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Multitarget-ribozyme directed to cleave at up to nine highly conserved HIV-1 env RNA regions inhibits HIV-1 replication—potential effectiveness against most presently sequenced HIV-1 isolates

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ABSTRACT

Several mono-, di-, tetra-, penta- and nonaribozymes were developed. These multitarget-ribozymes were targeted to cleave HIV-1 env RNA at up to nine different conserved sites. Each multitarget-ribozyme consisted of a chain of up to nine hammerhead motifs, each flanked by a different targeting sequence. The multitarget-ribozymes were functional in vitro and gave rise to multiple, specific partial and/or complete RNA digestion products. Per RNA copy, multitarget-ribozymes were more efficient than monoribozymes or ribozymes targeting a subset of the same sites. In contrast to monoribozymes, a 400nt nonaribozyme, targeted to cleave at nine different sites within a 1.3kb HIV-1 env RNA substrate, was active and showed the same specificity of cleavage when it was part of a large 3.3kb transcript. We conclude that multitarget-ribozymes retain the specificity of monoribozymes, but they are more efficient per ribozyme RNA copy and they remain active when they are part of a large transcript. A tetra-, penta- or nonaribozyme under control of the SV40 late promoter, the beta-actin gene promoter or the HIV-1 LTR, respectively, were cotransfected with the infectious HIV-1 DNA clone pNL4-3 into permissive HeLa T4 cells. Each cotransfection resulted in a specific inhibition of HIV-1 replication as determined by syncytia formation and p24 antigen release. In addition, coexpression of the nonaribozyme with an HIV-1 env RNA transcript resulted in the specific dramatic reduction of the env transcript. We conclude that the multitarget-ribozymes are also functional intracellularly. A nucleotide sequence comparison of the target sites indicates that the multitarget-ribozymes could potentially be effective against all thirty HIV-1 isolates presently sequenced. Their use may help to slow the selection of viral escape mutants and thereby prolong their effectiveness. We anticipate that multitarget-ribozymes will also be more effective in the successful targeting of less variable cellular RNAs.

INTRODUCTION

Ribozymes are small catalytic RNA molecules (1–3) which can be targeted to cleave a chosen substrate RNA at a specific nucleotide sequence containing, for example a GUX cleavage site, as is characteristic for hammerhead ribozymes (4). The use of ribozymes has been proposed for the selective inhibition of gene expression or as a potential antiviral agent (5–9). The genome or the transcripts of the human immunodeficiency virus type 1 (HIV-1), have recently become the prime viral target for the application of ribozymes (5, 10–13). The effective use of ribozymes against a rapidly replicating virus presents a challenge. The challenge is even greater with RNA viruses, like HIV, which continuously undergo mutational changes. This is caused by the error prone reverse transcriptase in the absence of proof reading mechanisms to correct the mistakes (14–16). Consequently, at all times during replication, new variants of the virus are generated.

For these reasons, any antiviral strategy which depends solely on the inhibition of viral replication by a single, sequence specific ribozyme, seems to be compromised from the beginning, since such a strategy requires that the sequence of the target RNA as well as that of the ribozyme do not change throughout the course of the infection. During the 5 to 10 years of the asymptomatic phase of an HIV-1 infection, the virus slowly replicates and the infection slowly progresses (for review see 17, 18). It is during this time that escape mutants could be selected. For example, AZT resistant HIV can be isolated from patients as early as 6 months after the initiation of AZT treatment (19). To decrease the chance for the selection of HIV mutants which can escape an antiviral ribozyme, one can first select relatively conserved target sites within the HIV genome. These sites should not be protected by proteins or by secondary structure intracellularly and must be accessible for cleavage. At this time it cannot be predicted which of these sites are accessible at all times and which sites would remain targets in most cells for a long period of time.

In this communication, we describe possible solutions to some of these major challenges which have to be confronted when ribozymes are targeted against viruses. We demonstrate for the first time that functional multitarget-ribozymes of the

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hammerhead type can be assembled. These multitarget-ribozymes are able to specifically and efficiently cleave a substrate RNA at multiple sites in vitro. Most important, when coexpressed with HIV-1 intracellularly, they are able to inhibit HIV-1 replication. Multitarget-ribozymes described here combine many desirable features, which are essential for their potential prolonged effective use as antivirals in a gene therapy.

MATERIALS AND METHODS

Construction and in vitro expression of ribozymes

All ribozymes were constructed by DNA fusion using between two and four, up to 60nt long, partially overlapping, synthetic oligonucleotides (20), which were annealed and amplified using polymerase chain reactions (21). The approximately 400bp nonaribozyme DNAs, clones 636 and 515, were assembled starting with two fused tetraribozyme DNA cassettes. All ribozymes were cloned into pGEM4XB downstream of the T7 RNA polymerase promoter (22) and the nucleotide sequences were determined and confirmed for each of the constructs. Some multitarget-ribozymes were also part of larger, up to about 3.3kb RNA transcripts and were transcribed using T7 RNA polymerase.

In vitro ribozyme cleavage reactions

The penv5 DNA (23) encodes a full length HIV-1 env gene of BH8 under the T7 polymerase promoter and was provided by Pat Earl (NIAID, NIH). The DNAs for the env RNA substrate and the ribozymes were linearized and transcribed using T7 RNA polymerase. Both RNAs were combined in 18 μl of 50 mM Tris-HCl, pH 7.5, 2 mM spermidine and 10 mM NaCl. The reaction mixture was heated for 1 min at 90°C, chilled on ice, and 2 μl 100 mM $MgCl_2$ was added to start the cleavage reaction (5). Reactions were carried out for various times at either 37°C or 55°C. Cleavage products were heated for 1 min at 90°C, and separated by electrophoresis on a 5% polyacrylamide-7M urea gel in Tris-borate-EDTA buffer.

Inhibition of HIV-1 replication

The tetraribozyme Rz41 (Xho I-RzE-RzF-RzG-RzH-BamH I) was cloned into the DNA vector pEUK-C1 under control of the SV40 late promoter (Clontech Inc., Palo Alto, CA). The pentaribozyme Rz52 (Hind III-Rz37-PL-RzE-RZF-RzG-RzH-BamH I) was cloned into pHBAPr DNA under control of the beta-actin gene promoter (24). The nonaribozyme Rz636 was cloned under control of the HIV LTR of pNL4-3 (25). The ribozyme DNAs were cotransfected with the infectious HIV-1 DNA clone pNL4-3 (25) into HeLa T4 cells (26). Syncytium formation was first observed starting two days after cotransfections. It was quantitated at day four. The release of p24 antigen was followed for 11 days and the amounts were determined by Elisa.

Disappearance of HIV-1 env RNA transcripts in the presence of nonaribozymes *in vivo*

HeLa-tat-III (27) cells were cotransfected using Lipofectin (Gibco BRL Inc., Gaithersburg, MD) with equal amounts of the pHenv (28) and the nonaribozyme Rz636 which had been cloned under control of the HIV LTR of pNL4-3 (25). This clone also contained Rz6 positioned 5′ of the nonaribozyme. In parallel, pHenv was cotransfected with the same DNA from which the nonaribozyme was removed but still contained the monoribozyme Rz6. In a third cotransfection an unrelated DNA was combined with equal amounts of pHenv DNA. Two days after transfection, total RNA was extracted from equal number of cells and was separated on formaldehyde gels. HIV-1 env transcripts were analyzed by Northern blot hybridization using a specific 300bp NheI-SspI fragment of the penv5 DNA as a ^{32}P labelled probe.

RESULTS

Multitarget-ribozyme constructs and target sites

All ribozymes used in this study contained the previously described hammerhead ribozyme motif flanked on either side by 8 nucleotides which were complementary to the target sequence in the substrate RNA (4). Some of the ribozyme constructs, ranging in size from about 60 to 400bp in length, are diagrammed in Figure 1. The complete sequence of one hammerhead ribozyme (Rz37) and the positions and sequences of all target sequences used in this study are shown in Figure 2. A total of ten different target sites were chosen from the gp120 encoding region of the HIV-1 envelope RNA. The selected sites all contain

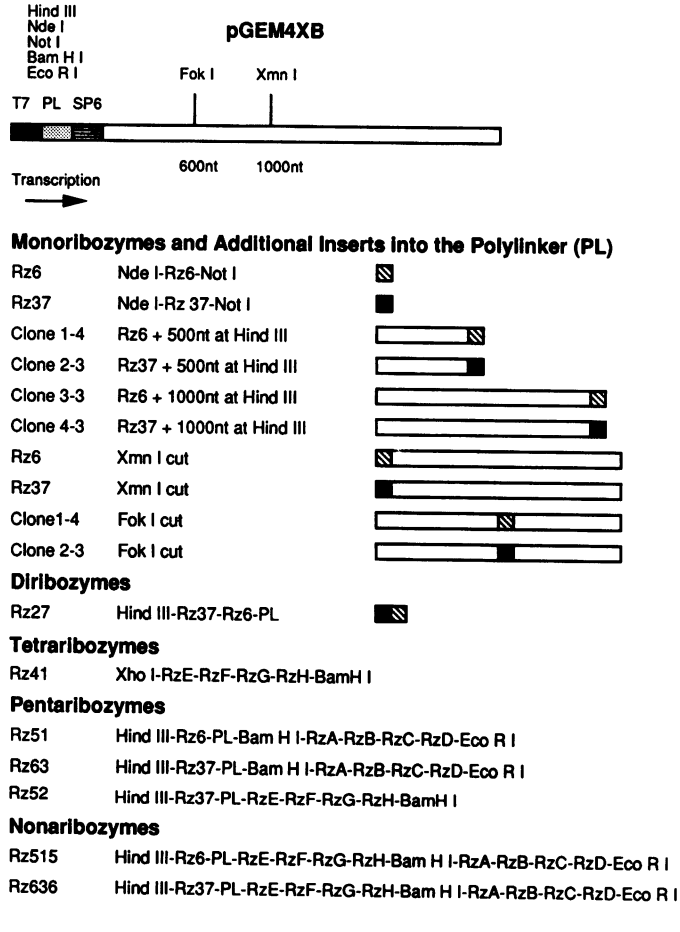

Figure 1. Ribozyme constructs. The multitarget-ribozyme DNAs were constructed by gene fusion (20) using synthetic overlapping oligonucleotides and polymerase chain reactions. The ribozymes were cloned into the polylinker site of the pGEM4XB vector (22). For clones 1-4 through 4-3, 500nt and 1000nt long extraneous sequences were inserted upstream of the ribozymes at the Hind III site. Linearization of the DNA with either EcoR I, Fok I or Hind III, respectively, allowed to generate T7 RNA polymerase runoff transcripts with the ribozyme at different positions within the RNA. The sequential order of the individual ribozymes within the multitarget-ribozymes is indicated by RzA through H as well as Rz6 and 37. They correspond to the individual selected target sites listed in Figure 2.

the typical cleavage site GUX with X being either a C, U or A (4). This triplet together with the flanking 6 upstream and 8 downstream nucleotides, respectively, were completely conserved between five different HIV-1 isolates (29). The sites are all located within short, relatively conserved domains of gp120. Each cloned ribozyme construct was expressed using T7 RNA polymerase. To study the effect of the location of the monoribozyme within a large transcript, approximately 500 or 1000bp long, unrelated sequences were placed in front of and/or behind four of the monoribozyme constructs as shown for clones 1-4 through 4-3 in Figure 1.

Specificity of multitarget-ribozymes

The monoribozymes Rz37 and Rz6, listed in Figure 1, were initially used to confirm that the optimal temperature for the cleavage reaction in vitro was about 55°C, that the reactions were relatively insensitive to NaCl and that the reactions required a relatively long time to reach completion. Increasing the ratio of ribozyme to substrate above 10 :1 did not significantly improve the rate of the cleavage reaction.

Monoribozymes were placed at various positions within a 500 or 1000nt transcript as shown in Figure 1: clones 1-4 through 4-3 and Rz6 and Rz37 with different sized runoff transcripts as indicated. In every case, increasing the size of the flanking regions of the ribozyme resulted in a significant reduction of its cleavage efficiency. We also observed a reduction in cleavage efficiency when the size of the substrate was increased to about 1kb or more. These data demonstrated that monoribozymes were most efficient when both ribozyme and substrate RNA were small molecules.

The intended target RNAs, the HIV genome and the env mRNA, however, are both large and extend to more than 9kb in length. With our antiviral strategy, however, in which a defective HIV genome itself would function as a ribozyme (see below), the RNA transcript would also be large, possibly more than 3.5kb. We were further concerned that single ribozymes against HIV would not be effective for a prolonged period of time, because HIV continuously evolves under selective pressure. It can be expected that it would be able to generate escape mutants rapidly. We therefore doubled the number of target sites and constructed a diribozyme Rz27 as shown in Figure 1 to see whether multiple sites could be targeted simultaneously. This diribozyme was about 80nt in length and consisted of Rz37 and Rz6 covalently linked. It was used to cleave a 1kb HIV env transcript and we detected a specific double cleavage of the HIV env RNA. This double digestion demonstrated, for the first time, that two individual ribozymes could be functional in a tandem configuration and that there was no apparent interference between the two cleavage sites and/or the target sites.

The two pentaribozymes Rz51 and Rz63 (Figure 1) consisting of chains of five different 38nt monoribozymes, each directed against a different site within the HIV env gp120 region were then constructed as outlined in Figures 2 and 3. Four of the five ribozymes were the same in Rz51 and Rz63 (RzA-RzB-RzC-RzD), while the fifth, Rz37 or Rz6, respectively, was different. For the generation of the substrate RNA, the HIV env gene DNA (penv5) was linearized in separate reactions with five different restriction enzymes as indicated in Figure 3, panel A. Five T7 RNA polymerase runoff transcripts were synthesized which were used as substrates. If the multitarget-ribozymes were functional, this combination of substrates should allow an evaluation of cleavage specificity and efficiency for the individual target sites.

The five undigested runoff RNA substrates, ranging in length from 178 to 1335nt, are shown in Figure 3B, lanes 1 through 5. The cleavage products from digestions with equal amounts of either one of the two pentaribozymes Rz51 or Rz63 are shown in Figure 3C. With both pentaribozymes, many of the cleavage reactions were incomplete and many of the fragments were present in both digestions. The difference in one ribozyme target site caused a slight difference in the digestion patterns. All digestion fragment sizes were consistent with the predicted fragment sizes. These data demonstrated that the multitarget-ribozymes did maintain cleavage site specificity. In addition, with both pentaribozymes, the largest substrate RNA which contained all five potential target sites was cleaved at least once, as indicated by the complete absence of undigested substrate RNA in Figure 3C, lanes 5.

The use of a nested set of substrates together with two pentaribozymes, which shared 4 out of 6 ribozymes, allowed us to evaluate the efficiency of some of the ribozymes and the target site usage. A detailed analysis of the digestion patterns surprisingly revealed, that all sites except target site C were cleaved by either one of the two pentaribozymes. All other sites were cleaved but with a slightly different efficiency. We, therefore, resequenced the target and ribozyme sequences, but

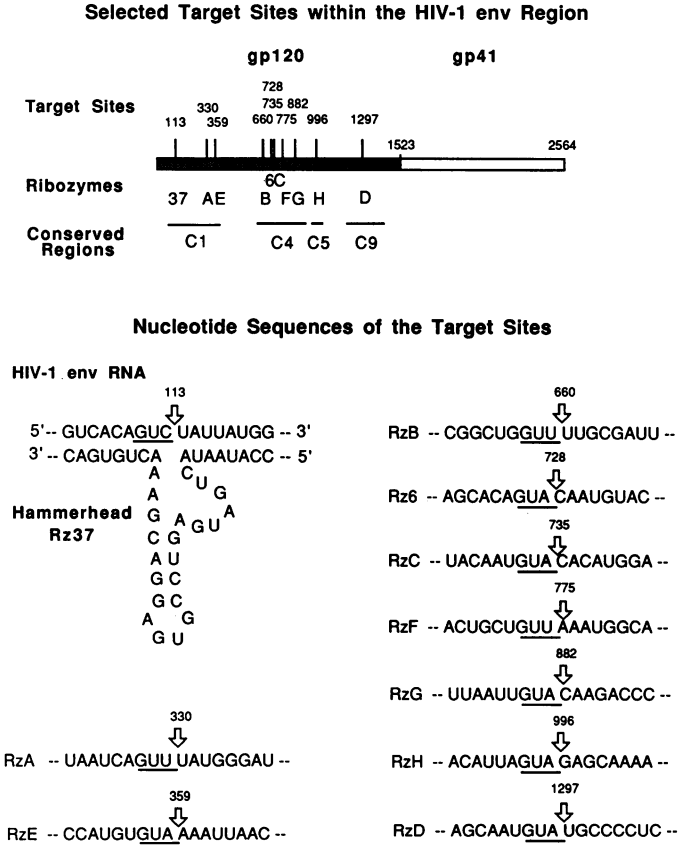

Figure 2. Selected target sites within the HIV env RNA. The sequence comparison of HIV env genes from five different virus isolates (27) was used for the selection of conserved ribozyme target sites within the gp120 region of the HIV env RNA. The ten sites are part of relatively conserved regions (C1 through C9). The precise sequences of the cleavage sites with the essential GUX triplet are listed. The complete sequence and secondary structure of Rz37 containing the conserved hammerhead ribozyme motif (4) is shown as an example. This same motif, but each motif with different targeting sequences, was repeated nine times in the case of the nonaribozymes.

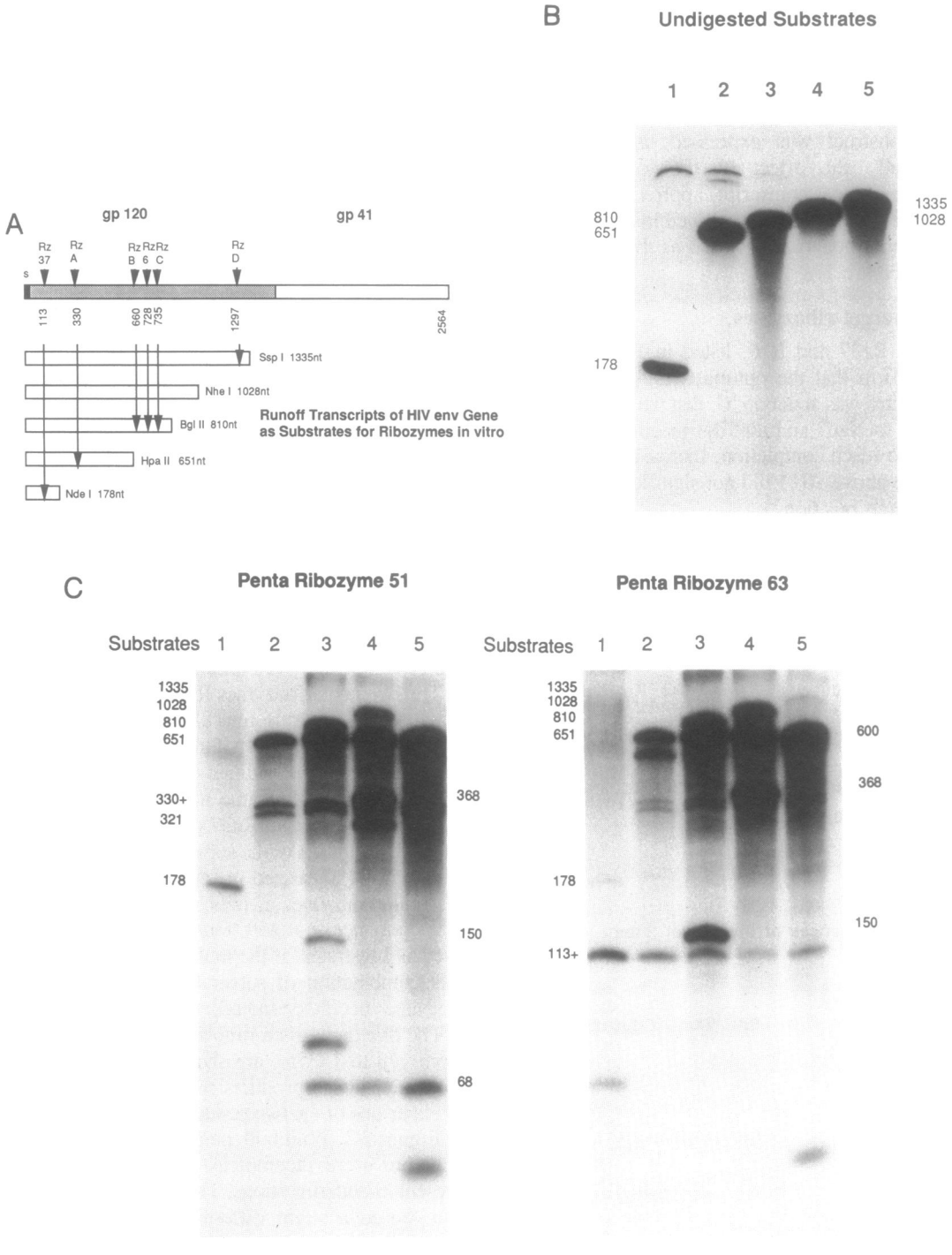

Figure 3. Specific cleavage of HIV env RNAs by pentaribozymes. HIV env DNA (23) was linearized using five restriction enzymes and a nested set of five different runoff transcripts were synthesized with T7 RNA polymerase (panel A). The undigested substrate RNAs are shown in panel B. The two pentaribozymes Rz51 and Rz63 were added in separate reactions to each of these substrate RNAs at a ratio of 1:1. In this particular experiment, the mixtures were incubated for 1 hr at 37°C and 30 min at 55°C. In most cases the cleavage reactions were carried out with a higher efficiency only at 55°C for 1 hr which resulted in approximetely the same amount of cleavage as shown here. The digestion products were separated on a polyacrylamide gel (panel C). With both pentaribozymes, the largest 1335nt substrate which contained all five potential cleavage sites was completely degraded.

we did not detect any nucleotide differences which could explain this resistance. This indicated that both pentaribozymes were functioning only as tetraribozymes. A potential intramolecular secondary structure, which could possibly be protecting the cleavage site on the substrate, was not obvious. The target site for Rz6, which was located only 7 nucleotides upstream of the site for RzC and which did partially overlap it, was efficiently used by the monoribozyme Rz6 as well as by the pentaribozyme Rz51. Since pentaribozyme Rz63 did not contain ribozyme Rz6 which was replaced by Rz37, an inhibitory effect through

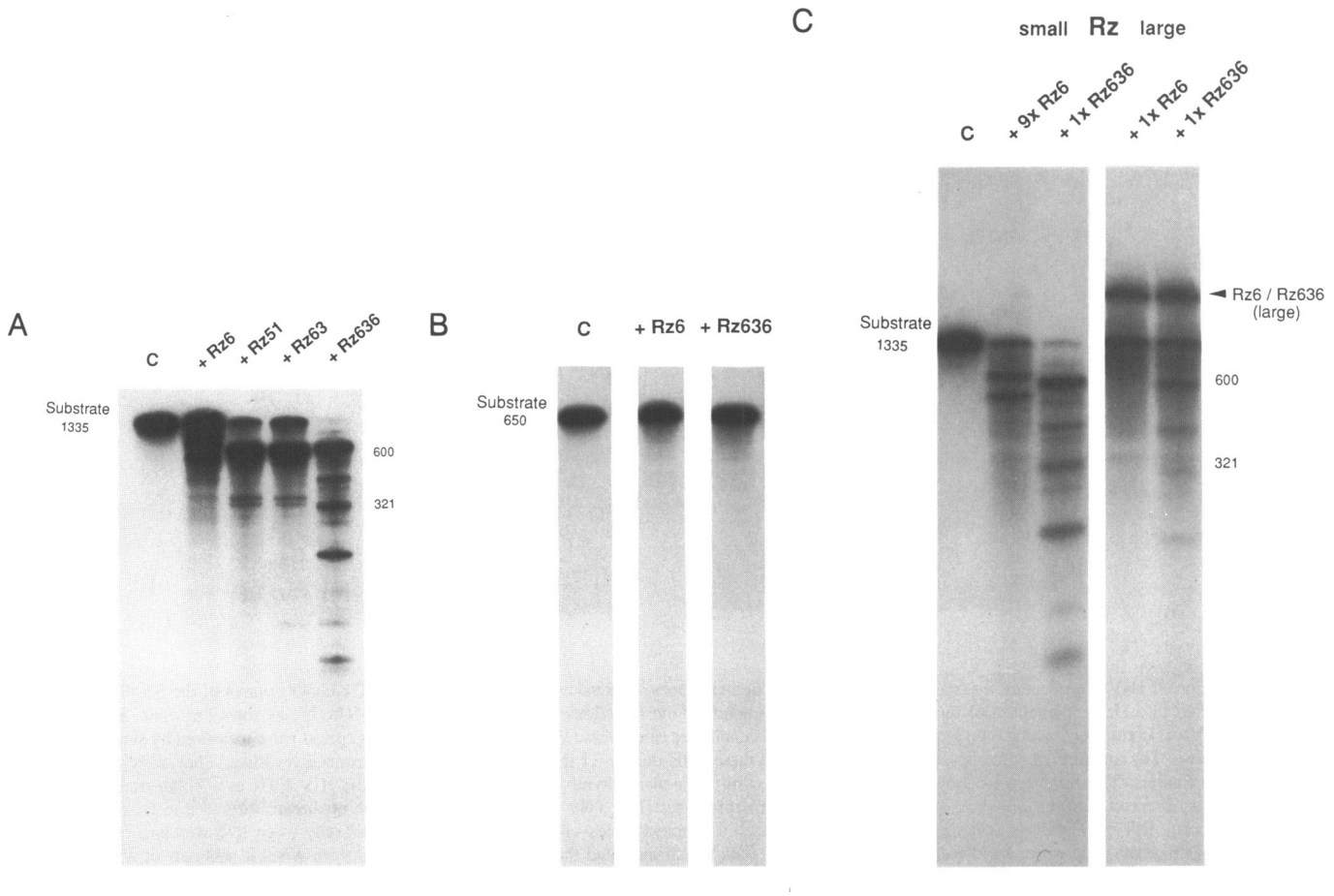

Figure 4. Cleavage specificity and efficiency of multitarget-ribozymes. The largest 1335nt substrate as shown in Figure 3, panel B, was incubated with equal molar amounts of monoribozyme Rz6, pentaribozymes Rz51 and Rz63 and nonaribozyme Rz636. Incubations were carried out for 60 min at 55°C (panel A). Panel B: A 650nt RNA transcript which does not contain a target sequence for any of the ribozymes was incubated for 1 hr at 55°C with Rz6 (60nt) or Rz636 (400nt). Panel C: Rz6 and Rz636 were expressed as small 60 and 400nt long T7 RNA polymerase transcripts, respectively. They were used to digest the 1335nt env RNA transcript. Large 2.9kb Rz6 and 3.3kb Rz636 transcripts were also transcribed. They were isotopically labeled like the substrate RNA and are visible on the gel. The amount of the small monoribozyme Rz6 was nine fold higher than the amount of the substrate RNA. All other ribozymes were added at equal molar ratios to the substrate RNA. Incubations were carried out for 1hr at 55°C.

competition between the two ribozymes Rz6 and RzC in pentaribozyme Rz51 could be excluded. The resistance to cleavage of site C even in the absence of Rz6 could not be explained at this time.

Efficiency of mono- vs. multitarget-ribozymes

The goal of our studies is the design of an antiviral ribozyme which would show a prolonged efficiency against HIV-1 in vivo and which would be effective against many if possible against all wild type HIV-1 strains. The improved efficiency of pentaribozymes suggested that ribozymes targeted to even more sites may be feasible. We, therefore, constructed two nonaribozymes (Rz636 and Rz515), designed to cleave HIV env RNA at nine different sites. A comparison of the cleavage efficiency of the same substrate by multitarget-ribozymes versus monoribozymes is shown in Figure 4A. A 1335nt long HIV-1 env substrate RNA was incubated for 30 min at 55°C and at equal molar ratios with the monoribozyme Rz6, with the two pentaribozymes Rz51 and Rz63, and with the nonaribozyme Rz636. From the digestion patterns and the amounts of undigested substrate RNA, we concluded that the digestion by the nonaribozyme was not only site specific, but it was also more efficient than with any of the other ribozymes, including the pentaribozymes. With monoribozymes as well as with multitarget-ribozymes incubations at 55°C always resulted in a faster cleavage than at 37°C. Longer incubation times were required to obtain the same extent of cleavage at 37°C.

Incubation of any of the multitarget-ribozymes with an unrelated RNA did not result in any specific or random cleavage products detectable on polyacrylamide gels, suggesting that the multitarget-ribozymes maintain sequence specificity (Figure 4B). Further experimentation will be required for the evaluation of any potential toxic effect on the cell with its diversity of different RNA species.

Multitarget-ribozymes are functional when part of a large RNA transcript

Reduction in cleavage efficiency when either the substrate RNA or the monoribozyme were part of larger RNA transcripts was observed early in these studies. The lowest efficiency was observed when both RNAs were large as shown in Figure 4C. In fact, when the monoribozyme Rz6 was expressed as a large 3kb transcript, no specific digestion products were observed after incubation with the 1335nt substrate RNA. The same Rz6, when

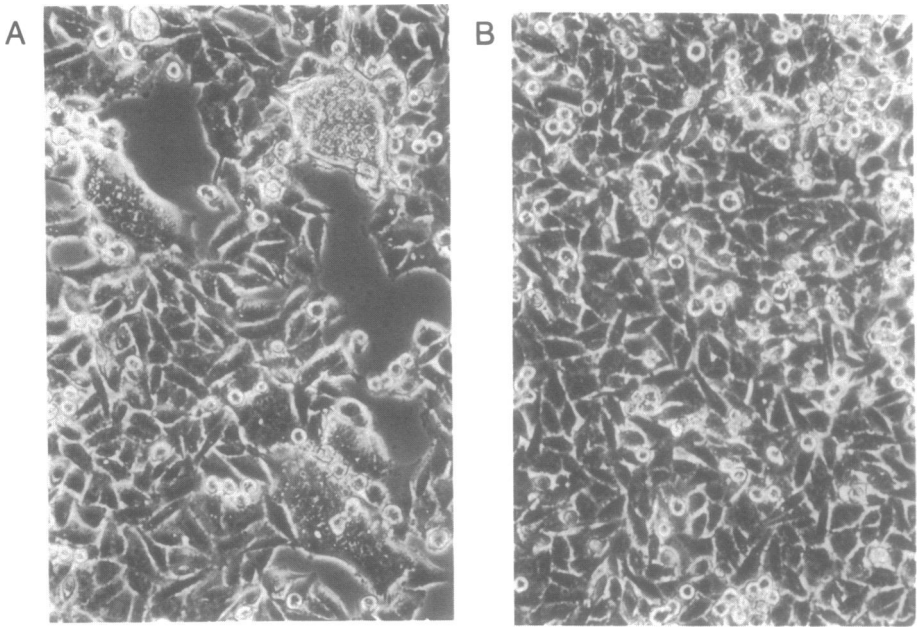

Figure 5. Inhibition of HIV-1 replication by multitarget-ribozymes. The tetraribozyme Rz41 was inserted into pEUK-C1 under control of the SV40 late promoter. It was cotransfected into HeLa T4 cells (26) together with an equal amount of the infectious HIV-1 clone pNL4-3 DNA (25) as shown in panel B. As a control an unrelated DNA was cotransfected as shown in panel A which did not contain the ribozymes. Virus replication and virus spread were measured by syncytia formation and day 4 is shown. The amounts of viral p24 antigen released from these cells during 11 days in culture were determined by Elisa. They were proportional to the extent of cell fusions. The same level of inhibition was observed with the nonaribozyme Rz636 expressed from the HIV LTR as a 3.3kb transcript. Slightly less inhibition was observed with Rz52 expressed from a beta-actin gene promoter (24). This was in contrast to the use of monoribozyme Rz6 from the HIV LTR as a 2.9kb transcript. HIV replication was not inhibited by Rz6 although it efficiently cleaved HIV-1 RNA in vitro but only when Rz6 was part of a small RNA transcript. The in vivo data, therefore, are consistent with the in vitro data which indicated that Rz6 was ineffective in vitro when it was part of a large transcript as shown in Figure 4, panel C.

expressed as a small 60nt ribozyme, efficiently cleaved the substrate when present in a nine fold excess. It also digested the substrate at a 1:1 molar ratio but somewhat less efficiently when compared with the digestion in Figure 4A. The nonaribozyme when expressed as a small 400nt transcript was highly efficient in cleaving the same substrate. In contrast to the monoribozyme, however, the nonaribozyme was still active when it was part of a 3.3kb transcript, and the same cleavage products were observed. These data demonstrate that multitarget-ribozymes can be active against large RNA substrates, even when they are also part of larger RNA transcripts.

Inhibition of HIV-1 replication

Constitutive expression of a monoribozyme directed to the HIV-1 gag region has previously been shown (5) to inhibit HIV-1 replication in tissue culture. To determine whether the HIV-1 env multitarget-ribozymes described here are able to inhibit HIV-1 replication, the infectious HIV-1 DNA clone pNL4-3 was cotransfected into HIV-1 permissive HeLa T4 cells together with a mono-, tetra-, penta- or nonaribozyme construct. Each multitarget-ribozyme was transiently expressed from a different promoter. In contrast to cotransfections with an unrelated control DNA as shown in Figure 5A, the formation of syncytia was drastically reduced by the expression of the multitarget-ribozymes. The inhibition of HIV-1 replication by Rz41 is shown in Figure 5B.

In the absence of the nonaribozymes but in the presence of the monoribozyme Rz6 expressed as a large 2.4kb transcript from the HIV-1 LTR promoter, there was no inhibition of HIV-1 replication. The majority of the cells participated in cell fusion. In each case at day four after cotransfections every viewing field contained ten or more syncytia involving ten or more cells each and p24 antigen levels were high in the supernatant. In contrast, cotransfections of the infectious DNA clone with either the plasmid DNA encoding the tetraribozyme Rz41 under control of the SV40 late promoter or the nonaribozyme Rz636 expressed as a large 3.3kb transcript from the HIV LTR, only one syncytium was observed on the average in ten viewing fields. With the pentaribozyme Rz52 expressed from the beta-actin gene promoter, approximately one syncytium per two viewing fields was observed on the average, suggesting that Rz52 expressed from the beta-actin promoter was less effective than the tetraribozyme Rz41 which contains four of the five ribozymes of Rz52 and which was expressed from the SV40 late promoter. For each culture the relative amounts of HIV-1 p24 antigen released into the medium during eleven days paralleled the frequency of cell fusion. They ranged from approximately 10% for Rz41 and Rz636 to 30% for Rz52, as compared to the total amount of p24 antigen released from the control culture without inhibition of HIV-1 replication. Peak p24 antigen values at day four were more than 40 ng/ml in the control culture as compared to 4 to 11 ng/ml in the presence of the ribozymes.

Effect of nonaribozyme expression on HIV-1 env expression

All of the ribozymes described in this study are targeted to the gp120 region of the env RNA of HIV-1. If the ribozymes were responsible for the inhibition of HIV-1 replication, there should be a substantial degradation of the env RNA in the presence of

the ribozymes. HeLa-tat-III cells (27) which permit high levels of transcription from an HIV-1 LTR promoter were cotransfected with pHenv DNA (28) which encodes the HIV-1 env target gene under control of the HIV-1 LTR promoter. A major, approximately 7kb RNA transcript and some minor RNA bands from pHenv were detected after Northern blot analysis using a specific ^{32}P labelled probe from the env region (Figure 6, lane 1). Cotransfection of the pHenv DNA with an equal amount of Rz6 DNA, which expresses Rz6 from the HIV-1 LTR promoter, resulted in a partial reduction of the total amount of env transcripts in the cell (Figure 6, lane 2). In contrast, coexpression of pHenv DNA with an equal amount of the same DNA which contained in addition the nonaribozyme Rz636, resulted in an almost complete absence of env RNA (Figure 6, lane 3). The extent of env transcript reduction is consistent with the inhibition of HIV-1 replication by Rz636. The limited reduction of env RNA was consistently observed in the presence of only the monoribozyme (lane 2). This result explains why the same vector DNA with only Rz6 does not significantly inhibit HIV-1 replication unlike the DNA containing both Rz6 and Rz636. In the presence of only Rz6, HIV-1 particles released from these cells were still infectious when permissive HeLa T4 cells were used for transfections, despite the fact that the particles would most likely carry a reduced number of env protein molecules.

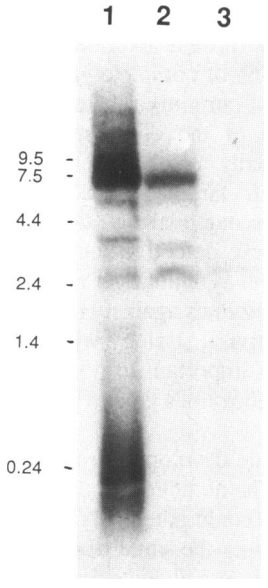

Figure 6. Absence of HIV-1 env transcripts after cotransfection with the nonaribozyme encoding plasmid. HeLa-tat-III cells (27) which efficiently support mRNA expression from the HIV-1 LTR promoter were transfected with pHenv plasmid (28) encoding the HIV-1 env mRNA. Total RNA was extracted from the cells, separated by formaldehyde gel electrophoresis, blotted onto membrane and hybridized to a specific ^{32}P labelled env DNA probe (lane 1). Lane 2: the same amount of pHenv DNA as in lane 1 was cotransfected with the same amount of a plasmid encoding the monoribozyme Rz6 under control of the HIV-1 LTR promoter. Lane 3: pHenv DNA was cotransfected with the same plasmid encoding the monoribozyme Rz6 as in lane 2 but encodes in addition the 400nt nonaribozyme Rz636.

DISCUSSION

The complete synthesis of small genes from synthetic oligonucleotides has become a reality for almost every laboratory. This technology, together with the polymerase chain reaction, allowed us to produce multitarget-ribozymes, which cleaved RNAs at multiple selected sites. The systematic search for high efficiency ribozymes has only begun within the last few years, and has focused mainly on different ribozyme motifs, target sites and flanking sequences. Observations of ribozyme action in tissue culture are still few and a potential therapeutical application of catalytic RNAs for the destruction of certain cellular or viral RNAs still requires more detailed investigations. Besides an efficient delivery system, each application also requires specific adjustments of the level of ribozyme expression, its target site selection and its cleavage efficiency in vivo.

The results described in this communication increase the options for adequately addressing these questions. They are especially important when confronting a mixture of quasi species like HIV. We have developed second generation ribozymes, multitarget-ribozymes which have highly desirable properties as compared to monoribozymes. Multitarget-ribozymes, as described here, not only maintained the target specificities of the individual ribozymes but also significantly raised the overall cleavage efficiency per catalytic RNA molecule. These data are encouraging for the successful future use of multitarget-ribozymes against cellular RNAs. For any antiviral strategy which employs ribozymes, the use of multitarget-ribozymes may be essential. It is likely to increase the chance for a prolonged effectiveness and multitarget-ribozymes could be further developed to be effective against many wild type HIV-1 variants in different cell types.

Error rates of RNA polymerases or reverse transcriptases reaching up to about 10^{-4} (16) obviously represent a serious problem for the successful use of any nucleotide sequence dependent antiviral strategy. It is highly probable that the selective pressure exerted by a single ribozyme would be overcome very fast. We earlier reported a very high frequency of mutations between different cDNA clones of vesicular stomatitis virus, a lytic negative strand RNA virus, which had been plaque purified just prior to the cDNA cloning (30). These data suggest that a virus with an altered and possibly resistant cleavage site may already exist within the virus population after a single replication cycle. It is likely to be selected. The use of multitarget-ribozymes therefore appears crucial.

Multitarget-ribozymes could be used in a gene therapy to inactivate the expression of a gene. It could, for example, be used in an intracellular immunization of stem cells (31). Multitarget-ribozymes could also be designed to target several RNAs simultaneously. At first glance our choice of the gp120 region of HIV env mRNA as the target for the multitarget-ribozymes as described here, does not seem to be the most effective way for specifically inhibiting HIV gene expression and replication. Targeting the expression of regulatory proteins, like Tat and Rev, or conserved regions within overlapping reading frames may potentially be more effective (18). It is important to note, however, that although the ribozyme target sites described here are all directed against the HIV-1 env exon, this region also represents an intron of the unspliced mRNA precursors for the regulatory proteins Tat and Rev. Their expression could also be affected if the ribozymes are functional in the nucleus. Future detailed experimentation will be required to distinguish the effects of the ribozymes on the levels of individual mRNAs like for env, tat and rev, but also on the level of HIV-1 genomic RNA itself. They could all contribute to the inhibitory effect of the multitarget-ribozymes on HIV-1 replication as demonstrated in Figure 5. Our

reason for choosing the gp120 region as the target was to selectively inhibit Env protein expression and to decrease the synthesis and packaging of complete genomic RNA, which are both important parts of our antiviral strategy. The less impaired continued expression of the other structural and regulatory proteins may potentially provide helper functions to allow propagation of novel, targeted defective interfering HIV-1 particles. In fact, it is our goal that the approximately 3.5 kb RNA genomes of these defective interfering HIV-1 particles themselves will function as multitarget-ribozymes. Therefore, the use of multitarget-ribozymes against HIV-1 has to be viewed in the context of this antiviral strategy which will be described in detail elsewhere. It is important to note that this strategy does not require that the ribozymes cleave every single target RNA to completion.

The largest ribozyme developed in our laboratory contained nine different units. The number of individual ribozymes within multitarget-ribozymes could potentially be further increased. On the other hand, repeating the same ribozyme motif many times within the same RNA is less likely to result in a dramatic increase in cleavage efficiency. This increased cleavage efficiency of multitarget-ribozymes may in part be the result of a cumulative efficiency of all individual ribozymes. A potential increased accessibility of the multitarget-ribozymes for the substrate may explain why multitarget-ribozymes were still active when they were part of a large RNA transcript. Additional studies are required to evaluate any potential effect the sequential order of the monoribozymes within the multitarget-ribozyme might have on cleavage efficiency.

The proposed use of multitarget-ribozymes against a retrovirus as described here, should help maintain effectiveness for a longer period of time. It will be less likely that the population of viral RNAs will escape from all potential cleavages at once. It is currently unknown, however, how long this time might be and how much time a potential stepwise selection might require. The search for more efficient ribozymes which could be combined into such multitarget-ribozyme constructs, may in the future significantly contribute to the length of time they will be effective against HIV.

The ten conserved target sites were originally chosen based on the sequence information from only five HIV-1 isolates (29), however, within the gp120 region not many more conserved sites were available. A comparison of the target sequences of all, approximately thirty HIV-1 isolates is shown in Table 1. The number of nucleotide changes each isolate carries within each of the ten target regions are listed. Surprisingly, most of the ribozymes described here would be effective against every North American isolate presently sequenced. The effects of some of these changes on multitarget-ribozyme activity are indicated. Any ribozyme activity which can be expected to be completely abolished by the changes are marked by boxes. These changes would affect the essential GU cleavage site directly. Changes which are listed in parenthesis do not affect activity. All other nucleotide changes can be expected to affect ribozyme activity to some extent, however, it is currently unknown by how much ribozyme activity would be decreased, if at all. It is possible that some of the mismatches may actually increase ribozyme activity. With the more distantly related African HIV-1 isolates, the majority of the viral RNAs would still be cleaved by at least two of the ten ribozymes described here and possibly by one or two more ribozymes. These comparisons are encouraging for the potential effective use of multitarget-ribozymes against different

Table 1. Effect of nucleotide differences in HIV-1 target regions on multitarget-ribozyme activity.

	Virus Isolate	Rz37	RzA	RzE	RzB	Rz6	RzC	RzF	RzG	RzH	RzD	Conserved
North America	HIVNL43	-	-	-	-	-	-	-	-	-	-	10
	HIVLAI	-	-	-	-	-	-	1	-	-	-	9
	HIVJH3	-	-	-	-	-	-	-	-	2	-	9
	HIVSC	-	-	-	5	-	-	1	1	-	-	7
	HIVALA1	-	-	-	-	-	-	-	-	-	-	10
	HIVBRVA	-	-	-	-	-	-	1	-	2	-	8
	HIVMFA	-	-	-	-	-	-	-	-	-	-	10
	HIVMN	-	-	-	-	-	-	-	-	-	-	10
	HIVADA	(1)	-	-	-	-	-	-	-	1	-	8
	HIVJRFL	-	-	-	-	-	-	1	-	-	-	9
	JRCSF	-	1	-	-	-	-	1	1	1	1	5
	HIVBAL1	-	-	-	-	-	-	-	-	1	-	9
	HIVHXB2	-	-	-	-	-	-	-	-	-	-	10
	WMJ22	-	-	-	-	-	-	-	-	-	-	10
	HIVJFL	-	-	-	-	-	-	2	-	3	-	8
	HIVRF	-	-	-	-	-	-	-	-	2	-	9
Africa	HIVNY5	-	-	-	-	1	-	1	-	1	-	7
	SF162	-	-	1	-	-	-	-	-	2	-	8
	HIVSF2	(1)	-	-	-	-	-	-	1	1	-	7
	HIVHAN	-	-	-	1	1	1	-	-	1	-	6
	HIVSF33	(1)	1	-	-	-	-	-	-	-	-	8
	CDC42	-	1	1	3	-	-	-	-	1	1	5
	HIVOYI	-	-	-	-	-	-	-	4	2		9
	HIVELI	(1)	-	-	1	-	-	-			2	5
	HIVZ2Z6	1	-	-	2	-	-	-	2	6		6
	HIVNDK	(1)	-	-	2	-	-	-	1	3		6
	HIVJY1	(1)	-	-	2	1	-	2	1	5	1	3
	HIVU455	2	1 + (1)	1	1	-	-	-	3	6	-	4
	HIVMAL	1 + (1)	-	2	2	1	-	1	1	6	1	3
	HIVZ321	2	-	1	1	1	1	1	3	5	-	2

Ribozyme inactivated (1) silent - conserved

The nucleotide sequences of thirty HIV-1 isolates from North America and Africa were provided by Bette Korber and Alan Perelson at the AIDS and Human Retrovirus Database and Theoretical Biology and Biophysics Group, Los Alamos National Laboratory. The sequences were compared with those of the ten ribozyme target sites. The selection of conserved target sites was initially based on sequence information from five HIV-1 isolates (27). Sequence differences which can be expected to inactivate a single ribozyme target activity are boxed. Nucleotide differences which can be expected to have no effect on activity are listed in parenthesis. With all other differences the effect is uncertain but differences may not necessarily lead to an inhibition of ribozyme activity, in contrary, the activity could in some cases even be increased. From all selected sites RzH showed the greatest heterogeneity even among the North American isolates. Most chosen sites were surprisingly well conserved especially with all North American isolates and to a lesser degree with the African isolates.

wild type HIV-1 strains. The data suggest that a further increase in the number of conserved target sites together with their combined multitarget-site activities may allow to develop an 'inescapable' multitarget-ribozyme which may be able to withstand the challenge of every wild type strain.

The use of multitarget-ribozymes against cellular RNAs which contain highly conserved target sequences may prove to be the method of choice, since it will most likely be the more effective per RNA copy as compared to antisense or monoribozymes. It can also be anticipated that the random choice of multiple target sites and the synthesis of multitarget-ribozymes from synthetic oligonucleotides may prove to be faster and more effective in destroying a specific cellular RNA than evaluating the efficiency of every single target site in vivo.

The inability of the multitarget-ribozyme to cleave a 650nt unrelated RNA at optimal incubation conditions in vitro is encouraging for the potential future use of multitarget-ribozymes in vivo. Until now, there is a lack of evidence describing a toxic effect of ribozymes on cells. The population of cellular RNAs,

however, consists of several thousand unique transcripts which all differ in abundance depending on cell type, the state of cell differentiation, etc. Therefore, the remote possibility that ribozymes could be toxic to a particlular cell still has to be evaluated carefully. This will also be the case for any antisense approach. Unlike the specific cleavage of target RNAs by ribozymes as demonstrated in vitro, the mechanism(s) of antisense effects which are only found intracellularly are less clear (32). The question of whether the multitarget-ribozymes could have potentially inhibited HIV-1 replication through antisense effects rather than through ribozyme cleavage was not answered here. Mutational changes of the ribozymes would be required which retain antisense sequence but abolish ribozyme activity. Since the individual ribozyme motifs within the multitarget-ribozyme are not targeting adjacent sites there is no increase in the size of any continuous antisense sequence. The longest continuous antisense sequence is only 8 nucleotides long for the monoribozyme as well as the multitarget ribozyme described here. It is unlikely that an RNA duplex of 8 bp in length would be able to induce antisense effects because such an interaction would have a low specificity and would most likely be detrimental to the cell itself. We conclude that the multitarget-ribozymes as described here can be highly effective in vivo. The use of multitarget-ribozymes is especially important and probably essential for the targeting of any RNAs which are continuously changing, like the RNA genome of HIV-1.

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