozyme gains its independence upon transcription. Even though, in our constructs, all trans-acting ribozymes were embedded in tRNAs (24), we do not believe that this structural feature had any detrimental effects on the connected-type expression system. Instead, the tRNA motif should favor the connected-type ribozymes because structural studies suggest that the mature tRNA part of the precursor has approximately the same conformation as the mature tRNA (28). Thus, as a result of the formation of discrete tRNA structures with ribozymes embedded in them, inappropriate base pairings between various portions of ribozymes within a long RNA transcript can be avoided.

The use of such self-cleaving ribozymes *in vivo* is intriguing. However, such a construct is not suitable for the use of a retroviral vector, at least in its present form, since the ribozymes will be excised during formation of recombinant viral stock. If a retroviral vector is to be used, one may modulate the stem II region in such a way that CARs become inactive forms (incorrect base pairings) and that, upon binding of activator proteins such as Tat and Rev, the inactive form is converted to an active one. Alternatively, a DNA viral vector such as adeno-associated virus vector may be used in order to avoid cis cleavage during infection (29).

In conclusion, the superiority, in terms of kinetic effectiveness, of shotgun-type ribozymes to connected-type ribozymes has been clearly demonstrated. Therefore, it appears that we should try simultaneously to target several

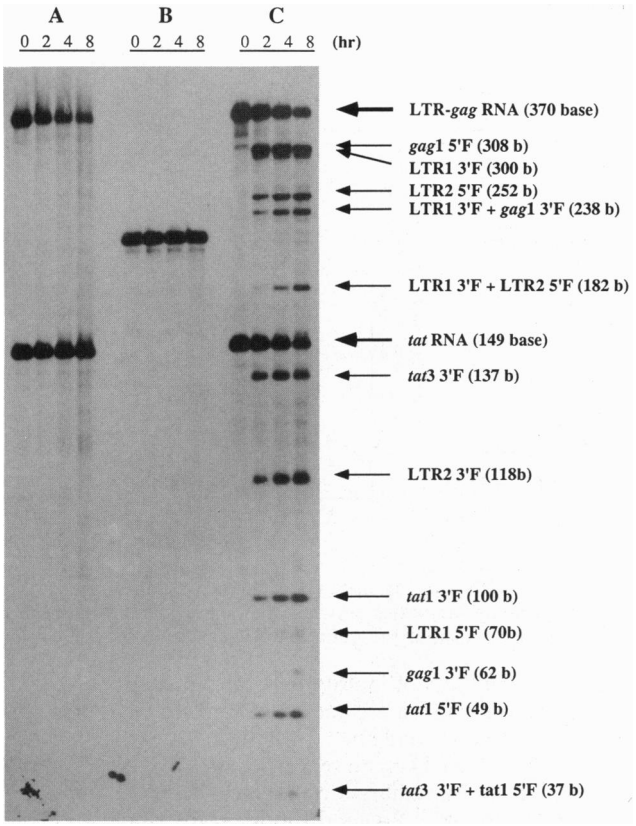

FIG. 5. Cotranscriptional cleavage of two types of HIV-1 RNA (*tat* RNA and LTR-*gag* RNA in Fig. 1*B*) by a shotgun-type ribozyme expression vector that encoded five different ribozymes targeted to five different sites, as shown in Fig. 1. (*A*) Two types of ^{32}P-labeled HIV-1 RNA (*tat* RNA and LTR-*gag* RNA, indicated by thick arrows) alone were incubated in the absence of the shotgun-type ribozyme expression vector. (*B*) ^{32}P-labeled RNA substrate lacking the target sites was incubated with the shotgun-type ribozyme expression vector under the conditions for transcription. (*C*) Two types of ^{32}P-labeled HIV-1 RNA (*tat* RNA and LTR-*gag* RNA) were incubated with the shotgun-type ribozyme expression vector. Cleavage products are indicated by thinner arrows, and 5'F and 3'F denote 5'- and 3'-fragments, respectively, with their sizes in parentheses.

conserved sites in HIV RNA by using shotgun-type ribozymes, provided that the trans-acting ribozymes can be adequately stabilized *in vivo*. Moreover, the usefulness of the shotgun-type expression system is not limited exclusively to ribozymes; any independent antisense RNA can be similarly produced. With a combination of several different target sites, one shotgun-type expression system might protect against both HIV-1 and HIV-2, or against several subgroups of HIV-1 (30). Indeed, even for specific treatment of infection by HIV-1, the targeting of several or all conserved sequences should be very effective in overcoming the genetic variability of the virus. As such, the applicability of shotgun-type constructs is not limited exclusively to HIV infection but may also be useful for curing other human diseases and for controlling gene expression in other organisms.

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