Activation of HIV-specific ribozyme activity by self-cleavage

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ABSTRACT

A hammerhead ribozyme designed to cleave in *trans* the R region of HIV-1 RNA was inserted into a eukaryotic expression vector. This ribozyme was studied *in vitro* using the T3 RNA polymerase promoter located upstream of the eukaryotic promoter. The ribozyme showed no activity against its specific target sequence under any condition tested. To decrease the influence of potential *cis* inhibitory sequences in such a ribozyme transcript, a specific target sequence was inserted upstream of the ribozyme-coding sequence. This insertion allowed the release by *cis* cleavage of a short RNA bearing ribozyme activity and able to cleave in *trans* an external RNA target. The *cis* cleavage reaction generated two RNA molecules: the shorter RNA species, which included the catalytic domain, showed a *trans* cleavage reaction. This self-cleavable ribozyme was active *in vitro* at 37°C against three distinct HIV-1 transcripts sharing the specific target sequence. Ribozyme activity was thus attained by self-cleavage of the ribozyme-containing sequence from the longer vector transcript.

INTRODUCTION

Ribozymes are small RNA molecules that catalyse RNA cleavage. The use of these catalytic RNAs is considered here for the specific inhibition of the expression of an HIV RNA target. Such an inhibitory effect has previously been sought against HIV expression. Indeed, genetically engineered ribozymes have already been shown to be active in transfected cells (1, 2, 3, 4, 5) and to inhibit HIV replication (6, 7, 8, 9, 10, 11). However a very large excess of ribozyme RNA seems to be required *in vivo*, in the few cases where it has been measured, and clearly improvements are necessary before anti-HIV ribozymes could be promoted as a form of anti-retroviral therapy.

The use of ribozymes *in vivo* will probably require the transcription of ribozyme genes from eukaryotic promoters. This could produce overlong RNAs whose catalytic domains could potentially interact with the *cis* surrounding sequences, hampering ribozyme activity. It is thus important to define a ribozyme structure that is not only efficiently expressed but that also possesses a high level of catalytic activity. Improvement of ribozyme structure could also be achieved by modification of either the catalytic domain itself (12) or the sequence recognition

(13). In this report we focus on the influence of the sequences present in the chimaeric vector RNA that bears the ribozyme activity.

The design of the ribozyme used in this study was based on the structure of the hammerhead ribozyme found in the satellite RNA of the Tobacco Ringspot Virus (14, 15). This type of ribozyme, redefined by Haseloff and Gerlach (16), contains a 22-nucleotide (nt) catalytic domain (the sequence responsible for cleavage activity) which must be flanked by sequences complementary to those surrounding the cleavage site of the target RNA. No ribozyme activity was detected after inserting this so-called standard ribozyme downstream of the RNA polymerase III promoter. Therefore a specific target was inserted into the ribozyme construct, upstream of the catalytic domain, so that upon *cis* cleavage, the RNA transcript would be shortened towards the 5' end of the ribozyme. We show here that the introduction of this self-cleavage activity suppresses the inhibitory effect of the *cis*-surrounding sequences and is essential for ribozyme activity on *trans* target RNA.

MATERIALS AND METHODS

Construction of targets

A target RNA containing the R region of HIV-1 was constructed from HIV-LAI (17) using the pBluescript KS+ plasmid (pKS+; Stratagene, La Jolla, USA) as shown in Fig 1 part A. In the RH3RV plasmid, the 416 nt long HIV-LAI EcoRV–HindIII fragment (8793–9209) was cloned between the EcoRV and HindIII sites of the pKS polylinker. This fragment lacked 14 nt of the 3' end of HIV viral RNA. In RH3RV the ribozyme cleavage site (GTC) shown in Fig 1 part A, is located 415 nt downstream of the T7 promoter. The RH3RV-del plasmid was derived from RH3RV by deleting 228 nt, to bring the cleavage site 215 nt from the T7 promoter. This deletion was obtained by excision of the SmaI–ScaI fragment (see Fig 1 part B), SmaI being located in the pKS polylinker 56 nt from the T7 promoter and ScaI being in the HIV fragment 210 nt from the EcoRV site. To obtain an *in vitro* transcript whose 3' end would be similar to the 3' end of the viral RNA, plasmid RH3RV+ was constructed from the RH3RV plasmid by the addition of 16 nt to the 3' end of the EcoRV–HindIII fragment. An oligonucleotide of 28 nt (AGCTTGCTTGAGTGCTTCATATGAGCT) was obtained from GENSET (Paris, France). It was composed of 16 nt restoring the HIV sequence flanked by a standard HindIII

adaptor at the 5' end and the HindIII adaptor preceded by a NdeI site, at the 3' end. This sequence was inserted into the HindIII site of RH3RV and led to the generation of an NdeI site (Fig 1 part B) that was used to obtain an *in vitro* transcript whose sequence was similar to the 3' end of HIV RNA.

Construction of ribozymes

The anti HIV-1 ribozyme was inserted into a modified Va gene of Adenovirus 2. This gene, transcribed *in vivo* by RNA

polymerase III, allows the synthesis of short RNA molecules. The polymerase III promoter includes two internal recognition sequences (Box A and Box B) which are transcribed (Fig 2A).

The Va gene, obtained from Human Adenovirus 2, was inserted into the pKS+ polylinker and modified at its 3' end. These modifications comprised the insertion of a multicloning site downstream from Box B and insertion of a polymerase III terminator used for *in vivo* transcription. Plasmid pVaR8 was constructed by inserting a ribozyme structure into the Va construct (Fig 2A). The ribozyme core sequence (25) was targeted to the R region present at the 5' and 3' ends of HIV-1 RNA. A single strand oligonucleotide (46 nt), composed of the 22 nucleotide catalytic domain, 8 flanking nucleotides, a HindIII adaptor site at the 5' end and a PstI adaptor site at the 3' end, was synthesised (Applied Biosystems, Roissy, France) and purified on a 12% acrylamide gel. It was inserted between the HindIII and PstI sites of the Va polylinker 13 nt from the 3' end of the polymerase III promoter Box B (Fig 2A). After excision of the HindIII–PstI fragment from the pVa plasmid polylinker, single strand oligonucleotide was ligated using T4 DNA ligase and the complementary strand was synthesised using the Klenow fragment of DNA polymerase I. The T3 RNA polymerase promoter, 69 nt upstream of the initiation site of the Va promoter, was used to transcribe ribozyme RNA for *in vitro* experiments.

To obtain a shorter RNA and to produce a *cis* self-cleavable ribozyme, we inserted a 17 nt oligonucleotide encoding the specific target, surrounded by BamHI adaptors, at the BamHI site of pVaR8, 11 nt upstream of the 5' end of the ribozyme. This construct (pVaR8-C) is shown in Fig 2A. In this internal target the GTC was positioned off-centre, towards the ribozyme insert to minimise the energy of the complementary strand after cleavage. Fig 2B shows a schematic representation of the cleavage mechanism. pVaR8-Cr was constructed in the same way with the specific target inserted in the reverse orientation.

Figure 1. Construction of the HIV target. A: Schematic representation of the target construction from HIV-LAI. B: RH3RV (top) is the standard target, RH3RV-del was deleted by 228 bp (middle) and RH3RV+ had 16 bp inserted at its 3' end using HindIII adaptors (not indicated). GTC represents the cleavage site of the HIV target (9128–9145, UACUGGGUCUCUCUGGU) for the ribozyme, the T3 and T7 boxes represent the T3 and T7 RNA polymerase promoters, used for *in vitro* transcription.

Figure 2. Construction of ribozymes. A: Schematic representation of the Va gene (top), construction of ribozyme VaR8 (middle) and construction of the self-cleavable ribozyme VaR8-C (bottom). Boxes A and B represent the polymerase III promoter. The T3 box represents the T3 RNA polymerase promoter used for *in vitro* transcription. The start site for the T3 transcript is located at nucleotide 1 (broad arrow), the start site for the Va transcript is located 69 nucleotides downstream of the T3 promoter (arrowed). The R8 ribozyme is located at nucleotide 161 in VaR8 and 185 in VaR8-C and internal target is located at nucleotide 140 in VaR8-C. B: Schematic representation of the self-cleaving mechanism in VaR8-C: the VaR8-C ribozyme, transcribed as a 242 nt long RNA (upper panel), is self-cleaved to generate a 92 nt long RNA (lower panel). Sequences located upstream from the GUC cleavage site are excised and only 6 nucleotides, downstream from the GUC, represent the complementary strand of the ribozyme recognition sequence.

All constructions were confirmed by sequencing using a Sequenase kit and the plasmids were doubly purified in a caesium chloride gradient.

In vitro transcription

In vitro transcription reactions were carried out using T3 (for the ribozyme) or T7 (for targets) RNA transcription kits (Promega) with 1 μ g of linearised plasmids. In the ribozyme transcriptions, the start site of the T3 transcript was located 69 nt upstream of the start site of the Va transcript. Radiolabelling was performed using 10 μ M 35 S-ATP (3000 Ci/mmol) and the resulting RNA was phenol/chloroform purified, and resuspended in 50 μ l of water. The size and the quality of the RNA were verified on 5% polyacrylamide urea gels. The radiolabelled RNA was quantified by measuring the total 35 S incorporated and comparing this to the specific activity of the 35 S-ATP used in its synthesis. For cleavage reaction studies, only the substrate RNA was radiolabelled. In this case, the quantity of ribozyme RNA was estimated on 1% agarose gels in the presence of ethidium bromide.

Ribozyme cleavage studies

Equal amounts of ribozyme and substrate RNA were heated at 90°C in a solution of Tris-HCl (20 mM, pH 7.5), MgCl₂ (20 mM) for 1 min and brought to the reaction temperature (37°C or 50°C) for variable periods of time. In cases where the reaction cycles were repeated, this was carried out in a DNA thermal cycler (Perkin Elmer, St Quentin en Yvelines, France) in a final volume of 5 μ l. After incubation, 3 μ l of formamide loading buffer were added, samples were heated for 2 min at 90°C, and 4 μ l were loaded on 6% acrylamide gels. Competition assays were performed at 50°C: 5 nM of 35 S-ATP-labelled RH3RV and 20 nM of VaR8-C were heated at 90°C in 10 μ l of Tris-HCl (20 mM, pH 7.5), MgCl₂ (20 mM) for 1 min and brought to the reaction temperature, then 5 μ l of non-radiolabelled RH3RV substrate were added to the reaction mixture which was then incubated for 30 min. Cleavage intensity was measured by densitometric scanning of autoradiograms.

Figures 3 to 6 were produced by scanning of autoradiograms on a Microtek 600Z scanner, and were processed using the Image-in program (Image-in, Pierrefite sur Seine, France).

RESULTS

Self-cleavable ribozyme

Standard hammerhead ribozyme was inserted downstream of a polymerase III promoter (pVaR8, Fig 2, see Materials and Methods). Such a construct failed to demonstrate any cleavage activity. We thus attempted to decrease the potential for interaction between the sequences directly involved in ribozyme activity and the *cis*-surrounding sequences by inserting a synthetic target upstream of the ribozyme structure (pVaR8-C, Fig 2). Self-cleavage of the RNA, by a reaction in *cis* at this internal target, should yield a shortened transcript harbouring the catalytic site.

Transcription (using T3 polymerase) of pVaR8 obtained from insertion of the catalytic structure of the standard hammerhead ribozyme into the Va gene (Fig 2A), revealed a unique band of 216 nt (Fig 3A, lane A). When the same ribozyme sequence was preceded by its specific target (as in pVaR8-C), transcription led to three bands of expected sizes (Fig 3A lane B). The largest band (242 nt) corresponds to leftover undigested transcript, and

the two smaller bands correspond to fragments (150 and 92 nt) resulting from a *cis* cleavage reaction. The 92 nt fragment bears the catalytic site. Thus, as expected, the RNA transcript bearing both ribozyme activity as well as the specific target in *cis* displayed self-cleavage activity. RNA carrying the ribozyme containing a target cloned in the reverse orientation (transcribed from pVaR8-Cr) did not show any such cleavage activity (Fig 3A, lane C). The weak 150 nt band in lane C was most likely due to non-specific degradation of VaR8-Cr or to slight contamination by the contiguous strong band since no product could be seen at 92 nt.

Ribozyme activity

We then compared the cleavage efficiency on a *trans* RNA target of the self-cleavable VaR8-C ribozyme with that of the non-cleavable VaR8. The target RH3RV RNA containing the R region of HIV-1 (497 nt) synthesised with T7 polymerase was 35 S-labelled and mixed in equal amounts with either of the ribozyme RNAs. The reaction mixtures were incubated at 90°C for 1 min followed by 30 min at 50°C. As shown in Fig 3B lane B, no catalytic activity towards the RH3RV target could be detected with VaR8 ribozyme whereas the VaR8-C ribozyme (lane C) digested the target RNA, yielding the expected 415 and 82 nt bands (Fig 3B lane C). A 1 in 10 dilution of VaR8-C (lane E) in the reaction still resulted in significant cleavage, VaR8 remaining inactive (lane D). The ribozyme transcript in which the internal target was inserted in the reverse orientation (VaR8-Cr) was also unable to cleave RH3RV target (lane F). These results demonstrated that the recovery of *trans* ribozyme activity could be achieved by the elimination of *cis*-surrounding sequences by self-cleavage.

In the transcription of pVaR8-C, leading to active VaR8-C ribozyme, two bands were expected to harbour the catalytic domain: the 242 nt long uncleaved complete transcript and the 92 nt released fragment. We purified all three bands in polyacrylamide gels, and tested their activities on the RH3RV target RNA as described above. The results obtained are presented in Fig 3C. The 92 nt band (lane B) showed effective *trans* cleavage whereas the 150 nt band (lane C) was inactive. The complete transcript (lane D) was also able to cleave the RH3RV target, apparently to an even greater extent than the 92 nt fragment. The activity of the uncleaved 242 nt fragment was in fact due to further *cis* cleavage of this fragment under *trans* cleavage reaction conditions, as monitored by the concomitant appearance of a 92 nt long band (data not shown). As this *cis* self-cleavage activity occurred under standard experimental conditions, the *trans* activity of the complete transcript (if any) could not easily be evaluated.

Temperature requirement for cleavage

To study the potential use of this ribozyme in *in vivo* experiments, the activity of the self-cleavable ribozyme was studied at 37°C versus 50°C. The 35 S-labelled targets RH3RV and VaR8-C were mixed as described in Materials and Methods and the samples were incubated at 37°C or 50°C for various times. Fig 4 shows the results obtained. At 37°C, after 1 min incubation the 2 bands resulting from RH3RV cleavage were observed, 26% of the target being cleaved. The plateau was reached after only 5 min (28% cleavage). After 30 min incubation, significant degradation of all bands was observed. When the experiment was performed at 50°C, the reaction kinetics were much slower, maximum

Figure 3. Panel A: Ribozyme transcripts present *in vitro*. Lane A: VaR8, lane B: VaR8-C and lane C: VaR8-CR. Each RNA was transcribed from the T3 RNA polymerase promoter, in the presence of ^{35}S -ATP, phenol/chloroform purified and loaded onto polyacrylamide sequencing gels. Unrelated sequence was added at the left of the panel to evaluate the fragment sizes. Panel B: Cleavage activity of VaR8 and VaR8-C. RH3RV target RNAs were transcribed from the T7 promoter, in the presence of ^{35}S -ATP, whereas ribozyme RNA, transcribed from the T3 RNA polymerase promoter, was not labelled. 25 nM RH3RV target was mixed with 25 nM of VaR8 ribozyme (lane B), 25 nM of VaR8-C (lane C), 2.5 nM of VaR8 (lane D) or 2.5 nM of VaR8-C (lane E). Lanes A and F represent control target RNA. Panel C: Cleavage activity of purified bands after self-cleavage of VaR8-C. Self-cleavable ribozyme was transcribed and bands were purified on 15% polyacrylamide gels. The radiolabelled RH3RV target was mixed with the band of 92 nt (lane B), 150 nt (lane C) or the complete transcript (lane D). No ribozyme products were added in lane A.

cleavage being observed only after 30 min (11% after 1 min; 31% after 30 min).

Although cleavage was quicker at 37°C than at 50°C, only partial cleavage of the target was observed at both temperatures. Two hypotheses could account for this result. Firstly, the structure of the ribozyme and/or the target RNA may change during the experiment to yield an inactive conformation(s), or the ribozyme may be rapidly degraded under these reaction conditions.

To address these questions, reaction mixtures were subjected to thermal denaturation at 90°C for 30 sec, followed by incubation at 37°C for 5 min. When this was repeated 5 times in a DNA thermal cycler, we observed a significant increase in the intensity of the cleaved bands (Fig 5, lane B) as compared with only 1 cycle (lane D) with a concomitant decrease in the quantity of the complete RNA target. After 5 cycles, 38% of the *trans* RNA target was cleaved using 25 nM VaR8-C ribozyme (28% with 12.5 nM, lane C), while one cycle gave only 10% cleavage (3% with 12.5 nM, lane E). Since we observed that more than 5 cycles led to a significant non-specific degradation of the RNA target (data not shown), we did not continue beyond 5 cycles. The lower cleavage activity observed here after one cycle (10%) compared to that in the previous experiment (28%) could be partially explained by the shorter denaturation step used here to minimise degradation of the target RNA (30 sec versus 2 min). Maximum cleavage was reached after 5 cycles (Fig 5), indicating that ribozyme activity could be restored after a denaturation step at 90°C. If the ribozyme were degraded, one would not expect to see this increase in activity observed on re-denaturation.

Figure 4. Cleavage activity of VaR8-C at 37°C and 50°C. After transcription of radiolabelled target RNA and ribozyme RNA, the target and ribozymes were mixed and incubated for various times at 37°C or 50°C. RH3RV target (25 nM) was mixed with 25 nM of VaR8-C except for the first lane (37°C panel) where no ribozyme was added. The reaction was carried out for 1, 3, 5, 7, 9, 15, 30, and 60 min as mentioned on the top of each panel. The mixture in the two extreme right hand lanes was incubated for 60 min at 4°C.

Figure 5. Variation in VaR8-C cleavage activity with cycle number. Ribozymes and target RNA were mixed, then incubated in a DNA thermal cycler. For each cycle, the mixture was heated at 90°C for 30 sec then incubated 5 min at 37°C. In lanes B and D, 25 nM of VaR8-C was used compared with 12.5 nM in lanes C and E. The reaction was carried out for 5 cycles (lanes B and C) or 1 cycle (lanes D and E) with 25 nM of RH3RV at 37°C. No ribozyme was added in lane A.

Incomplete cleavage might thus be explained by stabilisation into an inactive conformation of the secondary or tertiary structure of the ribozyme, the target or the complex, rather than by degradation of the ribozyme.

Figure 6. Effect of target structure on cleavage activity. VaR8 and VaR8-C ribozyme activity was tested against 3 RNA targets: RH3RV+ (left), RH3RV (centre), RH3RV-del (right). 25 nM ribozyme substrate was mixed with 25 nM VaR8 (lanes B and F), 2.5 nM VaR8 (lanes D and H), 25 nM VaR8-C (lanes C and G) or 2.5 nM VaR8-C (lanes E and I). No ribozyme was added in lane A. The reaction was performed for 5 cycles (lanes B to E) or for one cycle (lanes F to I). The sizes of the cleavage products were as follows: RH3RV+, 415 nt and 102 nt; RH3RV, 415 nt and 82 nt; and for RH3RV-del, x nt and 82 nt.

Influence of the target structure

Since our observations show that the secondary or tertiary structure of the substrate RNA is likely to interfere with the cleavage reaction, we studied the VaR8-C ribozyme *trans* activity towards 3 targets whose sizes were modified to generate potentially different structures. RH3RV was the native target, RH3RV-del was deleted by 228 nt at its 5' end and RH3RV+ represented the complete sequence of the 3' end of HIV-1 RNA (Fig 1 part B). Fig 6 shows that the cleavage efficiency of VaR8-C was very similar for these 3 substrates. No significant difference was detected in the cleavage reactions with VaR8-C using a ribozyme/target ratio of 1:1 (lanes C, G) or 1:10 (lanes E, I), for either 5 cycles (lanes C, E) or 1 cycle (lanes G, I).

The decrease in intensity of each substrate after cleavage was correlated to an increase in the cleavage product (see lane C in all panels). The slight differences observed in the intensities of the cleavage products was not correlated to a variation in the cleavage efficiency. Thus, the apparently weak cleavage efficiency observed when VaR8-C was incubated with RH3RV-del for 5 cycles (lane E, right panel) was not confirmed in other experiments (results not shown). The variable intensity of the

Figure 7. Competitive effect of denatured and native RNA target on ribozyme cleavage activity. 20 nM of VaR8-C was mixed with 5 nM of ^{35}S -ATP-labelled RH3RV in 10 μl (20 mM Tris-HCl pH 7.5, 20 mM MgCl_2), heated 1 min at 90°C, then cooled to 50°C. Denatured (open squares) or native (filled circles) RH3RV was added in 5 μl of the same buffer and the reaction was incubated for 30 min at 50°C. The cleavage efficiency was calculated for each competitor concentration (5, 10, 20, and 40 nM) by densitometric scanning of autoradiograms, and expressed as percentage of the total substrate. The initial cleavage value was obtained by addition of 5 μl of buffer in the same conditions.

cleavage products between the different panels may be due to variations in the specific activities of the radiolabelled targets.

The best results were always obtained using equimolar amounts of ribozyme and target for 5 cycles. These results indicate that targets of potentially different structures could be efficiently cleaved by VaR8-C. The non-self-cleavable VaR8 was unable to cleave *in trans* any of these targets under any of the conditions tested (Fig 6, lanes B, D, F, and H).

To study the influence of the structure of the *trans* RNA target on the cleavage reaction, we examined the competitive effect of the presence of denatured or native non-radiolabelled *trans* target. The cleavage reaction was carried out under the same conditions as those described above, mixing the ^{35}S -labelled target RH3RV with a 4-fold excess of VaR8-C at 90°C. For these experiments, 50°C was chosen in preference to 37°C in view of the slower reaction kinetics at 50°C. At 37°C, the ribozyme activity was such that after only 1 min, almost maximal cleavage was obtained, rendering such competition experiments difficult. When the temperature had descended to 50°C, non-radiolabelled RH3RV at 50°C, either initially denatured for 2 min at 90°C or not, was added to the reaction, and the mixture was incubated for 30 min. Under these conditions, the percentage of cleavage of the radiolabelled product in the absence of unlabelled RH3RV target was significantly lower (Fig 7, 13%) than that observed in previous experiments (Fig 4, 31%). This could be due to the slight differences in the experimental procedure used here, which could lead to momentarily uncontrollable temperature changes, and may also be accounted for by differences in reaction volume. Nevertheless, the results reported in Fig 7 show that the unlabelled *trans* target could compete with the labelled target in the cleavage reaction. This competitive effect was observed even if the unlabelled *trans* target was not initially denatured. Addition of an 8-fold excess (40 nM) of denatured unlabelled target led to a decrease in the cleavage intensity from 13% in the absence of target to 3%, as opposed to 1% on addition of the same quantity of native unlabelled target. This difference could be due to the decrease in the concentration of available denatured unlabelled target resulting from its degradation during heating,

as a decrease in intensity corresponds to an increase in the competitive effect.

As the native RH3RV *trans* target can compete successfully with the labelled target in the cleavage reaction, it would seem that the secondary structure of the RH3RV *trans* target is less important for the efficiency of the cleavage reaction than the structure of the VaR8-C ribozyme itself.

DISCUSSION

We show in this paper that a ribozyme sequence produced from an adenovirus-based expression vector may have its activity masked when included within a chimaeric RNA. We were able to recover this potential activity by reducing the size of the transcript. Thus, whereas no cleavage in *trans* of target RNA was observed in any tested conditions with the VaR8 ribozyme, the self-cleavable VaR8-C ribozyme displayed a cleavage activity under standard conditions. The *cis* self-cleavage reaction took place during the transcriptional process (Fig 3A) but cleavage of the full-length transcript was never complete, as already described for the virusoid from Lucerne Transient Streak Virus (LTSV; 18). Ribozyme *trans* activity was shown for the small band harbouring the ribozyme sequence, as expected, and for the uncleaved full-length transcript. This seems to be incompatible with the fact that non self-cleavable VaR8 and VaR8-Cr molecules were inactive. However, as already described by Forster and Symons (18), we found that the full-length transcript could be further self-cleaved after a new round of denaturation at 90°C (data not shown). Therefore, under the reaction conditions used to assay cleavage of the heterologous target, self-cleavage of this full-length transcript occurred, allowing the release of new shorter RNA species bearing ribozyme activity. Although the ribozyme in which the internal target had been inserted in the reverse orientation remained inactive, we could not formally exclude the possibility that the uncleaved transcript VaR8-C still demonstrated a ribozyme activity. Nevertheless, from our results it was clear that reducing the size of the ribozyme led to a dramatic increase in the cleavage activity.

Cleavage of the external *trans* target was partial at both temperatures used (37°C and 50°C; Fig 4). Interestingly, cleavage efficiency could be improved by increasing the number of denaturation cycles (Fig 5). Therefore, partial cleavage did not seem to reflect ribozyme degradation but rather the existence of active and inactive forms for either or both the ribozyme and the *trans* RNA target. The presence of such active and inactive structures has already been investigated by Forster and Symons (19) in the case of the *cis* cleavage of LTSV. These authors showed that the self-cleavage efficiency could be increased by heating and snap-cooling the LTSV transcript to generate alternative (inactive) and active conformations. In the case of the *trans* target cleavage, inactive forms might also be reactivated upon new cycles by re-denaturing inactive or inhibitory secondary structure. In our case, it was unclear whether the structure of the ribozyme or that of the target played a more important role. To partially answer this question, competition experiments were performed, where mixtures of ribozyme and radiolabelled target RNA were incubated with an excess of unlabelled denatured or native target. The results (Fig 7) show that native target RNA competes successfully in the cleavage reaction, indicating that the secondary structure of the RH3RV *trans* target is perhaps less involved in cleavage efficiency than the secondary structure

of the VaR8-C ribozyme itself. Thus, partial cleavage is more likely to be due to a change in ribozyme structure to an inactive conformation.

Incomplete cleavage can alternatively be caused by reversibility of the cleavage reaction, as has been demonstrated in the case of Tetrahymena ribozyme (20). In this case, there exists a reversible trans-esterification reaction resulting from an equilibrium between the cleavage and ligation reactions. Although trans-esterification ligation has not yet been demonstrated for hammerhead ribozymes on a *trans* target, the hammerhead ribozyme of the satellite RNA of tobacco ringspot virus shows a ready reversibility, particularly for the products of the minus RNA self-cleavage (21). Thus, the partial cleavage by VaR8-C might be due to the displacement of such an equilibrium towards ligation under the conditions used.

The formation of inactive structures, or the equilibrium displacement towards ligation, could be modulated by the choice of internal target for self-cleavage, as self-cleavage efficiency is dependent on the transcript structure (15, 18, 22). For example, it has been shown that reducing the degree of base-pairing immediately 3' to the cleavage site can increase ribozyme activity (13, 23). Such a modification of the internal target might thus allow us to increase the ribozyme activity.

We have shown that ribozyme activity is more efficient at 37°C than 50°C (Fig 4). Koizumi et al (24) report a similar optimal cleavage temperature, between 30 and 37°C, using an 11 nucleotide substrate. These conditions for ribozyme activity are obviously more compatible for *in vivo* use. Many of the experiments presented in this study include multiple denaturation steps that would restrict ribozyme use in a clinical setting. However, we show that self-cleavage occurs during *in vitro* transcription (Fig 3) to induce the formation of a ribozyme active at 37°C, without the need for denaturation, and preliminary results obtained in *ex vivo* experiments indicate that active ribozyme is indeed generated in an intracellular context.

In vitro experimental studies have often been carried out with short RNA ribozyme and/or short RNA targets (12, 13, 16, 24). The constraints imposed by *in vivo* expression of ribozyme RNA may require the synthesis of longer molecules whose conformation would either block the accessibility of the target sequences or decrease the catalytic activity of the ribozyme structure. This might explain the weak catalytic activity of the ribozymes in experiments performed *in vivo* (1, 2). Reducing the size of the ribozyme RNA should decrease potential intramolecular interaction and in most cases increase the activity. Some authors (26, 27, 28) have already investigated the possibility of shortening the ribozyme RNA transcript by using a second set of ribozymes together with their internal targets to reduce the lengths of both the 5' and 3' termini. In this study we introduced an internal target recognised uniquely by the ribozyme present in the vector and located 5' of the catalytic domain.

We have shown that the HIV-1 specific ribozyme used here could recognise an internal target and regulate its own size by 5' *cis* cleavage. Ribozyme activity was then able to occur on a *trans* RNA target. Therefore, it would be useful to design single ribozyme-containing vectors which include such a self-cleavage ability. Current studies are focused on the inclusion of this transcription unit within a eukaryotic expression vector, to test the biological function of this ribozyme in cell culture and to evaluate its potential *in vivo* use in the inhibition of HIV expression.

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