The subcellular localization and length of hammerhead ribozymes determine efficacy in human cells

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Received October 24, 1996; Revised and Accepted December 16, 1996

ABSTRACT

The length requirements of the antisense portion of hammerhead ribozymes for efficacy in living cells was investigated. The HIV-1 tat-directed asymmetric hammerhead ribozyme αYRz195 was used with a 195 nt 3'-antisense arm and a 3 nt 5'-antisense portion as well as a set of successively 3'-shortened derivatives thereof. In the 3'-antisense arm a minimum length of 20 complementary nucleotides was required for efficient association with a 645 nt target RNA transcript in vitro (for all constructs $k_{ass}$ ranged between 0.3 and $1.8 \times 10^4$/M/s). The cleavage rate constants ($k_{cleav}$) were independent of the length of the antisense flank and ranged between 0.8 and $1.2 \times 10^{-4}$/s. However, the length of the antisense arms, as well as the mode of delivery and the subcellular location of the ribozymes, had a dramatic effect on efficacy in HIV-1-producing human cells. When proviral HIV-1 DNA and ribozymes were co-microinjected into the nucleus of human cells, a minimum length of 51 nt in the antisense arm was necessary for antisense- and ribozyme-mediated inhibition of HIV-1 replication. Ribozymes with shorter antisense arms were almost ineffective. Conversely, short chain ribozymes, including those with chemical modifications, were superior to long chain ribozymes when co-microinjected into the cytoplasm. When transfected, all ribozymes showed an antisense effect as well as an additional ribozyme-mediated increase in inhibition. Consequences for the design and application of ribozymes are discussed.

INTRODUCTION

Hammerhead ribozymes, originally found in some subviral plant pathogens (1), have been used successfully to inhibit gene expression and viral replication in prokaryotic and eukaryotic cells (2–6). When designed to cleave a target RNA in trans, hammerhead ribozymes usually consist of two antisense stretches on either side of a catalytic domain of 22 nt (7,8). Inhibition is thought to consist of at least two steps, binding of the ribozyme to the target RNA via its antisense sequences and, subsequently, site-specific hydrolysis of a phosphodiester bond 3' of the cleavable motif NUX (9–12). For a complete catalytic cycle, dissociation of the cleaved products has to occur before the ribozyme can enter another cycle. For efficient association as well as fast dissociation in vitro, the optimal length of the antisense flanks was estimated to be in the range 6–8 nt on either side of the catalytic domain (13,14). Much longer antisense flanks are assumed to prevent the dissociation step, resulting in only stoichiometric cleavage of the target. Surprisingly, when hammerhead ribozymes with antisense flanks of several hundred nucleotides were applied in living cells, the catalytic activity significantly improved the antisense-mediated inhibitory effects (15,16). However, several studies suggest that there is a significant influence of the length of antisense portions of hammerhead ribozymes on the inhibitory activity (17–20). Thus, the optimal length of antisense flanks for ribozyme-mediated inhibition in living cells may differ from predictions derived from in vitro studies.

Further, the co-localization of the ribozyme with its target, i.e. the subcellular distribution of ribozymes, is assumed to be crucial for efficacy. Hammerhead ribozymes can be delivered to living cells either endogenously by introduction of recombinant ribozyme coding genes or exogenously by transfection or simply by the addition to the cell culture medium, as with antisense oligonucleotides (3). In the latter case, chemically modified ribozymes are generally used to substantially increase the stability and efficacy of ribozymes in the cell (6, 21, 22). The size of chemically synthesized ribozymes is limited, however, to less than ~50 nt. Therefore, the size of the antisense flanks is restricted to 14 nt or less on either side of the catalytic domain.

In this work, we used conventional symmetric as well as asymmetric hammerhead ribozymes to systematically analyze the length requirements of the antisense flanks of hammerhead ribozymes for efficient association and cleavage in vitro as well as for efficacy in human cells. To address the influence of subcellular localization on efficacy against HIV-1, the RNAs
Figure 1. Schematic depiction of HIV-1 target sequences with functional elements (top) and sequences of hammerhead ribozymes used in this work. The numbering is according to Ratner et al. (48). (Lower right) The tat/rev coding region was targeted by asymmetric hammerhead ribozymes with successively shortened 3'-antisense flanks. The ribozymes were named according to the length of the 3'-antisense flank, which ranged from 195 nt in αYRz195 to 20 nt in αYRz20. Cleavage of the target occurs 3' of a GUC triplet between positions 5597 and 5598, as indicated by an arrowhead. The Sall-Kpnl restriction fragment was used as a template for transcription of the target SR6 RNA for in vitro studies (24). The complex between ωYRz60 and the complementary HIV-1 sequence stretch with helices I, II and III is also shown. The arrow points to the G12 deletion in all inactive ribozymes. (Lower left) The 5'-leader/gag region of HIV-1 was targeted by the long chain ribozyme 2as-Rz12 (15) and by short armed symmetric chemically synthesized ribozymes with 12, 10 or 7 nt respectively on either side of the catalytic domain. Cleavage occurs 3' of a GUA triplet at nt 505, as indicated by an arrowhead.

In vitro transcription of RNA

For the in vitro synthesis of 2as-Rz12, plasmid pBS-Rz12 (15) was linearized with SacI, followed by trimming the ends with T4 DNA polymerase. The plasmids ωYRz195† and ωYRz195− (16) were linearized with EcoRI for the in vitro synthesis of αYRz195 and αYRz195− respectively. For the synthesis of the shorter ribozyme αYRz60† and the control αYRz60− the same plasmids were linearized with DdeI. For the synthesis of ωYRz20/25/31/35/41/45/51 and the respective inactive ribozymes, PCR-generated templates were used directly for transcription of RNA in vitro. Catalytically inactive ribozymes were obtained by deletion of G12 in the catalytic core. For the synthesis of SR6 RNA, used as the HIV-1 target for the determination of cleavage and association rates in vitro, NotI-linearized plasmid pRC-CMV-SR6 was used (24). Seven micrograms of template DNA were incubated in a reaction mixture containing 18 mM Na2HPO4, 2 mM NaH2PO4, 5 mM NaCl, 20 mM diethanol, 8 mM MgCl2, 4 mM spermidine and 1 mM NTPs in a total volume of 300 μl. Reactions were started by adding 60 U T3 RNA polymerase in the case of linearized templates or T7 RNA polymerase when the PCR-generated templates were used. After 2 h incubation at 37°C the reactions were stopped by adding 200 μl 20 mM MgCl2 and 40 U DNase I. After a further incubation for 20 min at 37°C, the solutions were extracted with phenol and RNAs were precipitated by adding a 1/10 vol. of 3 M sodium acetate and a 2.0x vol. of ethanol.

were microinjected into the nucleus or cytoplasm of SW480 cells or transfected together with infectious proviral HIV-1 DNA followed by quantification of virus production.

MATERIALS AND METHODS

Plasmids and synthesis of DNA templates for in vitro synthesis of ribozymes

Plasmid pBS-Rz12 was used for the in vitro transcription of ribozyme 2as-Rz12 (Fig. 1, left side), in which the hammerhead domain has been inserted into the HIV-1-directed antisense RNA 2as (15). The plasmids ωYRz195† and ωYRz195− (deletion of G12 in the catalytic core; numbering according to 23) were described recently (16). The templates for the in vitro transcription of shortened derivatives of ωYRz195 and ωYRz195− respectively (Fig. 1, right side) were generated by PCR. The 5'-DNA primer oligonucleotide for PCR contained a T7 promoter sequence (5'-GCAGATCTCGAGTAATACGACT- CACATAGGGAACAAAAGCTTATCTC-3'). The sequences of the 3'-DNA primer oligonucleotides for PCR (20mers) for the successively shortened hammerhead ribozymes correspond to the last 20 nt of the respective 3'-antisense arm and can be derived from Figure 1.
pellet was dissolved in 10 mM Tris–HCl, pH 8.0, 1 mM EDTA and the RNAs were further purified by chromatography with Sephadex G-50 using the same buffer.

Chemical synthesis of short symmetric hammerhead ribozymes

The short chain gag-directed symmetric ribozymes Rz12x12+ and Rz12x12−, where guanosine at position 5 was replaced by adenine, and Rz7x7+ were chemically synthesized as described (25). In ribozymes Rz10x10mod all cytidines and uridines were replaced by their 2′-fluoro derivatives but uridine at position 4 was 2′-aminoanduridine and uridine at position 7 adenosine. The last internucleotide phosphate at the 5′-end and the last three at the 3′-end were phosphorothioates, as described previously (25).

Determination of association rate constants in vitro

For the determination of association rate constants, ribozymes and the complementary target RNA SR6 were annealed essentially under the conditions described previously (15). Briefly, the annealing reactions with 32P-labeled ribozymes and unlabeled target RNA SR6 were performed in a total volume of 30 μl in a buffer containing 100 mM NaCl, 20 mM Tris–HCl pH 7.4, 10 mM MgCl2 at 3 °C ribozyme and 20–100 nM SR6 (pseudo first order kinetics). Aliquots (3 μl) were withdrawn from the reaction mixtures and added to 30 μl pre-cooled (0°C) stop buffer (50 mM Tris–HCl, pH 8.0, 25 mM EDTA, 8 M urea). The single-stranded and double-stranded fractions were separated on 5% polyacrylamide gels containing 8 M urea in a Tris-borate EDTA buffer (89 mM Tris-borate, pH 8.3, 25 mM EDTA). The temperature was kept below 25°C during electrophoresis. Under these experimental conditions, RNA–RNA double strands of 16 bp or more remained stable (Homann, unpublished results). The amounts of RNA contained in bands representing the single-stranded ribozyme or the duplex were quantified using a PhosphorImager (Molecular Dynamics) and were used to calculate the association rate constants (kass).

Measurement of ribozyme activity in vitro

The ribozyme cleavage assays were performed under single turnover conditions as described recently (15). Briefly, the ribozymes (20 nM) were incubated with 32P-labeled target RNA SR6 (2 nM) at 37°C for 15 h to ensure complete annealing in a solution containing 100 mM NaCl, 20 mM Tris–HCl pH 7.4. The cleavage reaction was started by the addition of Mg2+ to a final concentration of 10 mM. Aliquots were withdrawn at different time points and added to stop buffer (see above). The reaction products were heated to 96°C for 5 min and separated in polyacrylamide gels containing 8 M urea. From quantification of the time-dependent decrease in substrate we derived the cleavage rate constants (kcleav) according to a single exponential decay.

Target sequences for hammerhead ribozymes

Ribozymes were directed against the 5′-leader/gag portion of HIV-1 or the tat/rev coding exon respectively (Fig. 1). The gag-directed ribozymes were "symmetric", i.e. both antisense arms had the same length in the case of the short chain constructs Rz7x7+, Rz10x10mod, Rz12x12+ and Rz12x12− and >100 nt for the long chain ribozyme 2as-Rz12 (Fig. 1). The tat/rev-directed ribozymes were derived from αYRz195+ (16), which was designed asymmetrically, i.e. when complexed with the target helix I consisted of only 3 bp whereas helix III almost exclusively contributed to binding of the target (Fig. 1).

HIV-1 inhibition studies

Replication of HIV-1 was measured by co-microinjection of in vitro synthesized ribozymes together with infectious proviral HIV-1 DNA pNL4-3 (26) into the nucleus or the cytoplasm of SW480 cells as described (27,28). SW480 cells were grown on glass coverslips (10 mm diameter) in DMEM medium supplemented with 10% fetal calf serum. The DNA and RNA was dissolved in 10 mM Tris–HCl, pH 8.0, 1 mM EDTA at concentrations of 10 ng/μl pNL4-3 and 70 ng/μl RNA. One day after microinjection, 2 × 105 MT-4 cells were added and the total volume was adjusted to 1 ml with RPMI medium supplemented with 10% fetal calf serum. Virus production was measured 5 days post-injection and quantified by a commercial p24 ELISA (Organon). For each ribozyme, 50 cells were microinjected and the experiment was repeated six to nine times. As a positive control, we used a cat RNA transcript (850 nt), tRNAbulk or the HIV-1 tat-derived ‘sense’ RNA se100, a 100 nt long sense transcript (29), respectively. Replication in the presence of cat RNA was set to 100%.

Ribozymes were also co-transfected together with the proviral DNA pNL4-3 as described (15). Briefly, in vitro synthesized RNA was transfected together with 40 ng pNL4-3 into SW480 cells grown semi-confluent (~5 × 103 cells) in 48-well plates by calcium phosphate co-precipitation (30). One day after transfection, 2 × 105 MT-4 cells were added and production of HIV-1 was measured as described above.

RESULTS

Association and cleavage rates in vitro

The tat/rev-directed hammerhead ribozyme (16) is characterized by an asymmetric design of the antisense arms that flank the catalytic domain (Fig. 1, lower right panel). Whereas the 3′-antisense region consists of only three complementary nucleotides, the 3′-terminal region of the largest ribozyme αYRz195+ contains 195 nt. Leaving the 5′-antisense region and the catalytic domain unchanged, derivatives of αYRz195+ were made with truncated 3′-antisense flanks of 60, 51, 45, 41, 35, 31, 25 and 20 nt (Fig. 1). For each of these RNA, we determined the rate of association (kass) with the target RNA SR6 (645 nt) and its rate of cleavage (kcleav). The association ranged between 1.8 × 106 M/s, whereas the intermediate chain lengths (αYRz20+ and αYRz25+) showed relatively fast annealing (kass = 1.5 × 107/M/s), whereas the intermediate chain lengths (αYRz25+ and αYRz41+) annealed ~3- to 4-fold slower (Table 1). For each of the above mentioned constructs, a catalytically inactive control RNA was prepared in which nucleotide G12 (23) was deleted (see Fig. 1, arrow). This deletion had no influence on the association rates in vitro (data not shown).
Table 1. Association ($k_{ass}$) and cleavage ($k_{cleav}$) rate constants for short chain ribozymes and αYRz195-derived ribozymes\(^a\)

<table>
<thead>
<tr>
<th>Ribozyme</th>
<th>Length of 3'antisense arm (nt)</th>
<th>$k_{ass}$ (per M/s)</th>
<th>$k_{cleav}$ (per s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>αYRz20(^+)</td>
<td>20</td>
<td>$0.3 \times 10^4$</td>
<td>$0.4 \times 10^{-4}$</td>
</tr>
<tr>
<td>αYRz25(^+)</td>
<td>25</td>
<td>$0.3 \times 10^4$</td>
<td>$0.5 \times 10^{-4}$</td>
</tr>
<tr>
<td>αYRz31(^+)</td>
<td>31</td>
<td>$1.4 \times 10^4$</td>
<td>$0.8 \times 10^{-4}$</td>
</tr>
<tr>
<td>αYRz35(^+)</td>
<td>35</td>
<td>$0.5 \times 10^4$</td>
<td>$1.0 \times 10^{-4}$</td>
</tr>
<tr>
<td>αYRz41(^+)</td>
<td>41</td>
<td>$1.5 \times 10^4$</td>
<td>$0.9 \times 10^{-4}$</td>
</tr>
<tr>
<td>αYRz45(^+)</td>
<td>45</td>
<td>$0.4 \times 10^4$</td>
<td>$1.2 \times 10^{-4}$</td>
</tr>
<tr>
<td>αYRz51(^+)</td>
<td>51</td>
<td>$1.6 \times 10^4$</td>
<td>$1.1 \times 10^{-4}$</td>
</tr>
<tr>
<td>αYRz56(^+)</td>
<td>60</td>
<td>$1.6 \times 10^4$</td>
<td>$1.2 \times 10^{-4}$</td>
</tr>
<tr>
<td>αYRz195(^+)</td>
<td>195</td>
<td>$1.8 \times 10^4$</td>
<td>$1.2 \times 10^{-4}$</td>
</tr>
<tr>
<td>Rz7x7(^+)</td>
<td>7(^b)</td>
<td>n.d.(^c)</td>
<td>$0.5 \times 10^{-4}$</td>
</tr>
<tr>
<td>Rz10x10mod</td>
<td>10(^b)</td>
<td>n.d.(^c)</td>
<td>$0.1 \times 10^{-4}$</td>
</tr>
<tr>
<td>Rz1x12(^+)</td>
<td>12(^b)</td>
<td>n.d.(^c)</td>
<td>$0.5 \times 10^{-4}$</td>
</tr>
</tbody>
</table>

\(^a\)Rate constants were determined at 37°C. The substrate RNA was SRK6 (24) for αYRz195-derived ribozymes and 2s (27) for Rz7x7\(^+\), Rz10x10mod and Rz1x12\(^+\).

\(^b\)Same length as 5'antisense arm.

\(^c\)n.d., not determined.

The cleavage rate constants for all ribozymes were determined under single turnover conditions (15). The values of $k_{cleav}$ were not correlated with the length of the 3'-antisense arm and ranged between 0.4 and $1.2 \times 10^{-4}$/s (Table 1). The factor of two to three between the $k_{cleav}$ values is almost within the error range of the measurements. These relatively slow cleavage rates were similar to those found for other long chain ribozyme-substrate complexes and seem to reflect a slow step after annealing and before product release (reviewed in 31).

**Efficacy on viral replication is dependent on the size of the antisense arms**

The influence of the length of the antisense arm of derivatives of αYRz195\(^+\) on the extent of inhibition of HIV-1 replication was measured in two different ways: by microinjection and by calcium phosphate co-precipitation. First, in vitro active and inactive αYRz195-derived ribozymes were microinjected together with proviral DNA into the nucleus of human SW480 cells. Regardless of whether catalytically active or not, essentially no inhibition of HIV-1 replication occurred with ribozymes with a 3'-arm of 45 nt or shorter (data summarized in Fig. 2). A weak reduction of virus production (46%) was observed with the in vitro inactive ribozyme αYRz51\(^-\) and virus production was further 2.5-fold decreased with the in vitro active ribozyme αYRz51\(^+\) (Fig. 2). A stronger antisense-mediated inhibition was observed with the inactive ribozyme αYRz60\(^-\) (31% virus production) and this inhibition was 5-fold increased with the active ribozyme αYRz60\(^+\) (7% virus production). The additional ribozyme-mediated inhibition was thus stronger than for the less inhibitory ribozyme αYRz51\(^-\). The ribozyme-mediated increase in antisense effects could only be observed if a significant antisense-mediated inhibition occurred; the antisense effect seemed to be necessary for the additional ribozyme effect.

The same set of ribozymes and proviral HIV-1 DNA was used with a different technique for delivery: transfection into human SW480 cells by calcium phosphate co-precipitation. Significant inhibition of HIV-1 was observed with all constructs (Fig. 3). The antisense effect observed with catalytically inactive constructs was strongly increased by the additional cleavage capability (Fig. 3). For most constructs, except for αYRz45\(^-\), the antisense effect, as well as the ribozyme-mediated increase in inhibition, was dependent on the dosage of RNA (Fig. 3A and B). An increase in the amounts of RNA by a factor of 1.75 enhanced the antisense effect by a factor ranging between 15 (αYRz20\(^-\)) and 2.5 (αYRz35\(^-\)), as well as the total extent of inhibition. However, in contrast to nuclear microinjection, the shortest ribozyme showed the strongest antisense effect (αYRz20\(^+\)), leading to almost complete inhibition. Longer ribozymes seemed to be less effective. For example, αYRz60\(^+\) was the weakest inhibitor at 160 ng RNA (Fig. 3B). Based on these findings, however, a general correlation between length and efficacy cannot be derived.

**Different inhibition after microinjection into nucleus versus cytoplasm**

In view of the above described different inhibition potentials of ribozymes with long and short helix III-forming regions, depending on the mode of delivery, we decided to compare direct microinjection into the nucleus with into the cytoplasm. In addition to the tatrev-directed ribozyme αYRz195\(^+\), we also used ribozymes directed against the 5'-leader/gag region of HIV-1. This included the long chain ribozyme 2as-Rz12 (Fig. 1; 15) and the short symmetric ribozymes Rz1x12\(^+\), Rz7x7\(^+\) and Rz10x10mod, which recognize the same target motif but contain only 12, 10 or 7 nt in each of their symmetric antisense arms (Fig. 1).

When directly delivered into the nucleus, both long chain ribozymes 2as-Rz12 and αYRz195\(^+\) respectively showed ~99%
The subcellular localization and length of chain ribozyme Rz12x12+ as well as the inactive control microinjected into the cytoplasm, weak inhibition was observed inhibition (Fig. 4). Conversely, when the same constructs were microinjected into the cytoplasm, weak inhibition was observed with ribozyme αYRz195+, with no significant inhibition with 2as-Rz12 and the antisense control αYRz195− (Fig. 4). The short chain ribozyme Rz12x12+ as well as the inactive control Rz12x12− completely abolished production of HIV-1 when microinjected into the cytoplasm, but did not show any inhibition when directly delivered into the nucleus (Fig. 4).

At the high RNA concentrations used in this experiment (70 ng/μl), no differences could be seen between the short inactive Rz12x12− and the catalytically active ribozyme Rz12x12+, because virus production was suppressed to background levels. Therefore, the ribozymes were injected at lower RNA concentrations of 10 and 0.1 ng/μl respectively. In addition, we tested two homologous constructs of Rz12x12+ with antisense arms further shortened to 7 nt on either side, termed Rz7x7+ (Fig. 1), and the chemically modified ribozyme Rz10x10mod.

When these short chain ribozymes were microinjected into the cytoplasm, a dosage dependence of inhibition of HIV-1 was observed. Under conditions that did not show any antisense effect (Rz12x12− at 0.1 and 10.0 ng/μl; Fig. 5), a modest reduction in HIV-1 was measured with the active ribozymes Rz12x12+ and Rz10x10mod at 0.1 ng/μl RNA (Fig. 5). At increased RNA concentrations (10.0 ng/μl), virus production was 21% (Rz12x12+) to 10% (Rz10x10mod). In either case, Rz12x12+ showed stronger inhibition than Rz7x7+, indicating that shortening to as little as 7 nt decreases inhibition. The chemically modified ribozyme inhibited at least equally as well as Rz12x12+ (Fig. 5). Neither Rz7x7+ nor Rz10x10mod showed any inhibition when microinjected into the nucleus at the highest RNA concentration of 70 ng/μl (data not shown).

**DISCUSSION**

To systematically study the influence of the length of helix III on association and cleavage rates in vitro and inhibitory activity in living cells, the antisense arm of αYRz195+ was systematically shortened from 60 to 20 nt. A set of tat/rev-directed ribozymes with antisense flanks of 60, 51, 45, 41, 35, 31, 25 and 20 nt was constructed and a minimum length of the 3′-antisense arm of 51 nt was found to be required for antisense-mediated inhibition of HIV-1 replication when microinjected into the nucleus (−50% virus production) with the catalytically inactive ribozyme αYRz51−. This inhibition was further increased with the active ribozyme αYRz51+ (Fig. 2). The minimum length required for inhibition of −50% does not correlate with association rates determined in vitro, which were comparable with those for...
shorter 3'-antisense flanks (Table 1). The HIV-1 inhibition data show that the mode of ribozyme delivery has an extraordinary influence on ribozyme effects. Secondly, the experimental data demonstrate that the presence of at least one long antisense arm in a hammerhead ribozyme is highly favourable and almost a requirement for activity in the nucleus. Similar observations have also been made in the use of a different set of HIV-1-directed hammerhead ribozymes (Thompson et al., submitted). It should be noted, however, that our work made use of two defined sets of ribozymes that add significantly to the understanding of ribozyme design and delivery but that may not allow the derivation of general conclusions. For example, in vitro transcribed hammerhead ribozymes with short antisense flanks can also show inhibition in the nucleus (Thompson et al., submitted).

It is noteworthy that earlier studies on HIV-1-directed antisense RNA showed that the association rates in vitro correlated with efficacy in living cells when transfected into the cytoplasm (32). However, in these earlier studies the k_{ass} values for slow and fast annealing antisense species differed by >100-fold, whereas here differences were maximally 6-fold. Thus, small differences in k_{ass} values may not be important for effectiveness in the cell. A similar observation has been made recently by W. James (personal communication). More importantly, the results shown in Figure 2 could be interpreted such that the length requirements in the nucleus reflect another critical parameter which is not relevant when RNA is delivered to the cytoplasm by transfection protocols. For example, the stability of duplex RNA formed between the ribozyme and the target in the nucleus of human SW480 cells could be over-estimated when compared with melting temperatures in vitro at 37°C and physiological ionic strength. This hypothesis is consistent with the complete lack of inhibition by the short chain chemically synthesized ribozymes (Fig. 4) that were proven to be effective in the cytoplasm. Recent experiments on the dissociation of long chain duplex RNA (56 bp) showed that strand exchange can occur at 37°C and can be increased by up to 10^2-fold in the presence of cetyltrimethylammonium bromide (33), a known facilitator of nucleic acid annealing (34). Functionally similar facilitators in the nuclei of mammalian cells, such as the hnRNP proteins (35–37) or the tumor suppressor protein p53 (38,39), could weaken interactions even between long chain RNA duplexes in the nucleus and make them more dynamic than expected from in vitro data. It has been consistently suggested that a ‘helix-destabilizing environment’ in the nucleus could shift the optimal length of the antisense arms from eight to 20 or more nucleotides (40). Accordingly, the observed minimal antisense length of ~50 nt for a ribozyme to be active in vivo could reflect the stability of interactions that are necessary for biological effectiveness in the nucleus. This is consistent with the earlier finding that a similar minimum length of ~60 complementary nucleotides was important for inhibition by endogenously expressed HIV-1-directed hammerhead ribozymes (17).

A completely different picture emerges from the transfection experiments. Here, the short ribozymes with antisense flanks of 20 and 25 nt were most effective (Fig. 3). In vivo active ribozymes showed greater efficacy than in vitro inactive derivatives in the cytoplasm. Consistently, the synthetic ribozymes Rz12x12++, Rz10x10mod and Rz7x7+ performed well in the cytoplasm after microinjection, whereas the long chain constructs 2as-Rz12 and αYRz195+ were not effective when tested at the same concentration of 70 ng/μl (Figs 4 and 5). When the long chain ribozymes 2as-Rz12, αYRz195+ or αYRz195– respectively were applied at the same molarity (4.6 μM, e.g. 640 ng/μl for 2as-Rz12) as the short ribozymes at 70 ng/μl (e.g. Rz12x12++, 4.6 μM; see Fig. 4), inhibition could not be measured unequivocally, since all controls also showed inhibition (data not shown). This non-specific RNA-mediated inhibition of HIV-1 seems to be due to a general toxicity of RNA at high concentrations (640 ng/μl). Thus, it remains open whether long chain ribozymes can elicit specific inhibition in the cytoplasm. A consequence for application is to direct long chain inhibitory RNA to the nucleus of target cells and to deliver short constructs to the cytoplasm. For endogenous expression from the chromosome especially, long constructs seem to be favourable.

It should be noted that the consistency of results obtained by calcium phosphate transfection and by cytoplasmic microinjection does not necessarily mean that transfection delivers the RNA in an active form into the cytoplasm. One could also imagine that endogenous transport of transfected ribozymes guides them into the nucleus in a form that is different from direct nuclear microinjection.

The stronger inhibition observed with Rz10x10mod than with Rz7x7+ and even Rz12x12++ shows that increased biological stability of a ribozyme by chemical modification can substantially increase efficacy in living cells. The increased efficacy of Rz10x10mod versus Rz7x7+ and Rz12x12++ could also reflect a stabilizing influence of the 2'-modification on stability of the ribozyme–substrate complex in the living cell. Since stabilization of a chemically synthesized short ribozyme (Rz10x10mod; Fig. 5) led to an increased inhibition, this finding strongly suggests the employment of therapeutic concepts based on exogenous delivery of chemically modified ribozymes. This concept has been shown to be successful in a number of applications (41,42). However, properties such as cellular uptake, prolonged half-life and high efficacy will have to be improved.

When delivering ribozymes into the nucleus, long antisense arms (>50 nt) seem to be required for effectiveness. Such ribozymes are most easily expressed endogenously in transduced cell lines from recombinant ribozyme genes or in transgenics. Assuming that the locus of ribozyme action is the nucleus, one
could think of improving the efficacy of ribozyme transcripts by preventing their nuclear export.

Regardless of the mode of delivery, once an antisense effect was observed, inhibition was further increased by a factor of two to 10 (for most RNA pairs) when an in vitro active ribozyme was used. Thus, based on the assumption that antisense effects are a necessary prerequisite for ribozyme-mediated inhibition, one should aim to combine effective antisense species with the catalytic hammerhead domain. The asymmetric design is well suited to this approach (16). The increased efficacy measured with ribozymes versus in vitro inactive controls has to be explained formally by their cleavage capability, although the cleavage rate constants measured in vitro seem to be too slow to play a role in the cell (43). Much higher cleavage rates were observed when higher Mg²⁺ concentrations were used (44), indicating that faster cleavage rates can in principle be achieved. In vivo, faster catalysis could be facilitated by cellular factors (45–47).

ACKNOWLEDGEMENTS

We thank H. zur Hausen for continuous support and W. Nedbal for critical comments on this work. We acknowledge financial support by the Deutsche Forschungsgemeinschaft (SC14/2-1), by the Bundesminister für Bildung, Forschung und Wissenschaft and by the European Union.

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