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Specific cleavage of target RNAs from HIV-1 with 5′ half tRNA by mammalian tRNA 3′ processing endoribonuclease

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ABSTRACT

Mammalian tRNA 3′ processing endoribonuclease (3′ tRNase) can be converted to an RNA cutter that recognizes four bases, with about a 65-nt 3′-truncated tRNA^{Arg} or tRNA^{Ala}. The 3′-truncated tRNA recognizes the target RNA via four base pairings between the 5′ terminal sequence and a sequence 1-nt upstream of the cleavage site, resulting in a pre-tRNA-like complex (Nashimoto M, 1995, *Nucleic Acids Res 23*:3642–3647). Here I developed a general method for more specific RNA cleavage using 3′ tRNase. In the presence of a 36-nt 5′ half tRNA^{Arg} truncated after the anticodon, 3′ tRNase cleaved the remaining 56-nt 3′ half tRNA^{Arg} with a 19-nt 3′ trailer after the discriminator. This enzyme also cleaved its derivatives with a 5′ extra sequence or nucleotide changes or deletions in the T stem-loop and extra loop regions, although the cleavage efficiency decreases as the degree of structural change increases. This suggests that any target RNA can be cleaved site-specifically by 3′ tRNase in the presence of a 5′ half tRNA modified to form a pre-tRNA-like complex with the target. Using this method, two partial HIV-1 RNA targets were cleaved site-specifically in vitro. These results also indicate that the sequence and structure of the T stem-loop domain are important, but not essential, for the recognition of pre-tRNAs by 3′ tRNase.

Keywords: HIV *env*; HIV *gag*; pre-tRNA; RNase 65; 3′ tRNase

INTRODUCTION

RNase 65 is a spermidine-dependent, sequence-specific endoribonuclease. It was first discovered by chance in extracts of mouse FM3A cells (Nashimoto et al., 1991a; Nashimoto, 1992). Although extracts of all the mammalian tissue cells so far tested have been found to contain this enzyme (Nashimoto, 1993), little is known about its physiological role and its substrate. RNase 65 activity requires not only a protein, but also a small RNA, which has been shown to be a 3′-truncated tRNA of about 65 nt (Nashimoto et al., 1991b). The sequence specificity of the substrate varies depending on the species of 3′-truncated tRNA, i.e., 67-nt 3′-truncated tRNA^{Arg} and 66-nt 3′-truncated tRNA^{Ala} direct RNA cleavage at a site 1-nt downstream of the sequences GCCC and CCCC, respectively (Nashimoto, 1993; 1995). A model in which RNA targets are recognized by the corresponding 3′-truncated tRNA via four base pairings, with consequent formation of a complex such as a 5′-processed pre-tRNA with a 3′ trailer (Fig. 1), has been verified by base substitution experiments (Nashimoto, 1995). This model suggested that the protein component of RNase 65 is tRNA 3′ processing endoribonuclease (3′ tRNase) itself. Indeed, 3′ tRNase purified from pig liver cleaved RNA substrates at the expected site only in the presence of the corresponding 3′-truncated tRNA (Nashimoto, 1995).

A number of studies have demonstrated that, in eukaryotic cells, the 3′ trailer after the discriminator nucleotide is removed from a pre-tRNA by endoribonuclease (3′ tRNase) activity (Hagenbuchle et al., 1979; Castano et al., 1985; Oommen et al., 1992; Levinger et al., 1995; Nashimoto, 1995). Not much, however, is known about the biochemical and biophysical properties of the enzyme or the mechanism of substrate recognition. Even for purified enzymes from *Xenopus laevis* ovaries and pig liver, no detailed description is yet available (Castano et al., 1985; Nashimoto, 1995).

Artificial endoribonucleases with a custom-made sequence specificity have been generated by RNA en-

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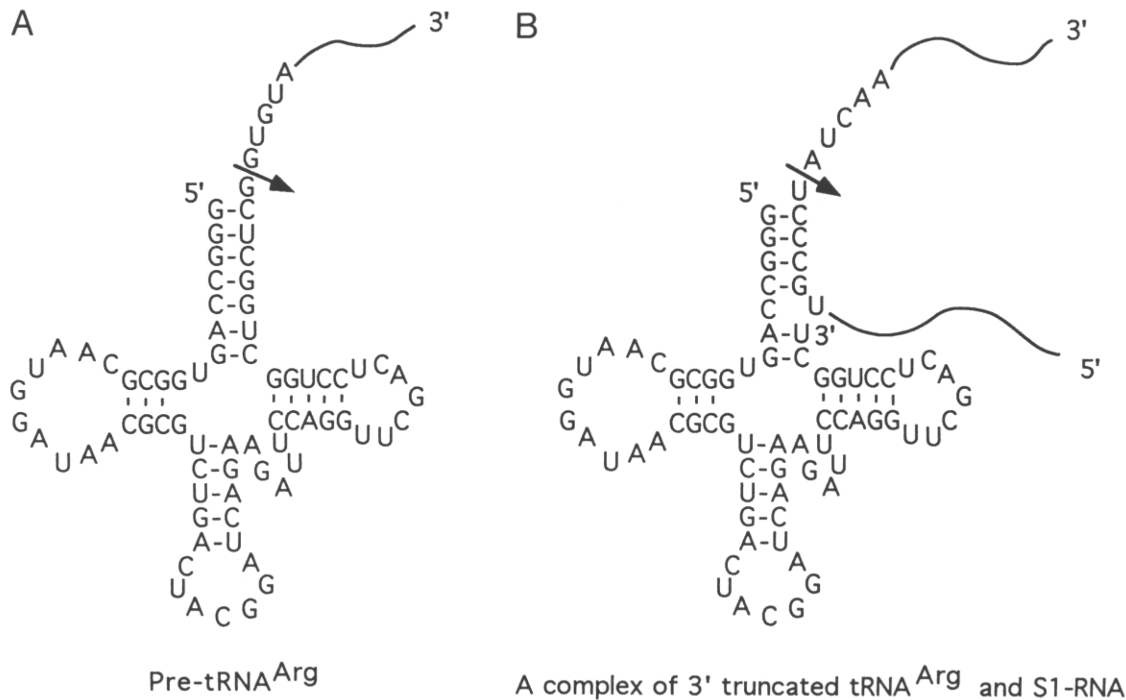

FIGURE 1. Plausible secondary structures of pre-tRNA^{Arg} (**A**) and a complex of 3′ truncated tRNA^{Arg} and S1-RNA (**B**).

gineering from natural RNA or ribonucleoprotein enzymes. These include a group I intron (Zaug et al., 1986), hammerhead (Uhlenbeck, 1987) and hairpin (Hampel et al., 1990) ribozymes, prokaryotic RNase P RNA (Forster & Altman, 1990), and human RNase P (Yuan et al., 1992; Yuan & Altman, 1994). In particular, the small ribozymes are used widely to cleave RNA targets at specific sites not only in vitro, but also in culture cells (Yu et al., 1993; Cantor et al., 1993). Research is under way to test them for their efficacy as therapeutic agents.

The RNase 65 activity suggested that 3′ tRNase can be converted to a highly specific endoribonuclease using an artificially further 3′-truncated tRNA. Here, I successfully developed a general method for the targeted cleavage of RNA with 3′ tRNase, and applied it to cleaving portions of HIV-1 RNA in vitro. This study also provided new insights into the mechanism of pre-tRNA recognition by mammalian 3′ tRNase.

RESULTS

Recognition and cleavage of a complex of two half tRNAs by 3′ tRNase

In this study, I tried improving the RNase 65 activity using an artificially further 3′ truncated tRNA to create a more specific RNA cutter. To start with, I selected the combination of a 36-nt 5′ half tRNA^{Arg} (GT7H) truncated after the anticodon and the remaining 56-nt 3′ half (T3H) with a 19-nt 3′ trailer. T3H and GT7H were synthesized by in vitro transcription from synthetic DNA templates in the presence and absence of [α-^{32}P]UTP, respectively. The target T3H was cleaved after the nucleotide corresponding to the discriminator in the presence of GT7H by mouse 3′ tRNase, generating 37- and 19-nt products (Fig. 2C, lanes 1–4). This shows that a complex of GT7H and T3H (Fig. 2A) can form a tertiary structure that can be recognized and cleaved at the expected site by 3′ tRNase, just as a complex of the 3′-truncated tRNA^{Arg} and its corresponding target S1-RNA can (Fig. 1B) (Nashimoto, 1995). This suggested that, if an RNA target can be made to form a pre-tRNA-like complex recognizable by mammalian 3′ tRNase, using the 5′ half tRNA modified to base pair with the target at two sites corresponding to the acceptor and anticodon stems, then the RNA target will be cleaved after the nucleotide corresponding to the discriminator specifically by the enzyme.

5′ extension of the target is tolerated

In general, any target RNA can form 7 and 5 base pairs, upstream of the tentative target site, with the 5′ half tRNA containing the D stem and loop, corresponding to the acceptor and anticodon stems, respectively. The target RNA also has an extra sequence upstream of the 5 base pairing region. On the other hand, a structure corresponding to the T stem and loop, which has the conserved nucleotides and structure, cannot generally be formed between the 7 and 5 base pairing sequences in contrast to the variable loop. To determine whether

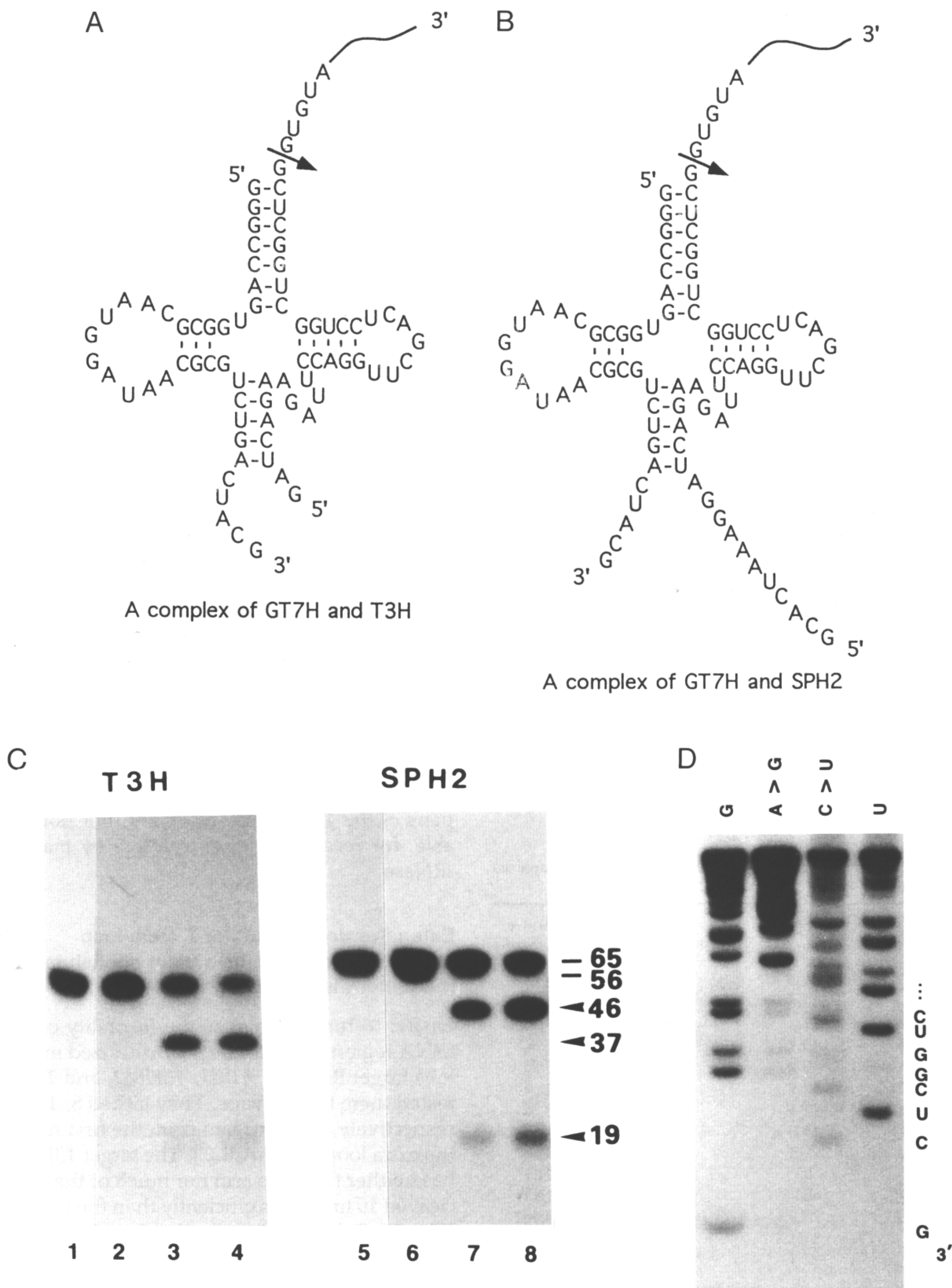

FIGURE 2. Specific cleavage of the 3′ half tRNA^{Arg} (T3H) and its derivative (SPH2) in the presence of the 5′ half tRNA^{Arg} (GT7H) by mouse 3′ tRNase. Plausible secondary structures of complexes of GT7H with the target RNAs T3H (**A**) and SPH2 (**B**) are shown. The sequence 5′-AGCAGGGUCGUUUU-3′ is omitted from the 3′ region of the target RNAs. Arrows indicate 3′ tRNase cleavage sites. **C:** Each assay using these ^{32}P-labeled targets (10 nM) is shown in the following four lanes: an intact RNA target (lanes 1, 5); RNA products after cleavage reactions by 3′ tRNase in the absence (lanes 2, 6) and presence of 0.5 μM (lane 7), 1.25 μM (lane 3), and 5 μM (lanes 4, 8) of GT7H. Bars and arrowheads with a nucleotide length denote substrates and cleavage products, respectively. **D:** The 3′ terminal sequence of the 5′ cleavage product of the target SPH2.

or not 3′ tRNase can recognize these general features of a complex of the target RNA with the 5′ half tRNA, the following four points were examined: (1) Can a 5′ region of the target RNA be extended? (2) Are the conserved nucleotides in the T loop, which are involved in the formation of the tRNA tertiary structure (Kim et al., 1974), replaceable? (3) Are the base pairs of the T stem dispensable? (4) To what extent can the T stem-loop and extra arm regions be shortened?

First, a 65-nt 3′ half tRNA^{Arg} with nine 5′ extra nucleotides (SPH2) (Fig. 2B) was tested and shown to be cleaved at the expected site in the presence of GT7H by 3′ tRNase, generating 46- and 19-nt products (Fig. 2C, lanes 5–8). The cleavage site was confirmed by determining the 3′ terminal sequence of the 46-nt cleavage product 3′-end-labeled with [5′-^{32}P]pCp (Fig. 2D). The values of K_m (the Michaelis constant) × K_d (the dissociation constant) and V_{max} (the maximum velocity) of SPH2 were comparable to those of T3H (Table 1), suggesting that 5′ extension of the target is tolerated in this two half tRNA system.

The conserved nucleotides in the T loop are replaceable

Secondly, three target RNAs, SPHM1, SPHM2, and SPHM3 (Fig. 3A,B,C), which had 1–5 nt substituted for the conserved and semi-conserved nucleotides in the T loop, were assayed. SPHM1 has U-to-A and C-to-G substitutions at conserved positions 55 and 56 (in the numbering system of tRNA nucleotides), respectively. They interrupt base pairs with guanines at positions 18 and 19 in the D loop and destabilize the tRNA tertiary structure (Kim et al., 1974). SPHM1 also has a G-to-U change at semi-conserved position 57, U-to-A and A-to-C changes at conserved positions 54 and 58, respectively, which disrupt the interaction with each other (Kim et al., 1974). SPHM2 has the U-to-A change at 55, and SPH3 has the U-to-A and A-to-U changes at 55 and 58, respectively. These three target RNAs were cleaved after the discriminator in the presence of GT7H by 3′ tRNase (Fig. 3D), although the values of $V_{max}/K_m \cdot K_d$ decreased with the increase in the number of substituted nucleotides (Table 1). These results indicate that the conserved nucleotides in the T loop are replaceable.

TABLE 1. Kinetic parameters of cleavage of pre-tRNA-like complexes by pig 3′ tRNase.

Substrate	$K_m \cdot K_d$ [a] (μM^2)	V_{max} [a] (pmol/min)	Relative $V_{max}/K_m \cdot K_d$
GT7H-T3H	9.9	1.1	1.0
GT7H-SPH2	12.6	1.0	0.72
GT7H-SPHM1	17.1	0.34	0.18
GT7H-SPHM2	13.1	0.53	0.36
GT7H-SPHM3	15.2	0.43	0.25
GT7H-T7HM1	12.9	0.93	0.65
GT7H-T7HM2	17.1	0.50	0.26
GT7H-T7HM3	18.6	0.26	0.13
GT7H-T3HM1	22.1	0.21	0.086
GT7H-T3HM2	ND[b]	ND[b]	ND[b]
GT7H-T3HM3	ND[b]	ND[b]	ND[b]
GT7H-T3HM22	24.2	0.11	0.041
GENV-Env	11.1	0.67	0.55
GGAG-Gag	17.1	0.14	0.075

[a] K_m and K_d represent the Michaelis constant of cleavage of a pre-tRNA-like complex by 3′ tRNase and the dissociation constant of the complex, respectively. Only the product of K_m and K_d was obtained from this study. The maximum velocity per nanogram of pig 3′ tRNase fraction after Mono Q column chromatography is represented. Each measurement was from averages of two trials with a standard deviation of 3–8%.

[b] Hardly any cleavage was detected. The lower limit of detection was about 0.01 pmol/min.

The base pairs of the T stem are important but not indispensable

Thirdly, I tested three other target RNAs, T7HM1, T7HM2, and T7HM3 (Fig. 4A,B,C) for cleavage. T7HM1 and T7HM2 were variants lacking one and two base pairs, respectively, in the T stem region. T7HM3 has four changed bases in the T stem and one at the semi-conserved position in the extra arm, which may prevent formation of any stable stem structure. The specific cleavage of these three targets occurred at the expected site (Fig. 4D), although the catalytic efficiency decreased as the number of base pairs of the T stem decreased (Table 1). These results indicate that the base pairs of the T stem are important, but not indispensable, for recognition and cleavage by mammalian 3′ tRNase.

Extensive deletion of the T stem-loop and extra arm domains almost abolishes the substrate activity

Lastly, to further analyze the flexibility of the 3′ half tRNA region to 3′ tRNase, I synthesized more anomalous target RNAs, T3HM1, T3HM2, and T3HM3, and tested them for cleavage. They lacked 8, 12, and 17 nt respectively, downstream from the first nucleotide of the extra loop (Fig. 5A,B,C). The target T3HM1, which has neither the extra arm nor much of the T stem, was cleaved 10 times less efficiently than the wild-type T3H (Fig. 5E; Table 1). On the other hand, the cleavage of T3HM2 and T3HM3, which have 10 and 5 nt, respectively, in the T stem-loop and extra arm regions, was hardly detected (Fig. 5E; Table 1). It is possible that the hardly detectable cleavage of T3HM2 was due to prevention of anticondon stem formation by self-pairing between UCAG in the anticodon stem and CUGG in the T stem region of T3HM2. Thus, I tested a derivative of T3HM2, T3HM22, which has two base substitutions in the T stem region to avoid the self-pairing

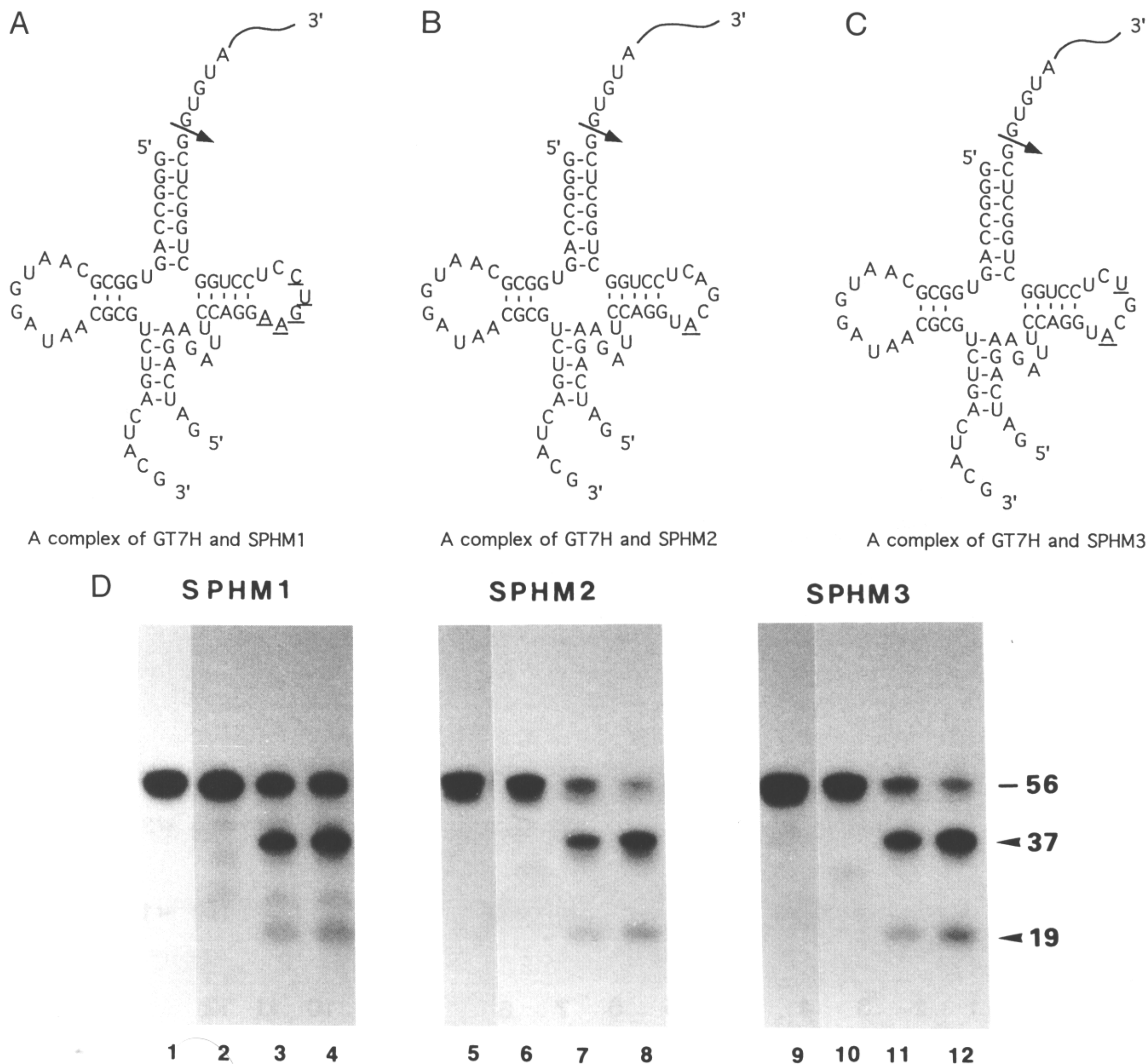

FIGURE 3. Specific cleavage of SPHM1, SPHM2, and SPHM3 in the presence of GT7H by mouse 3′ tRNase. Plausible secondary structures of complexes of GT7H with the target RNAs SPHM1 (**A**), SPHM2 (**B**), and SPHM3 (**C**) are shown. The bases substituted for the original bases in $tRNA^{Arg}$ are underlined. The sequence 5′-AGCAGGGUCGUUUU-3′ is omitted from the 3′ region of the target RNAs. Arrows indicate 3′ tRNase cleavage sites. **D:** Each assay using these ^{32}P-labeled targets (10 nM) is shown in the following four lanes: an intact RNA target (lanes 1, 5, 9); RNA products after cleavage reactions by 3′ tRNase in the absence (lanes 2, 6, 10) and presence of 0.5 μM (lanes 7, 11), 1.25 μM (lane 3), and 5 μM (lanes 4, 8, 12) of GT7H. Bars and arrowheads with a nucleotide length denote substrates and cleavage products, respectively.

(Fig. 5D). The cleavage of T3HM22 was more efficient than that of T3HM2, but it was half as efficient as that of T3HM1 (Fig. 5E; Table 1). These results show that extensive deletion of the T stem-loop and extra arm domains almost abolishes the substrate activity. They suggest that the anticodon stem structure is important for recognition by 3′ tRNase in the case of a pre-tRNA-like complex with extensive deletion of the T stem-loop and extra arm domains.

The results of the cleavage assays with the systematically altered 3′ half tRNAs suggest that any target RNA can be cleaved specifically by mammalian 3′ tRNase in the presence of a 5′ half tRNA modified to form a pre-tRNA-like complex with the target.

Specific cleavage of HIV-1 RNA

Next, I applied this specific RNA cleavage method using a modified 5′ half tRNA and mammalian 3′ tRNase to the in vitro cleavage of partial HIV-1 RNA sequences. Two portions of HIV-1 RNA were synthesized as target RNAs using T7 RNA polymerase. One

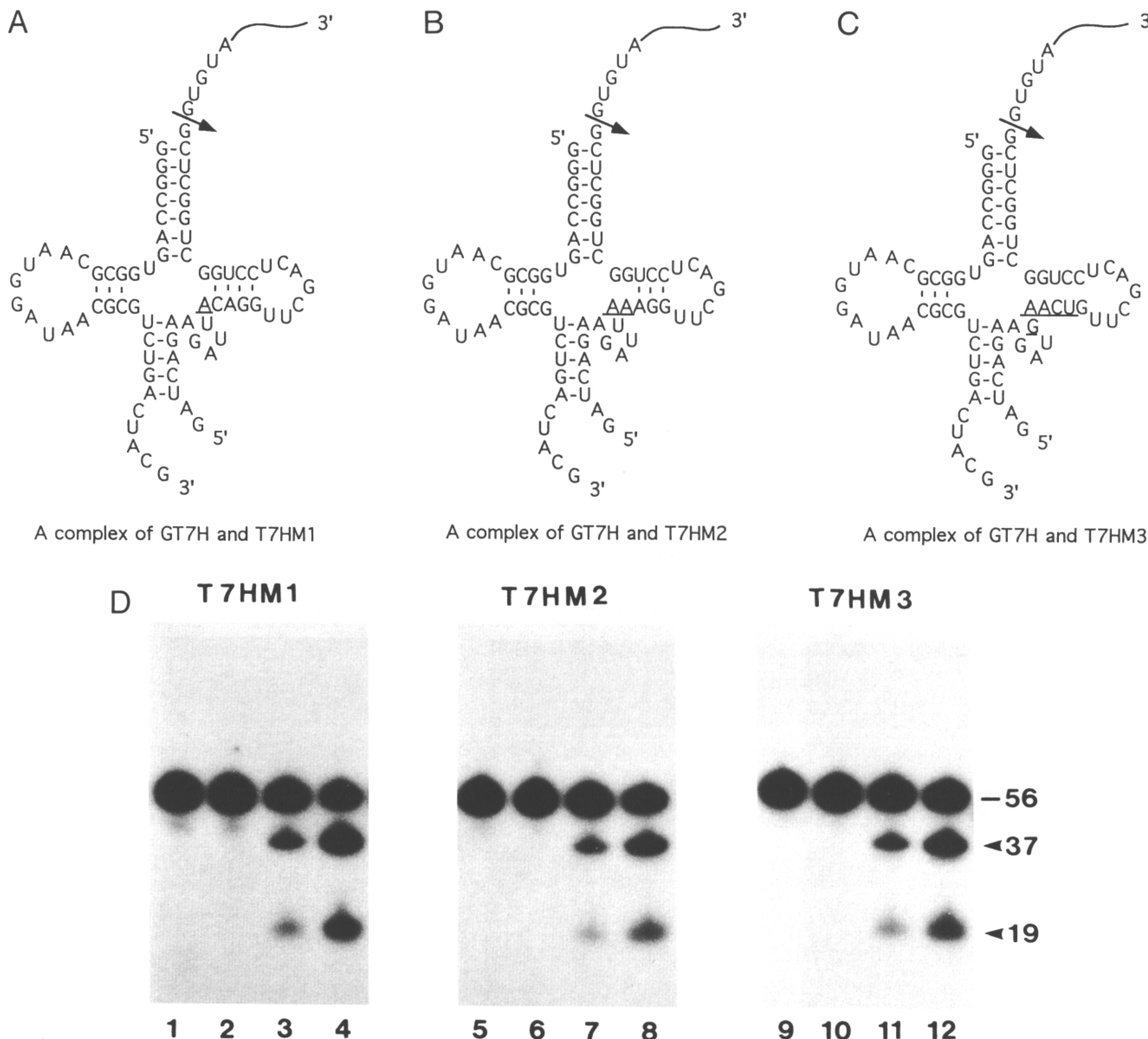

FIGURE 4. Specific cleavage of T7HM1, T7HM2, and T7HM3 in the presence of GT7H by mouse 3′ tRNase. Plausible secondary structures of complexes of GT7H with the target RNAs T7HM1 (**A**), T7HM2 (**B**), and T7HM3 (**C**) are represented. The bases substituted for the original bases in tRNA^{Arg} are underlined. The sequence 5′-AGCAGGGUCGUUUU-3′ is omitted from the 3′ region of the target RNAs. Arrows indicate 3′ tRNase cleavage sites. **D:** Each assay using these ^{32}P-labeled targets (10 nM) is shown in the following four lanes: an intact RNA target (lanes 1, 5, 9); RNA products after cleavage reactions by 3′ tRNase in the absence (lanes 2, 6, 10) and presence of 0.5 μM (lanes 3, 7, 11) and 5 μM (lanes 4, 8, 12) of GT7H. Bars and arrowheads with a nucleotide length denote substrates and cleavage products, respectively.

was a 60-nt target, Env, which contains the core of the Rev-binding element in the *env* gene region (Ratner et al., 1985; Bartel et al., 1991). The other was a 56-nt target, Gag, which is a part of the *gag* gene region (Ratner et al., 1985). Env contains a potential hairpin structure that imitates the T stem-loop and has a 6-nt stem. At the same time, I synthesized the 5′ half tRNAs GENV and GGAG, which are specific to the targets Env and Gag, respectively. GENV consists of the 7-nt sequence complementary to one portion of the target Env, the D stem and loop of tRNA^{Arg}, the 5-nt sequence complementary to another portion of Env, and a portion of the tRNA^{Arg} anticondon loop. GGAG was constructed by replacing the two Env-complementary sequences of GENV with two sequences complementary to two portions of Gag. The complex of GENV and Env, and the complex of GGAG and Gag were expected to form pre-tRNA-like structures (Fig. 6A,B). The target Env was cleaved in the presence of GENV by 3′ tRNase with an efficiency comparable to that of T7HM1 (Fig. 6C; Table 1). However, no cleavage was detected in the presence of either yeast total tRNA or the 5′ half tRNAs GGAG and T7H (Fig. 6C). 3′ tRNase cut the target Gag in the presence of GGAG, although

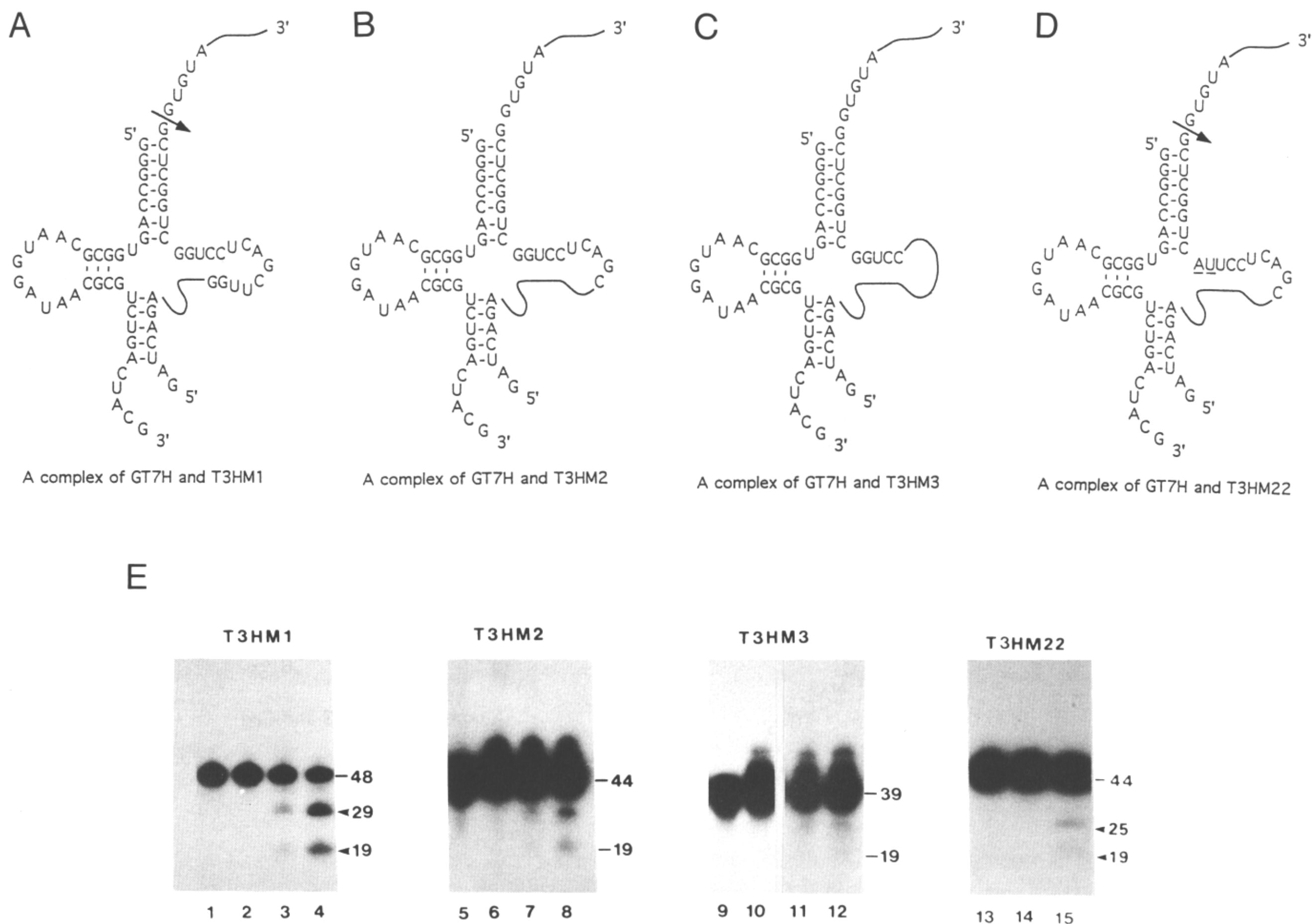

FIGURE 5. Specific cleavage of T3HM1, T3HM2, T3HM3, and T3HM22 in the presence of GT7H by mouse 3′ tRNase. Plausible secondary structures of complexes of GT7H with the target RNAs T3HM1 (**A**), T3HM2 (**B**), T3HM3 (**C**), and T3HM22 (**D**) are represented. The bases substituted for the original bases in $tRNA^{Arg}$ are underlined. The lines in the middle of the targets denote regions deleted from the original $tRNA^{Arg}$. The sequence 5′-AGCAGGGUCGUUUU-3′ is omitted from the 3′ region of the target RNAs. Arrows indicate 3′ tRNase cleavage sites. **E:** Each assay using these ^{32}P-labeled targets (10 nM) is shown in the following four lanes: an intact RNA target (lanes 1, 5, 9, 13); RNA products after cleavage reactions by 3′ tRNase in the absence (lanes 2, 6, 10, 14) and presence of 0.5 μM (lanes 3, 7, 11, 15) and 5 μM (lanes 4, 8, 12) of GT7H. Arrowheads with a nucleotide length denote cleavage products.

sevenfold less efficiently than Env; there was no cleavage at all in the presence of yeast total tRNA, GENV, or T7H (Fig. 6D; Table 1). The semi-conserved nucleotides found in tRNA at positions 37 and 60 were replaced in the target Env (Fig. 6A), and the conserved base pair at positions 53 and 61 was replaced in Gag (Fig. 6B). Together with the experimental results on the 3′ half $tRNA^{Arg}$ variants, these results suggest that the conserved and semi-conserved nucleotides in the 3′ half tRNA region are not indispensable. These experiments have also confirmed that the specific RNA cleavage method proposed here is effective on any RNA target.

The exact cleavage site of Env was determined by two-dimensional thin layer chromatography on a 3′ terminal nucleotide of the about 47-nt 5′ cleavage product that was 3′ end-labeled with [5′-^{32}P]pCp. Env was found to have been cleaved not only after the discriminator (33% of the total cleavage products), but also 1-nt upstream (13%) and downstream (54%) of the expected site (Fig. 6E). The 3′ terminal nucleotides of the about 43- and 45-nt 5′ products were analyzed in the same way. The cleavage of Gag occurred both after the discriminator (43%) and also 1-nt upstream (13%) and 2-nt downstream (44%) of the expected site (Fig. 6E). The phenomenon of multisite cleavage is discussed below.

DISCUSSION

Implications for mechanism of substrate recognition

Although the present study was primarily intended for application to targeted RNA cleavage, it also provides information on the features of the pre-tRNAs that are recognized by the mammalian endoribonuclease. One

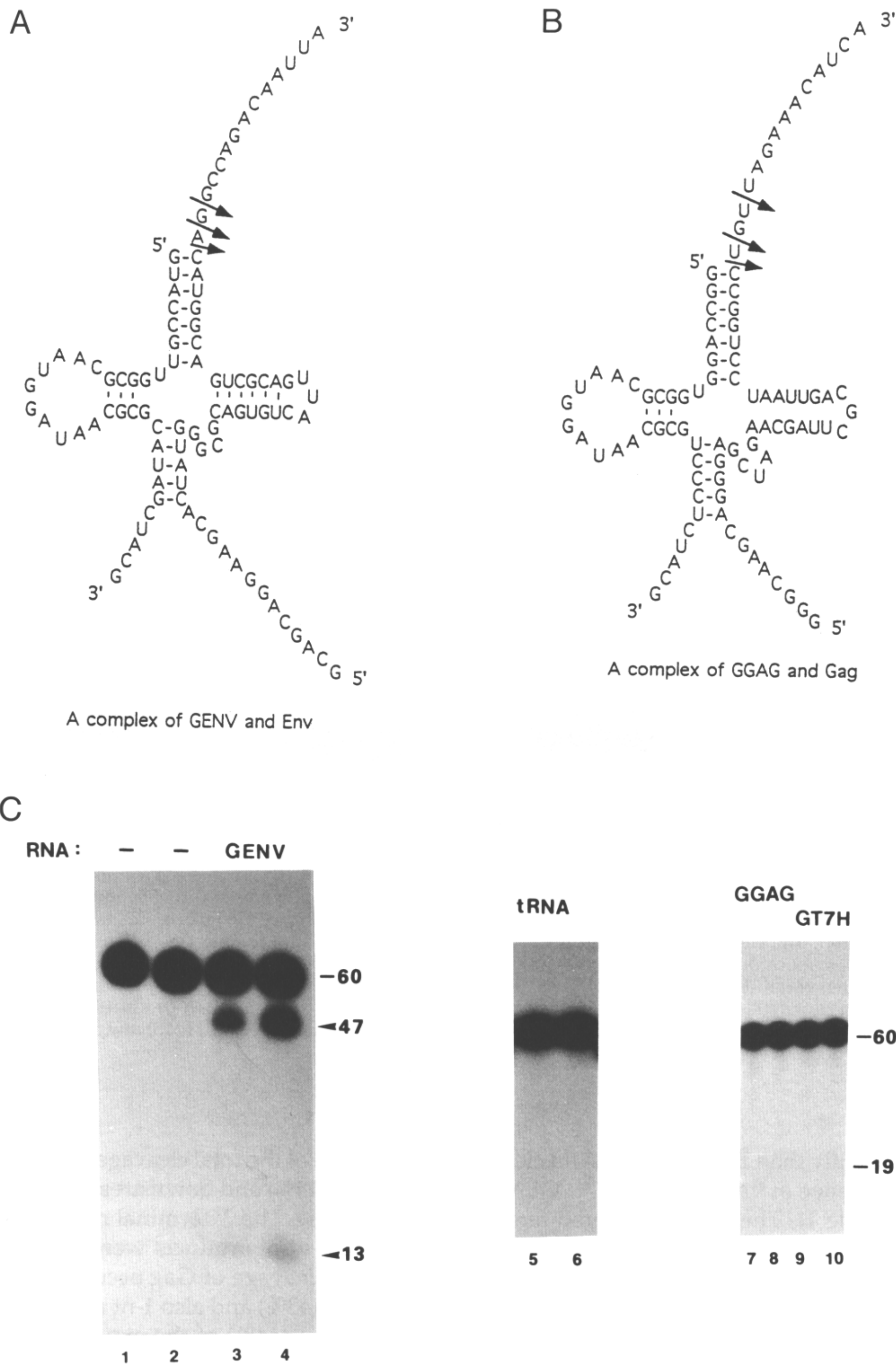

FIGURE 6. Specific cleavage of partial HIV-1 RNA targets. **A:** Plausible secondary structure of a complex of a target RNA (Env) from the *env* gene region with a modified 5′ half $tRNA^{Arg}$ (GENV) containing 7- and 5-nt sequences complementary to Env. **B:** Plausible secondary structure of a complex of a target RNA (Gag) from the *gag* gene region with a modified 5′ half $tRNA^{Arg}$ (GGAG) containing 7- and 5-nt sequences complementary to Gag. Arrows denote cleavage sites. **C:** 10 nM of the ^{32}P-labeled 60-nt target Env (lane 1) was assayed for specific cleavage by 3′ tRNase in the absence (lane 2) and presence of 0.05 μM (lanes 3, 5, 7, 9) and 0.5 μM (lanes 4, 6, 8, 10) of the indicated RNAs. **D:** 10 nM of the ^{32}P-labeled 56-nt target Gag (lane 1) was assayed for specific cleavage by 3′ tRNase in the absence (lane 2) and presence of 0.5 μM (lanes 3, 5, 7, 9) and 5 μM (lanes 4, 6, 8, 10) of the indicated RNAs. Arrowheads with a nucleotide length indicate cleavage products. **E:** Analyses of 3′ terminal nucleotides of the 5′ cleavage products by two-dimensional thin layer chromatography. The 3′ terminus of the about 47-nt 5′ product of Env was composed of A, C, and G at the indicated percentages (Env). The 3′ terminal nucleotides of the about 43- and 45-nt 5′ products of Gag were a mixture of C and U (5′ Gag) and U (3′ Gag), respectively. (*Continues on facing page.*)

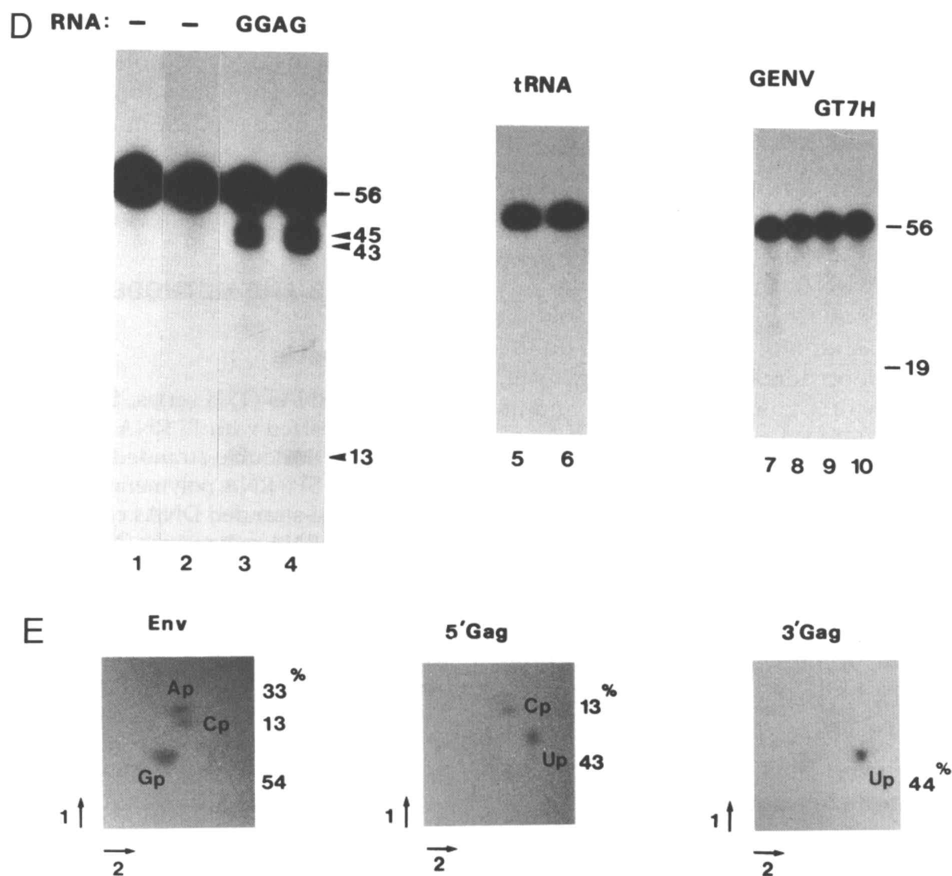

FIGURE 6. *continued.*

more RNA stalk composed of a sequence unrelated to tRNA and extending from the middle of the acceptor stem or anticondon loop (Figs. 1B, 2B) does not hinder the interaction between a pre-tRNA-like complex and 3′ tRNase. This is consistent with the fact that the 3′ processing of pre-tRNA^{Tyr} containing an intron in the anticondon loop occurs in *Xenopus* oocytes (Melton et al., 1980). This study demonstrated that both the conserved nucleotides in the T loop and the base-pairing of the T stem are important, but not essential, features for recognition and cleavage by 3′ tRNase. The anticodon stem also appears to be important for recognition in the case of a pre-tRNA-like complex with a shortened T stem-loop region (Fig. 5), whereas it is not in the case of one with an intact T stem-loop (M. Nashimoto, in prep.). Qualitative results obtained from base substitution experiments using in vitro *Drosophila* system (Levinger et al., 1995) are compatible with my results.

This two half-tRNA system will be useful for the further investigation of pre-tRNA-3′ tRNase interactions, because many pre-tRNA variants can be obtained through the combination of a small number of half tRNAs. Such investigations may help to elucidate both the features of a more effective 5′ half tRNA for specific cleavage and the mechanism of multi-site cleavage.

Multisite cleavage

The specific cleavage of the 3′ half tRNA^{Arg} variants occurred after the discriminator nucleotide, whereas both Env and Gag in the pre-tRNA-like complexes were cleaved at several sites by 3′ tRNase. The cleavage sites of the splicing endonuclease are determined by the length of the anticondon stem (Mattoccia et al., 1988; Reyes & Abelson, 1988). It has been suggested that *Xenopus* RNase P cleaves pre-tRNAs at a fixed distance from the reference points in the mature domain (Carrara et al., 1989). It has also been suggested that selection of the cleavage site by human RNase P involves measurements of both length of the acceptor T-stem extended helix and the position of the bulging nucleotide within this extended helix (Yuan & Altman, 1995). Both Env and Gag have potential base pairs that form a 3-nt T loop, although the base pairs are not stable (Fig. 6A,B). The multisite cleavage by mammalian

3′ tRNase might therefore reflect the feature of their T loop domains.

Target site selection

Here I demonstrated that this novel method for specific RNA cleavage using mammalian 3′ tRNase is effective in vitro. Although this method is, in principle, applicable to any site on any RNA, the rate of cleavage varies depending not only on the degree of RNA folding near the target site (Nashimoto, 1992), but also on the features of the T stem-loop domain 5′ to the target site. The cleavage efficiency of 3′ half $tRNA^{Arg}$ T3H and its derivatives T7HM1, T7HM2, and T7HM3, which have a T stem region containing 5, 4, 3, and no base pairs, respectively, decreased with the decrease in the number of the base pairs (Table 1). The 3′ half variants SPHM1, SPHM2, and SPHM3, which have base changes in the conserved region of the T loop, were worse substrates than wild-type T3H (Table 1). Therefore, one should take account of these observations when selecting a target site on an RNA substrate for the most efficient cleavage.

Prospects for targeted cleavage in vivo

This method should also be effective in both mammalian tissue culture cells and intact organisms, which possess a natural supply of 3′ tRNase, if a small 5′ half tRNA is introduced into them. A method has been developed for targeted cleavage of mRNA by human RNase P, which, unlike *Escherichia coli* RNase P, is composed of an RNA (H1 RNA) and one or more proteins indispensable to its activity (Yuan et al., 1992; Yuan & Altman, 1994). This method is very similar to mine in that both systems utilize a portion of tRNA as an "external guide sequence" that forms a complex with the target RNA like a pre-tRNA with a 5′ leader or 3′ trailer. Ribozymes such as the group I intron, the hammerhead, the hairpin, and *E. coli* RNase P RNA can also be exploited, with some RNA modification, for in vitro sequence-specific RNA cleavage (Zaug et al., 1986; Uhlenbeck, 1987; Forster & Altman, 1990; Hampel et al., 1990). Recently, the small ribozymes have also been shown to be very effective in tissue culture cells (Cantor et al., 1993; Yu et al., 1993). The inhibitory effects of the small RNA enzymes may be facilitated in vivo by nonspecific RNA binding proteins such as viral nucleocapsid and cellular hnRNP proteins (Tsuchihashi et al., 1993; Herschlag et al., 1994). The specific inhibition of gene expression in vivo by antisense RNA and DNA is thought not only to result from their simple binding to a target RNA, but also to be facilitated by the degradation of the target by means of double-stranded RNase and RNase H, respectively (Murray & Crockett, 1992). Whichever method is applied to the targeting of a specific RNA in cells, the inhibitory mechanism, which involves exogenous RNA or DNA and endogenous proteins, may be not straightforward, but very promiscuous. The conventional antisense techniques, which have been studied most intensively, succeeded in vivo in many cases (Murray & Crockett, 1992), whereas the method using 3′ tRNase need further improvement before use in cells.

MATERIALS AND METHODS

RNA synthesis

The target RNAs (T7H series, SPH series, and T3H series) were synthesized with T7 RNA polymerase (Takara Shuzo) from synthetic double-stranded DNAs containing a T7 promoter, with SP6 RNA polymerase (Takara Shuzo) from synthetic double-stranded DNAs containing an SP6 promoter, and with T3 RNA polymerase (Nippon Gene) from synthetic double-stranded DNAs containing a T3 promoter, respectively. The target RNAs (Env and Gag), the 5′ half $tRNA^{Arg}$ (GT7H), and the modified 5′ half tRNAs (GENV and GGAG) were synthesized with T7 RNA polymerase from synthetic double-stranded DNAs containing a T7 promoter. The transcription reactions were performed in the presence or absence of [α-^{32}P]UTP (Amersham Japan) under the conditions specified by the manufacturers. These RNAs were gel-purified prior to the specific cleavage assays.

Preparation of mouse 3′ tRNase

Mouse 3′ tRNase was prepared as described previously (Nashimoto, 1992, 1995). Mouse FM3A cells were cultured in ES medium (Nissui) containing 3% fetal calf serum and harvested at a density of 5×10^5 cells/mL. After the cells were washed twice with phosphate-buffered saline, they were incubated at 4 °C in twice packed cell volume of buffer A (10 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol) for 10 min and homogenized with 10 strokes in a Douce homogenizer with a tight-fitting pestle. After removal of the nuclei by low-speed centrifugation, I added one-tenth volume of buffer B (0.3 M Hepes, pH 7.9, 1.4 M KCl, 0.03 M $MgCl_2$) to the sample, and centrifuged it at $100{,}000 \times g$ for 60 min. The cytosolic supernatant was precipitated with ammonium sulfate (50% saturation) and dialyzed extensively against buffer 2 (20 mM Tris-HCl, pH 7.5, 0.2 mM EDTA, 1 mM dithiothreitol, 10% glycerol). The dialyzed sample (10 mL; 12.5 mg protein/mL) was applied to a 20-mL Q Sepharose Fast Flow column (Pharmacia) preequilibrated in buffer 3 (20 mM Tris-HCl, pH 7.5, 0.2 mM EDTA, 1 mM dithiothreitol). The column was washed with one column volume of buffer 3 and the protein was eluted with a 160-mL linear gradient from 0 to 1,000 mM KCl in buffer 3. 3′ tRNase fractions (10 mL) eluted around 350 mM KCl were loaded directly onto a 5-mL Blue Sepharose column (Pharmacia) preequilibrated in buffer 3 containing 500 mM KCl. After washing the column with 5 column volume of buffer 3 containing 500 mM KCl, active bound proteins were eluted with buffer 3 containing 2,000 mM KCl. This Blue Sepharose chromatography was repeated using the flow-through protein fraction. The second bound fraction (about 80 μg protein), which had a specific activity (5 units/μg) several-fold over the first one,

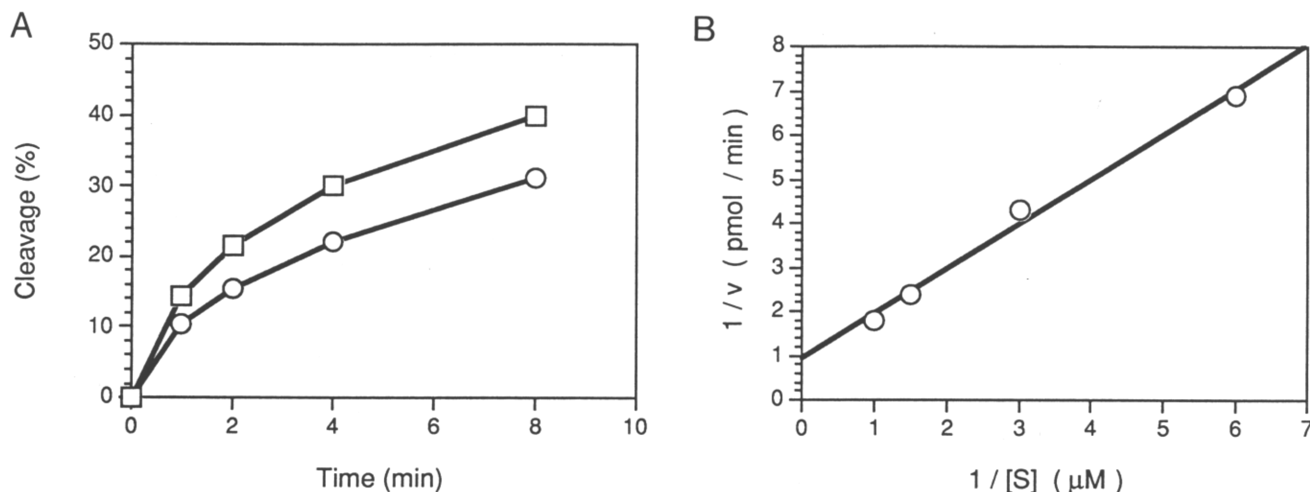

FIGURE 7. Kinetic analysis of cleavage of a complex of GT7H and T3H by pig 3′ tRNase. (**A**) Time course of cleavage of 0.67 µM (circle) or 0.17 µM (square) of T3H by pig 3′ tRNase in the presence of 10 µM of GT7H. (**B**) A lineweaver–Burk plot for 1.0–0.17 µM of T3H. Values of $K_m \cdot K_d$ and V_{max} were obtained from the best-fit line (Table 1).

was desalted, concentrated by ultrafiltration with buffer 3 containing 20% glycerol, aliquoted, and frozen at −80 °C. One unit of the enzyme is defined as the amount that converts 50% of the pre-tRNA^{Arg} (0.1 pmol) to to the 3′ processed product in 10 min under the conditions described below (Nashimoto, 1995).

Specific cleavage assay

The specific cleavage reactions for the ^{32}P-labeled target RNAs (0.1 pmol) were performed with the 3′ tRNase fraction (0.4 unit) from mouse FM3A cells after the second Blue Sepharose chromatography in the presence of an excess molar amount of 5′ half tRNA^{Arg} or its derivative in a mixture (10 µL) containing 10 mM Tris-HCl, pH 7.5, 0.5 mM dithiothreitol, 3.2 mM spermidine for 30 min at 37 °C. After resolution of the reaction products on a 10% polyacrylamide–8 M urea gel and their quantitative analysis with a Bio-Image Analyzer BA100 (FUJIX), the gel was autoradiographed.

RNA sequencing and analysis of 3′ terminal nucleotides

The 5′ cleavage product of the cold target SPH2 was 3′ end-labeled with [5′-^{32}P]pCp, gel-purified, and sequenced by the chemical RNA sequencing method (Peattie, 1979; Nashimoto, 1993).

The 5′ products generated by the specific cleavage of the cold target RNAs were 3′ end-labeled with [5′-^{32}P]pCp, gel-purified, and digested completely with RNase T2 (Sankyo Chemical). ^{32}P-labeled nucleoside 3′ monophosphates were analyzed by two-dimensional thin layer chromatography (Nashimoto, 1993) and quantitated with a Bio-Image Analyzer BA100.

Kinetic analysis

The cleavage of target RNAs in pre-tRNA-like complexes by 3′ tRNase was examined at various concentrations of substrate to obtain kinetic parameters. Because the concentration of the pre-tRNA-like complex can be expressed as $[S_{Target}] \times [S_{5'half}]/K_d$ (where $[S_{Target}]$, $[S_{5'half}]$, and K_d denote the concentrations of target RNA and 5′ half tRNA, and the dissociation constant, respectively), the Lineweaver–Burk equation becomes

$$\frac{1}{v} = \frac{1}{V_{max}} + \frac{K_m \cdot K_d}{V_{max} \cdot [S_{5'half}]} \times \frac{1}{[S_{Target}]}.$$

When $[S_{5'half}]$ is more than 10-fold larger than $[S_{Target}]$, $[S_{5'half}]$ can be regarded as a constant. Thus, the values of $K_m \cdot K_d$ and V_{max} can be obtained from double-reciprocal plots (Fig. 7B). The reaction mixture (6 µL) contained 10 mM Tris-HCl, pH 7.5, 0.5 mM dithiothreitol, 3.2 mM spermidine, 10 µM 5′ half tRNA, and 1.0–0.17 µM target RNA. After preincubation at 37 °C for 10 min, the reactions were started by adding pig 3′ tRNase fraction (1–5 ng) after Mono Q column chromatography (Nashimoto, 1995), and continued at 37 °C for various time periods. The reaction products were resolved on a 10% polyacrylamide–8 M urea gel and then quantitated with a Molecular Imager (Bio-Rad). The reaction velocity was linear with respect to the amount of enzyme in the above range (data not shown). The amount of cleavage products generated in 1 min was used to determine the initial velocity (Fig. 7A).

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