EMBO reports

scientific report

Selection of targets and the most efficient hairpin ribozymes for inactivation of mRNAs using a self-cleaving RNA library

Alicia Barroso-delJesus & Alfredo Berzal-Herranz+

Instituto de Parasitología y Biomedicina 'López-Neyra', CSIC, Ventanilla 11, 18001 Granada, Spain

Received May 10, 2001; revised October 22, 2001; accepted October 23, 2001

The identification of proficient target sites within long RNA molecules, as well as the most efficient ribozymes for each, is a major concern for the use of ribozymes as gene suppressers. *In vitro* selection methods using combinatorial libraries are powerful tools for the rapid elucidation of interactions between macromolecules, and have been successfully used for different types of ribozyme study. This paper describes a new method for selecting effective target sites within long RNAs using a combinatorial library of self-cleaving hairpin ribozymes that includes all possible specificities. The method also allows the identification of the most appropriate ribozyme for each identified site. Searching for targets within the *lacZ* gene with this strategy yielded a clearly accessible site. Sequence analysis of ribozymes identified two variants as the most appropriate for this site. Both selected ribozymes showed significant inhibitory activity in the cell milieu.

INTRODUCTION

Antisense RNA molecules in general, and ribozymes in particular, are good candidates for the development of specific gene suppressers and, therefore, the development of a new class of therapeutic agents (Oberstrass and Nellen, 1997). The potency of these molecules as specific gene silencers lies in their great specificity, achieved by sequence complementarity to the target RNA. Many successful experiments describing down-regulation of gene expression by ribozymes have been reported (Scalon, 1998; Rossi and Couture, 1999). The majority is focused on achieving efficient gene inactivation rather than developing general use strategies. So far, ribozyme design has been a matter of trial and error, and no clear rules have been defined. The most common methods used for selection of target sites within large RNA molecules and design of ribozymes are mainly theoretical. The sequence to be targeted is scanned to identify potential sites based on sequence restrictions for each type of ribozyme. In addition, strategies aimed at identifying accessible regions within the substrate molecule are also used. This involves experimental mapping of the RNA *in vitro* and computer predictions to identify regions of low folding potential. The ribozyme sequence is then designed assuming that perfect Watson–Crick interactions with the target sequence will yield the most efficient molecule. These methods have had limited success. More recently, other approaches have been developed based on the use of combinatorial ribozyme libraries (Campbell and Cech, 1995; Lieber and Strauss, 1995; Yu *et al.*, 1998). However, none provides information on both cleavable sites and the ribozymes that might cleave them. This paper describes a new experimental strategy for the direct identification of cleavable sites within an mRNA and the most proficient hairpin ribozymes for cleaving them.

RESULTS AND DISCUSSION

Library of putative self-cleaving molecules

The proposed method is based on the catalytic action of a combinatorial library of hairpin ribozyme sequences that includes all possible specificities. The 5′ half of the hairpin ribozyme (Chowrira and Burke, 1992) was attached by PCR to the 3′ end of the *lacZ* gene used in the development of the strategy (Figures 1 and 2). In addition, a T7 promoter sequence was linked to the 5′ end. Sequences that serve as primer-binding sites for amplification steps, and which provide a restriction site (*Bgl*II), were also introduced into the 3′ end of the gene upstream

+Corresponding author. Tel: +34 958 80 51 87; Fax: +34 958 20 33 23; E-mail: alba@ipb.csic.es

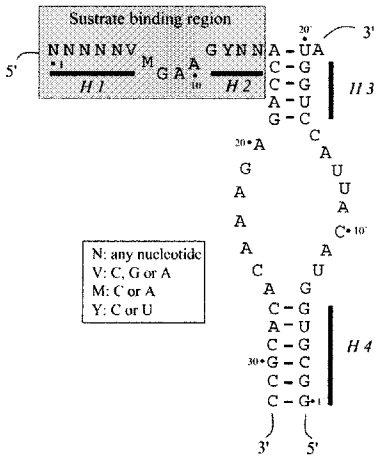

Fig. 1. Bimolecular hairpin ribozyme used in the selection procedure. H1–4 are ribozyme sequences involved in helical regions 1–4 of the ribozyme–substrate complex. Substrate interaction regions are shown with sequence variations corresponding to the initial RNA library. 5′ half ribozyme sequences are numbered 1–32; 3′ half ribozyme sequences are numbered 1′ to 21′.

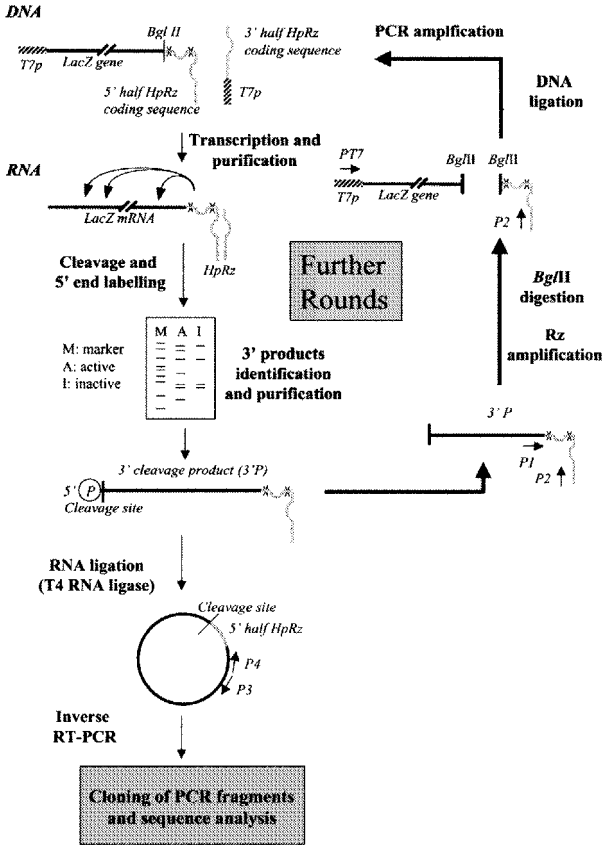

Fig. 2. Schematic of the *in vitro* selection procedure. Details are given in Methods. x, Degenerated sites within the substrate interaction regions of the ribozyme; P1–4 and PT7, primers used for cDNA synthesis and PCR amplification; T7p, T7 promoter; HpRz, hairpin ribozyme.

of the ribozyme sequence (Figure 2). During chemical synthesis of the ribozyme-template oligonucleotides, seven positions (nucleotides 1–5, 13 and 14) were randomized within the substrate-binding domain. Additionally, sequences at positions 6, 7 and 12 were mutated to V, M and Y, respectively (Berzal-Herranz *et al.*, 1993; Joseph *et al.*, 1993; Shippy *et al.*, 1998) (Figure 1). Thus, the library was designed to comprise all possible ribozyme specificities—theoretically >1.9 × 10^5 sequences. Incubation of gel-purified full-length RNA generated by *in vitro* transcription of the described template, together with the 3′ half of the ribozyme (Chowrira and Burke, 1992), yielded the library of self-cleaving RNA molecules (Figures 1 and 2). The two-piece ribozyme design allowed the separation of ribozyme catalysis from RNA synthesis.

Selection method

The selection method operates as follows (Figure 2). The library of self-cleaving RNA molecules is incubated under optimal cleavage conditions (see Methods). Only those ribozymes able to find and bind a target sequence can catalyze cleavage of the mRNA. Cleavage reactions yield RNA products with 2′-3′ cyclic phosphate and 5′-OH termini. Subsequent 5′ end-labeling of 3′ cleavage products by the T4 PNK allows their identification by gel electrophoresis. To distinguish 3′ cleavage products from degradation products, a control reaction must be carried out in parallel with a non-active ribozyme library lacking the 3′ half of the catalytic domain. After gel purification, each 5′ end-phosphorylated 3′ fragment is converted into a circular RNA molecule by ligation with T4 RNA ligase (Uhlenbeck and Gumport, 1982). This reaction provides a sense primer-binding site for RT–PCR amplification of selected 3′ fragments. PCR products obtained with primers P3 and P4 are cloned and sequenced. Analysis of individual clones gives accurate information about the cleavage site, as well as the ribozyme sequence responsible for cleavage. However, the 3′ cleavage products purified during the first round are expected to be a pool of molecules exhibiting a wide range of ribozyme sequence specificity. Sequence variability is increased since *trans*-cleavage cannot be prevented under these conditions. To reduce this, the ribozyme portion of each gel-purified 3′ cleavage product can be specifically retrotranscribed and amplified with primers P1 and P2, and introduced into new rounds of selection. Therefore, PCR products are digested with the appropriate restriction enzyme (*Bgl*II) and ligated to the 3′ end of the targeted gene. Thus, a DNA template pool of the same structure as the starting one is generated. The complexity of this pool should be significantly reduced, and decrease progressively with further selection rounds. In each round, the 3′ fragments can be analyzed as before.

The possibility of performing further selection steps permits the precise identification of those ribozymes that have promoted cleavage at each particular site, a feature that renders this method unique with respect to other strategies (Campbell and Cech, 1995; Lieber and Strauss, 1995; Yu *et al.*, 1998), which assume that the best ribozyme for an identified site is that with a complementary sequence (Watson–Crick interactions) to the

A. Barroso-delJesus & A. Berzal-Herranz

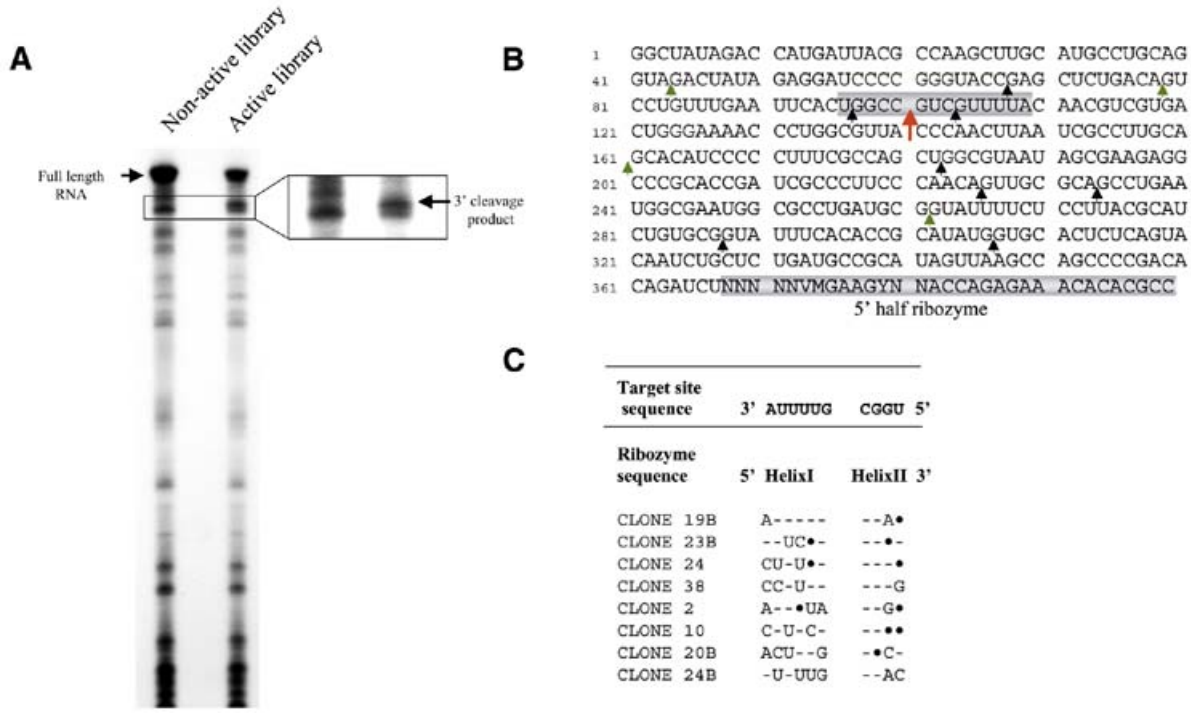

Fig. 3. First cycle of selection. (**A**) Autoradiography showing first-round cleavage. The total RNA library was subjected to cleavage as described in Methods. The reaction mix was incubated with T4 polynucleotide kinase in the presence of [γ-^{32}P]ATP and products resolved by 4% denaturing polyacrylamide gel electrophoresis. A magnification of the boxed area containing the 3′ cleavage product (→) is provided. (**B**) Sequence of the susRNA. ↑, thirteen potential cleavage sites in the mRNA; green arrows, substrates located within accessible mRNA regions as predicted by the mfold program (data not shown); red arrow, cleavage site experimentally mapped. The entire 14 nt long substrate sequence theoretically recognized by the ribozyme that cleaves at G_{101} is shown boxed with a gray background. (**C**) Helix I and II sequences of ribozymes selected after one cycle. –, Nucleotides forming a Watson–Crick base pair with the corresponding nucleotide within the target site shown on top; •, nucleotides forming wobble base pairs (G.U) with the target sequence.

target site. It has been shown that, at least for the hairpin ribozyme, this assumption is not necessarily correct (Yu *et al.*, 1998). The present achievement is based on two critical features in the design of the combinatorial RNA library. First, a self-cleaving RNA library is used. Thus, selected molecules provide information about the target site and the ribozyme. Secondly, ribozyme catalysis is separated from RNA synthesis using a two-piece ribozyme. Therefore, stringency of the selection process can be controlled without challenging the synthesis of the RNA.

Searching for cleavage sites within the LacZ-mRNA and most efficient ribozymes

According to the library design and the established requirements for the J2/1 region of the hairpin ribozyme substrate (Pérez-Ruiz *et al.*, 1999), 13 potentially cleavable sites were identified within the model target RNA (Figure 3B). Of these, only one clear 3′ cleavage product was experimentally detected after the first self-cleavage reaction with the 196 000 library variants (Figure 3A). Sequence analysis of individual clones demonstrated that cleavage had mainly occurred at the linkage between nucleotides C_{100} and G_{101} (Figure 3B). Two additional sites were detected at very low frequency (data not shown). This is probably not the only target that can be cleaved by hairpin ribozymes, but it should be the most proficient. In this regard, the sensitivity of the method plays an important role, discriminating multiple minor cleavage events of no interest from those that are more efficient. The results clearly demonstrate that this strategy allows the selection of cleavable sites different from those obtained using normal methods based on sequence searching and secondary structure prediction (Figure 3B).

Ribozyme sequences selected for the G_{101} site after the first round deviated substantially from those expected, showing low sequence complementarity with the identified target (Figure 3C). This low specificity may reflect the existence of *trans*-cleavage reactions, although the possibility that these ribozymes had been selected due to their own, potentially low activity cannot be ruled out. Further rounds of selection were performed with selected ribozymes to reduce sequence dispersion [up to five rounds, the last being more restrictive in terms of Mg^{2+} concentration (2 mM) and reaction time (15 min)]. Sequence analysis of 42 individual clones unambiguously identified the cleavage site immediately 5′ to G_{101}. It also revealed a significant reduction in ribozyme sequence variability in favor of stable interactions (Table I). Table I shows the percentage of each nucleotide at every position of the substrate-binding domain in the 42 clones (G_5) in comparison to the percentage at the initial population (G_0), demonstrating a strong bias for ribozymes forming stable helices I and II. Nucleotides forming Watson–Crick base pairs with the corresponding nucleotide on the target sequence were

Table I. Variation in nucleotide representation percentages for each ribozyme position throughout the selection procedure

S	A	U	U	U	U	G	C	G	G	U
	Helix I							Helix II		
Rz	1	2	3	4	5	6	7	12	13	14
	$G_0 \rightarrow G_5$	$G_0 \rightarrow G_5$	$G_0 \rightarrow G_5$	$G_0 \rightarrow G_5$	$G_0 \rightarrow G_5$	$G_0 \rightarrow G_5$	$G_0 \rightarrow G_5$	$G_0 \rightarrow G_5$	$G_0 \rightarrow G_5$	$G_0 \rightarrow G_5$
A	29 → 21	16 → 48	21 → 76	22 → 93	20 → 93	36 → 0	56 → 83		41 → 0	14 → 14
C	17 → 7	24 → 0	13 → 3	35 → 0	28 → 0	44 → 100	44 → 17	50 → 100	32 → 76	59 → 48
G	21 → 31	16 → 45	33 → 18	35 → 7	32 → 7	20 → 0			18 → 3	5 → 33
U	33 → 40	44 → 7	33 → 3	8 → 0	20 → 0			50 → 0	9 → 21	22 → 4

G_0, percentages in the initial population; G_5, percentages after five selection cycles; Rz, ribozyme position; S, identified substrate sequence.

positively selected at every position of each helix except for the most distal. In addition, G•U base pairs were also positively selected only at positions 2 in helix I, and 13 and 14 in helix II. Finally, a mismatch was selected at the first position in helix I, where a guanosine forming a G•A pair was apparently chosen. These results are in good agreement with those reported previously (Joseph *et al.*, 1993). Burke and co-workers, using short oligonucleotide substrates, demonstrated that the hairpin ribozyme tolerates mismatches well at the distal positions in both helices (Figure 1). In this regard, further analysis revealed that this G was positively selected between the first and fourth cycle, during which 60% of the population showed a G at position 1 (data not shown). However, a restrictive fifth round produced a dramatic reduction in G prevalence at this position. This indicates that the presence of a mismatch in a distal position of helix I might be tolerated in optimal reaction conditions, but becomes detrimental in suboptimal conditions. In the latter case, counter-selection in favor of stable interactions with the substrate is observed. Nevertheless, the last cycle was unable to bring values below the G_0 percentages, therefore showing an apparent overall positive selection for this nucleotide. These results demonstrate that the selection conditions can be easily modified and suggest that the use of more stringent reaction conditions might accelerate the process, allowing a reduction in the number of cycles needed. Additionally, raising the selection pressure would probably yield many less ribozyme variants. Rapid activity screening of the few deduced ribozyme variants would allow the identification of the most proficient catalytic motifs. These results also demonstrate positive selection of A at position 7 within the ribozyme sequence, across from a C in the target sequence (Table I). This agrees with the proposed A•C interaction between nucleotides at these positions (Shippy *et al.*, 1998).

Trans-activity of selected ribozymes

The proposed method allows the identification of cleavage sites and the selection of active ribozymes *in cis*. However, this will not be the case when *in vivo* targeting a specific mRNA of interest. The ability of three different ribozyme variants, derived from the selection process, to cleave *in trans* the same full-length mRNA was evaluated. The ribozymes tested were: *RzG101*, containing a perfectly matching sequence with substrate G_{101}; RzG101G1, which contains a G residue at position 1 within the helix I region, resulting in a mismatch with the substrate at this position; and *RzG101G14*, which codes for a G at position 14, forming a wobble interaction with the substrate sequence (Figure 4A). The results demonstrate that the three variants are able to cleave the full-length mRNA *in trans*.

Intracellular activity of selected ribozymes

Although this is an entirely *in vitro* selection process, there is no reason to believe that the results are not applicable to the *in vivo* situation (Yu *et al.*, 1998; zu Putlitz *et al.*, 1999). The ability of the above-described ribozymes to catalyze the inactivation of the targeted gene in an eukaryotic cell environment was therefore tested. For this, the HEK 293 human cell line was used. Cells were transiently cotransfected with two plasmids encoding the substrate (pβgal) and the ribozyme (pcRzG101, pcRzG101G1, pcRzG101G14 or pcRzG101Mut), respectively (see Methods). Measurements of β-gal activity were performed 48 h post-transfection. Colorimetric assays were carried out as described in Methods. Results in Figure 5A demonstrate that two out of the three potentially active ribozymes tested had a significant inhibitory effect *in vivo*. RzG101 and RzG101G14 showed similar capacity to silence β-gal expression (~40 and 46% of inhibition, respectively), while RzG101G1 yielded a very low inhibitory effect. These results highlight a strong correlation with those ones obtained from the *in vitro* selection procedure. Ribozymes selected after high selection pressure conditions were the ones exhibiting the highest *in vivo* activity, whilst almost no effect was observed with RzG101G1 counter-selected during the last cycle. The slightly higher *in vivo* activity of RzG101G14 is in good agreement with its higher representation in the final population of selected ribozymes (Table I). It has been described that a wobble interaction at the last position of helix II may favor the catalytic performance of the hairpin ribozyme (Yu *et al.*, 1998). Despite this favorable effect, the likely destabilization of Helix II may explain the lower *in vitro trans*-cleavage activity (Figure 4). This might be compensated for the different ionic *in vivo* environment and by tethering the target to the ribozyme during selection procedure. Northern blot analyses were carried out to determine intracellular levels of ribozyme. No relevant differences were found among the four constructs tested (Figure 5B). Therefore, percentages of inhibition measured for each ribozyme are in fact comparable.

A. Barroso-delJesus & A. Berzal-Herranz

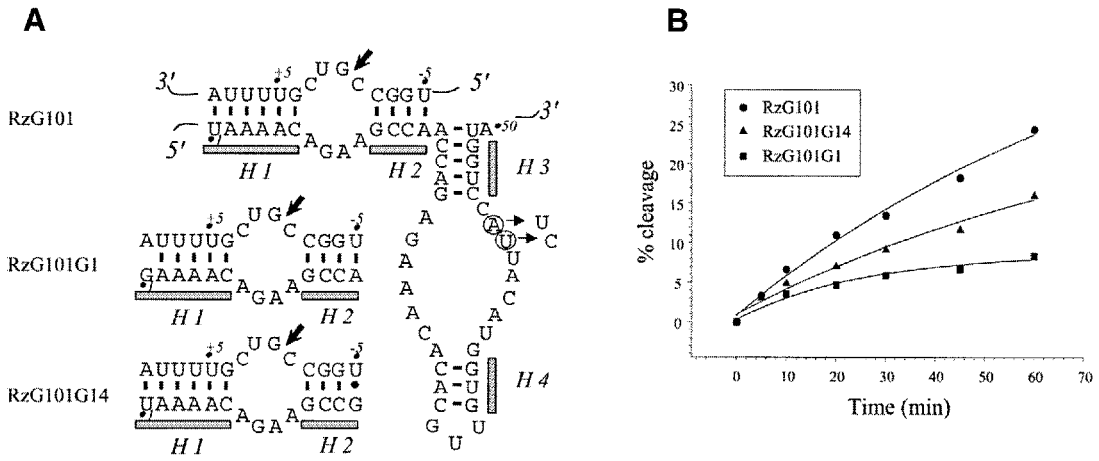

Fig. 4. *In vitro trans*-cleavage experiments. (**A**) Secondary structure of the hairpin ribozyme–substrate complex used for *in vitro trans*-cleavage experiments. Sequences of the RzG101 and the mapped substrate are indicated. Substrate binding domains of RzG101G1 and RzG101G14 accomplished with G101 substrate are also shown. An arrow indicates the cleavage site. Substrate nucleotides are numbered –5 to +9. Ribozyme nucleotides are numbered 1 to 50. Encircled nucleotides are those mutated in this work to generate an inactive ribozyme for *ex vivo* experiments. H1–4 are as described in Figure 1. (**B**) Graphic representation of *in vitro* cleavage activity of RzG101, RzG101G14 and RzG101G1 under ribozyme excess conditions.

The strategy described in this work is a rapid and efficient method for the identification of proficient hairpin ribozymes. With this approach, all possible targets within the mRNA as well as all possible ribozymes for each site are simultaneously assayed, providing accurate information about real target sites and ribozymes. This method should allow the negative selection of any specific nucleotide which, when stably interacting with the substrate, is deleterious for catalytic activity (e.g. because of the induction of incorrect folding of the ribozyme–substrate complex). This feature seems to be especially important when designing hairpin ribozymes (Yu *et al.*, 1998). We believe it could be of great use in the targeting of large, structured RNAs, and are currently using this approach to identify efficient hairpin ribozymes against different viral RNAs.

METHODS

Eukaryotic cells and transfections. HEK 293 cells (ATCC: CRL 1573) were grown in RPMI medium supplemented with 10% FBS, 2 mM L-glutamine and 40 mg/l gentamycin. One day prior to transfection, cells were seeded in 6-well plates at a density of 3×10^5 cells/well and grown overnight to 60–80% confluence. Transfections were performed using FuGene 6 Reagent (Roche) following the manufacturer's instructions. A 3-fold excess of the Rz encoding plasmid (1.5 μg) over that encoding β-gal (0.5 μg) was used to ensure that ribozymes would be present in every cell containing the substrate RNA.

DNA oligonucleotides and RNA synthesis. Oligodeoxyribonucleotides (ODNs) were synthesized in an Oligo 1000 DNA synthesizer (Beckman Instruments) and purified as described previously (Pérez-Ruiz *et al.*, 1999). RNA molecules were all generated by *in vitro* transcription using T7 RNA polymerase and gel purified as described previously (Barroso-delJesus *et al.*, 1999).

Construction of DNA templates. A template for the transcription of the RNA library was obtained by PCR amplification of pUCsWT with primers SELT7 (TAATA CGACT CACTA TAGGC TATGA CCATG ATTAC G) and SEL5'Rz (GGCGT GTGTT TCTCT GGTNN RCTTC KBNNN NNAGA TCTGT GTCGG GGCTG GCTTA). pUCsWT is a modified pUC19 plasmid containing the 14 nt cognate substrate of the sTRSV hairpin ribozyme. The resulting amplicon coded for the so called 'Sus gene' that contains the α peptide portion of the *lacZ* gene flanked by the sequences coding for the 5' half of the hairpin ribozyme and the T7 promoter (underlined in the primer sequence) at the 3' and 5' ends, respectively. Its corresponding RNA molecule is here referred to as 'SusRNA'. The same construct was used as a template for the synthesis of the RNA substrate processed in *in vitro trans*-cleavage reactions. RNA coding for the 3' half of the ribozyme was obtained by *in vitro* transcription of SEL3'Rz (TACCA GGTAA TATAC CACGC CATAT GTGAG TCGTA TTA) previously annealed to PT7 (TAATA CGACT CACTA TA; Milligan and Uhlenbeck, 1989). Ribozymes for *trans*-cleavage experiments were transcribed from ODNs RzG101 (TACCA GGTAA TATAC CACAA CGTGT GTTTC TCTGG TTGGC TTCTG TTTTA CCCTA TAGTG AGTCG TATTA), RzG101G1 (TACCA GGTAA TATAC CACAA CGTGT GTTTC TCTGG TTGGC TTCTG TTTTC CCCTA TAGTG AGTCG TATTA) and RzG101G14 (TACCA GGTAA TATAC CACAA CGTGT GTTTC TCTGG TCGGC TTCTG TTTTA CCCTA TAGTG AGTCG TATTA), prior to annealing with PT7. Templates for eukaryotic *in vivo* expression of ribozymes were assembled by annealing and extension of two overlapping ODNs: G101 (AGATA AAACA GAAGC CAACC AGAGA AACAC ACGTT) and 3'RzHp (TCCTA CCAGG TAATG TACCA CAACG TGTGT TTCTC) for RzG101, and 3'RzHpMUT (TCCTA CCAGG AGATG TACCA CAACG TGTGT TTCTC) for RzG101Mut. Ribozymes G101G1 and RzG101G14 were obtained similarly with oligonucleotides G1 or G14 and 3'RzHp, respectively. ODNs G1 and

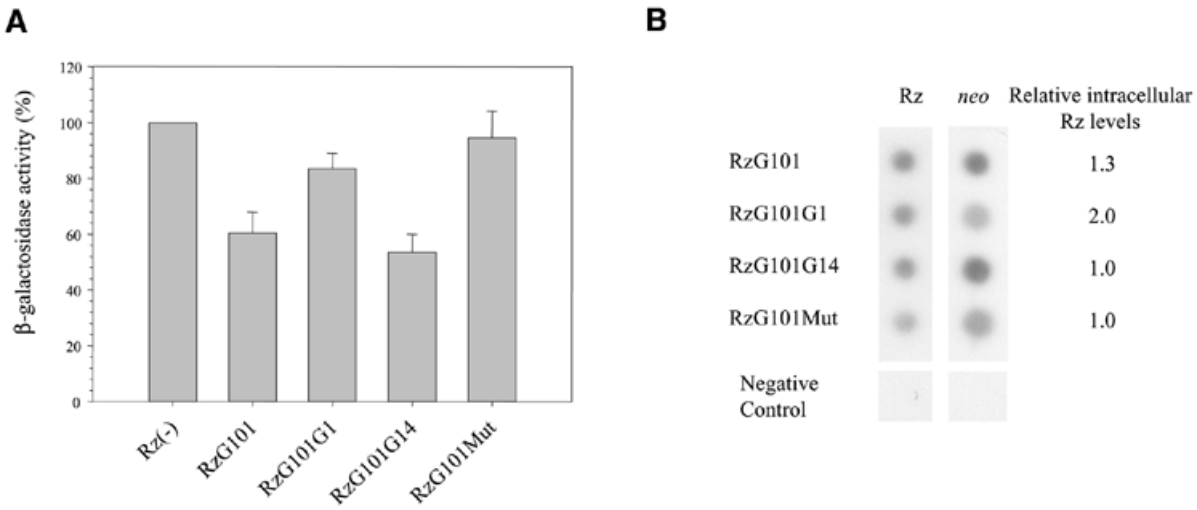

Fig. 5. *In vivo* experiments. (**A**) Intracellular activity of selected ribozymes. Inhibitory effect in HEK 293 cells expressed as a percentage of β-galactosidase activity. Data are the average of three independent experiments. Measurements were performed as described in Methods. (**B**) Intracellular levels of ribozyme. Ribozyme levels were determined in transient transfected HEK 293 cells by northern analysis. Transfection efficiency and RNA loading were normalized using *neo* mRNA levels. Intracellular amounts of each ribozyme were normalized to the lowest value.

G14 have the same sequence as ODN G101 except for substitutions T:G and A:G, respectively (substituted nucleotides are underlined in sequence G_{101}). Templates were then included between the unique *Eco*RV site of pcDNA3 vector (Invitrogen). Plasmid pβgal (a kind gift from Dr Javier Oliver), which encodes for the β-galactosidase gene under the control of the CMV promoter, was chosen for eukaryotic *ex vivo* expression of substrate mRNA.

Identification of self-cleavage products. Self-cleaving RNA molecules for the initial population were assembled by hybridization of 30 pmol of susRNA (*lacZ* messenger plus 5′ half hairpin ribozyme) and 50 pmol of the 3′ half hairpin ribozyme molecule. In further selection rounds, RNA quantities were decreased to 5 and 15 pmol, respectively. Annealing was performed by heating at 65°C for 4 min and then cooling on ice for 15 min. Cleavage reactions were started by the addition of cleavage buffer at a final concentration of 50 mM Tris–HCl pH 7.5, 12 mM $MgCl_2$, with incubation at 37°C for 90 min (unless otherwise indicated). Reaction products were 5′-end labeled with 25 U of T4 polynucleotide kinase (New England Biolabs) in the presence of 25 µCi of [γ-^{32}P]ATP in a total volume of 10 µl. The reaction mix was incubated at 37°C for 45 min. 3′-cleavage products were resolved on 4% (w/v) polyacrylamide–7 M urea gels, excised, and eluted as described previously (Barroso-delJesus *et al.*, 1999).

RNA ligation. RNA ligation was performed at 4°C overnight with 24 U of T4 RNA ligase (USB) in the presence of 50 mM Tris–HCl pH 7.5, 5 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, 100 µg/ml BSA and 10 U of RNAguard™ (Amersham Pharmacia Biotech), in a total volume of 10 µl.

RT–PCR and sequence analysis. cDNA synthesis of ribozymes entering further rounds of selection was initiated by annealing primer P2 (GGCGT GTGTT TCTCT GGT) to the gel-purified 3′ cleavage product. Annealing was performed by heating at 95°C for 2 min followed by slow cooling to 42°C in 50 mM Tris–HCl pH 8.3, 10 mM $MgCl_2$, 10 mM DTT, 75 mM KCl and 0.6 mM dNTPs. AMV reverse transcriptase (RT) (2 U/µl) (Promega) was then added and the reaction mix incubated at 42°C for 30 min. AMV RT was then inactivated for 5 min at 99°C and cDNA was PCR amplified after addition of primer P1 (TGAAT TCTTT AAGCC AGCCC CGACA C) and 0.02 U/µl of *Taq* polymerase (Biotools). The resulting amplicon was ligated to the 3′ end of the *Sus* gene after *Bgl*II digestion. The ligation product was finally PCR amplified using primers PT7 and P2 to generate the DNA template on entering a new selection cycle. Similarly, circular RNAs were reverse transcribed and amplified as described using primers P3 (TCTAT GCATC CGCAT CAGGC GCCAT) and P4 (TCTCC ATGGT TTCTC CTTAC GCATC TGT). Amplicons were *Nco*I–*Nsi*I digested and cloned into the pGEM®-5Zf(-) vector (Promega) for sequence identification of cleavage sites and ribozymes.

***Trans*-cleavage reactions.** *Trans*-cleavage experiments were carried out under single-turnover conditions with 10 nM substrate and 300 nM ribozyme. Both RNAs were mixed, denatured at 65°C for 4 min, renatured on ice for 15 min, and brought to reaction temperature (37°C) for 10 min. The reaction was initiated by adding the correct amount of 10× cleavage buffer, and followed over a time course of 60 min. RNAs were resolved on 5% (w/v) polyacrylamide–7 M urea gels and quantified using a β-scan radioanalytical imaging instrument (Instant Imager, Packard Instrument Co.).

β-galactosidase measurements. HEK 293 cells were collected 48 h post-transfection, washed with PBS and lysed with 80 µl of CCLR1x buffer (Promega). A volume of 50 µl from each cell lysate was diluted in buffer Z (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$ and 40 mM 2-mercaptoethanol) up to 200 µl and incubated at room temperature for 5 min. To start reactions, 50 µl of ONPG buffer (4 mg/ml ONPG

in buffer Z) were added. Mixtures were then transferred to a 96-well plate and incubated at 37°C with moderate shaking. Measurements of absorbance at 420 nm were performed at 60 and 90 min using a Biomek Plate Reader instrument (Beckmann). OD values were normalized to total protein concentration in cell lysates determined by the Bio-Rad protein colorimetric assay. Absorbance values of mock transfected cells samples were used as the background to spectrophotometric measurements, and values obtained from β-gal expressing control cells were considered as 100% β-gal activity.

Northern analysis. RNA extraction was performed with the RNAqueus™ kit (Ambion) following the manufacturer's instructions. Total RNA was isolated from HEK 293 cells that had been transfected with individual Rz expression vectors as described above. Fifteen micrograms of total RNA were loaded per dot on a Hybond-N nylon membrane (Amersham Pharmacia). The membrane was hybridized overnight at 65°C with a ^{32}P internally labeled RNA probe complementary to the Rz sequence. Transcripts were not detected in control transfectants in which DNA was omitted. RNA amounts were quantified on a β-scan radioanalytic imaging instrument (Instant Imager, Packard Instrument Co.).

Hybridization with a DNA probe complementary to the *neo* mRNA (encoded in the same plasmid than the Rz) was used both as a DNA transfection and an RNA loading control. Levels of *neo* mRNA were used to normalized Rz levels.

ACKNOWLEDGEMENTS

We thank V. Augustin for excellent technical assistance. This work was supported by grants from the Spanish DGES and the Fundación laCaixa (to A.B.-H.), and the Junta de Andalucía.

REFERENCES

Barroso-delJesus, A., Tabler, M. and Berzal-Herranz, A. (1999) Comparative kinetic analysis of structural variants of the hairpin ribozyme reveals further potential to optimize its catalytic performance. *Antisense Nucleic Acid Drug Dev.*, **9**, 433–440.

Berzal-Herranz, A., Joseph, S., Chowrira, B.M., Butcher, S.E. and Burke, J.M. (1993) Essential nucleotide sequences and secondary structure elements of the hairpin ribozyme. *EMBO J.*, **12**, 2567–2574.

Campbell, T.B. and Cech, T.R. (1995) Identification of ribozymes within a ribozyme library that efficiently cleave a long substrate RNA. *RNA*, **1**, 598–609.

Chowrira, B.M. and Burke, J.M. (1992) Extensive phosphorothioate substitution yields highly active and nuclease-resistant hairpin ribozymes. *Nucleic Acids Res.*, **20**, 2835–2840.

Joseph, S., Berzal-Herranz, A., Chowrira, B.M., Butcher, S.E. and Burke, J.M. (1993) Substrate selection rules for the hairpin ribozyme determined by *in vitro* selection, mutation, and analysis of mismatched substrates. *Genes Dev.*, **7**, 130–138.

Lieber, A. and Strauss, M. (1995) Selection of efficient cleavage sites in target RNAs by using a ribozyme expression library. *Mol. Cell. Biol.*, **15**, 540–551.

Milligan, J.F. and Uhlenbeck, O.C. (1989) Synthesis of small RNAs using T7 RNA polymerase. *Methods Enzymol.*, **180**, 51–62.

Oberstrass, J. and Nellen, W. (1997) Regulating genes with antisense RNA. In Weiss, B. (ed.), *Antisense Oligodeoxynuleotides and Antisense RNA*. CRC Press, Boca Raton, FL, pp. 171–195.

Pérez-Ruiz, M., Barroso-delJesus, A. and Berzal-Herranz, A. (1999) Specificity of the hairpin ribozyme. Sequence requirements surrounding the cleavage site. *J. Biol. Chem.*, **274**, 29376–29380.

Rossi, J.J. and Couture, L.A. (eds) (1999) *Intracellular Ribozyme Applications. Principles and Protocols*. Horizon Scientific Press, Norfolk, UK.

Scalon, K.J. (ed.) (1998) *Therapeutic Applications of Ribozymes*. Humana Press, Totowa, NJ.

Shippy, R., Siwkowski, A. and Hampel, A. (1998) Mutational analysis of loops 1 and 5 of the hairpin ribozyme. *Biochemistry*, **37**, 564–570.

Uhlenbeck, O.C. and Gumport, R.I. (1982) T4 RNA ligase. In Boyer, P.D. (ed.), *The Enzymes*. Academic Press, New York, NY, Vol. 15B, pp. 31–60.

Yu, Q., Pecchia, D.B., Kingsley, S.L., Heckman, J.E. and Burke, J.M. (1998) Cleavage of highly structured viral RNA molecules by combinatorial libraries of hairpin ribozymes. *J. Biol. Chem.*, **273**, 23524–23533.

zu Putlitz, J., Yu, Q., Burke, J.M. and Wands, J.R. (1999) Combinatorial screening and intracellular antiviral activity of hairpin ribozymes directed against hepatitis B virus. *J. Virol.*, **73**, 5381–5387.

DOI: 10.1093/embo-reports/kve247