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Optimization of *trans*-splicing ribozyme efficiency and specificity by *in vivo* genetic selection

Brian G. Ayre*, Uwe Köhler[1], Robert Turgeon and Jim Haseloff[1]

Plant Biology Department, Cornell University, Ithaca, NY 14853, USA and [1]Department of Plant Sciences, University of Cambridge, Downing Street, Cambridge CB2 3EA, UK

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ABSTRACT

***Trans*-splicing ribozymes are RNA-based catalysts capable of splicing RNA sequences from one transcript specifically into a separate target transcript. In doing so, a chimeric mRNA can be produced, and new gene activities triggered in living cells dependent on the presence of the target mRNA. Based on this ability of *trans*-splicing ribozymes to deliver new gene activities, a simple and versatile plating assay was developed in *Saccharomyces cerevisiae* for assessing and optimizing constructs *in vivo*. *Trans*-splicing ribozymes were used to splice sequences encoding a GAL4-derived transcription activator into a target transcript from a prevalent viral pathogen. The transcription activator translated from this new mRNA in turn triggered the expression of genes under the regulatory control of GAL4 upstream-activating sequences. Two of the activated genes complemented metabolic deficiencies in the host strain, and allowed growth on selective media. A simple genetic assay based on phenotypic conversion from auxotrophy to prototrophy was established to select efficient and specific *trans*-splicing ribozymes from a ribozyme library. This simple assay may prove valuable for selecting optimal target sites for therapeutic agents such as ribozymes, antisense RNA and antisense oligodeoxyribonucleotides, and for optimizing the design of the therapeutic agents themselves, in higher eukaryotes.**

INTRODUCTION

RNA molecules with catalytic activities, commonly referred to as ribozymes, are potential tools for genetic manipulation. Various naturally occurring ribozymes have been dissected and used as endoribonucleases to cleave specific target transcripts (1–3). In general, however, these ribozymes demonstrate poor efficiency *in vivo*, and optimization has been hampered by the need to detect small reductions in target-gene activity (4,5). An alternative approach to ribozyme technologies might exploit the coupled cleavage and ligation reactions of naturally occurring RNA catalysts involved in splicing. In splicing reactions, intervening regions that disrupt the coding sequence of a gene are removed, and result in gene activation. Gene activation presents tremendous potential for the development of RNA-based technologies since detection of a *de novo* gene activity above negligible background is intrinsically easier to study than reductions in the levels of an existing gene, and the new activity can have profound effects on the phenotype of the host organism.

Group I introns are natural splicing catalysts that autocatalyze the removal of intron sequences and ligation of the exon sequences via two sequential transesterification reactions (6). Group I introns have been bisected, and co-expression of the individual components *in vivo* resulted in *trans*-splicing of exon sequences from one molecule into sequences of another, to create a unique chimeric transcript (7–9). Translation of the fusion RNA resulted in *de novo* gene activation. As one example, wild type globin sequences were *trans*-spliced into sickle globin mRNA to create 'repaired' transcripts *in vivo* (10). In a second example, an endotoxin was activated by *trans*-splicing endotoxin sequences into the coat protein mRNA of a prevalent plant virus (11). The translated product of this fusion mRNA resulted in inhibition of cellular proliferation, and was limited to cells expressing the viral target sequences.

The latter example incorporated design parameters shown to improve the specificity of the ribozyme for its target, and the efficiency of *trans*-splicing, relative to other designs (7,11,12). The inclusion of an 'extended antisense sequence' (EAS) at the 5′ end of the ribozyme improved both the efficiency and specificity for the intended target (11). *Trans*-splicing efficiency was further improved by retaining a potential P10 duplex at the 3′ end of the catalytic core (7). Notwithstanding these improved design parameters, it was recognized that *trans*-splicing efficiency and specificity could be further improved.

In this paper, an *in vivo*, genetic approach for selecting optimized ribozymes from a library of constructs is described. Selection is by a simple *Saccaromyces cerevisiae* plating assay, and is based on ribozyme-mediated conversion from auxotrophy to prototrophy. Start-codon-deficient mRNA encoding a GAL4-derived transcription activator was *trans*-spliced in frame with the desired target transcript, which, for

*To whom correspondence should be addressed. Tel: +1 607 255 8826; Fax: +1 607 255 5407; Email: bga2@cornell.edu
Present address:
Uwe Köhler, Medigenomix GmbH, Lochhamer Strasse 29, D-82152 Martinsried, Germany

the experiments described, was derived from the coat protein of cucumber mosaic virus (CMV) (13). The translated product of this chimeric transcript activated downstream genes that were under the regulatory control of GAL4 upstream activating sequences (UASs): the *HIS3* gene to confer histidine prototrophy, the *ADE2* gene to confer adenine prototrophy and the *lacZ* gene to confer β-galactosidase activity.

Constructs demonstrating efficient splicing *in vivo* were selected by their ability to complement the metabolic deficiencies in the host strain, and allow growth on selective media. These constructs were then subjected to a second plating assay that assured that the observed prototrophy was the result of *trans*-splicing with the desired target transcript. A more rigorous test for specificity, in which the GAL4-derived sequences are replaced with sequences encoding a potent cytotoxin, is also described. This *in vivo* genetic selection scheme, unlike existing computer-based and *in vitro* screening approaches (14), enables rapid identification of ribozymes optimized for a particular target from large libraries in the complex cellular environment.

MATERIALS AND METHODS

Plasmid construction

Plasmids were constructed by standard procedures (15,16). A CMV coat protein (CP)-green fluorescent protein (GFP) target gene and a mutant derivative were isolated as *Bam*HI–*Sac*I fragments from plasmids pCMV-GFP and pMUT-GFP, respectively (7,11). Each was sub-cloned in to the same sites of pVT103-U (17) between the *ADH1* promoter and terminator sequences to create pCMV-GFP(URA3) and pMUT-GFP(URA3). For expression of the ribozymes, an *Sph*I fragment containing the *ADH1* promoter and terminator sequences from pVT103-U was inserted into the *Pvu*II sites of pRS424 (18) by blunt-end ligation. In the resulting plasmid, pADH424, the *ADH1* promoter directs transcription from the f1- strand (i.e. transcription is from the same strand as the *TRP1* allele). The previously described ribozymes genes (11) with 3′ exon sequences derived from diphtheria toxin A (DTA) chain were inserted into pADH424 as *Bam*HI–*Sac*I fragments to create p2ATcisRzDTA, p2ATΔP5RzDTA, p2AT9RzDTA, p2AT54RzDTA and p2AT302RzDTA. A new 3′ exon encoding amino acids 2–147 of the yeast GAL4 protein fused to amino acids 412–490 of the herpes simplex virus VP16 protein (19) was PCR amplified from plasmid pBin35S-mGAL4/VP16+UASmGFP5er (J. Haseloff, unpublished) using a 5′ primer with sequence ACTA*GGTACC*-CAACAATAAAGCTCCTGTCCTCC and a 3′ primer with sequence ACTAA*CTAGT*GGATCCTACCCACCGTAC-TCG. The PCR product was digested with *Kpn*I and *Spe*I (italicized), sub-cloned into the same sites of pET17B (Novagen), and subsequently digested with *Kpn*I and *Pst*I. This gene fragment then replaced the *DTA* 3′ exons in the above plasmids as *Kpn*I–*Pst*I fragments to create pcisRzGVP, pΔP5RzGVP, p9RzGVP, p54RzGVP and p302RzGVP.

A *Xho*I site internal to the *GAL4*-VP16 3′ exon (GVP) was removed from p54RzGVP by site-directed mutagenesis (20) using an oligonucleotide with sequence CCTCCTGATCTT-CCCTaGAGAGGACCTCGACATGATCC (mutated nucleotide is in lower case). In doing so, codon usage was improved for yeast, without altering the amino acid sequence. The RzGVP fusion gene was then PCR amplified with a 5′ primer corresponding to a sequence in the *ADH1* promoter, GCA-CAATATTTCAAGCTAT, and a 3′ primer with sequence CCAGCTG*CTGCAG*ACCCACCGTACTCGTCAATTCC-AAG, removing the *Bam*HI site from the 3′ end of the gene fragment. This PCR product was digested with *Xho*I (downstream of the 5′ primer binding site in the template) and *Pst*I (italicized), and sub-cloned into the same sites of pcisRzDTA (11) to create pcisRzGVP(ΔXΔB). This *cis*-splicing construct was introduced into yeast, and the GVP 3′ exon was functionally tested (not shown). The functional exon was then sub-cloned into p54RzGVP as a *Kpn*I–*Pst*I fragment to create p54RzGVP(ΔXΔB).

Ten additional ribozyme constructs, pARzGVP, pBARzGVP, pCBARzGVP, pDCBARzGVP, pBRzGVP, pCBRzGVP, pDCBRzGVP, pCRzGVP, pDCRzGVP and pDRzGVP, were created by introducing new EAS regions into p54RzGVP(ΔXΔB). The new sequences were created by PCR using pMUT-GFP as the template, and appropriate combinations of the following oligonucleotides: A, GAC-TTAA*CTCGAG*ACCAGTGCTGGTCGTAACCG; B, GAC-TTAA*CTCGAG*AAAACTACCTGTTCCATGGC; C, GAC-TTAA*CTCGAG*CCCTCGTCAACAGGATCGAGC; D, GAC-TTAA*CTCGAG*AATACTCCAATTGGCGATG; A′, CTT-AA*GGATCC*TCCAGTAGTGCAAATAAATTTAAGG; B′, CTTAA*GGATCC*TGTCTCCCTCAAACTTGAC; C′, CTT-AA*GGATCC*TTTTGTTGATAATGATCAGCG; and D′, CT-TAA*GGATCC*CTTATTTGTATAGTTCATCC (i.e. A and A′ created the 'A' EAS, A and B′ created the 'BA' EAS, A and C′ created the 'CBA' EAS, etc.). The resulting PCR products were digested with *Bam*HI and *Xho*I (italicized), and sub-cloned into the same site of p54RzGVP(ΔXΔB) (see Fig. 1 for a graphic representation of target and ribozyme genes used in this study).

Test for prototrophy

Yeast manipulations were by standard protocols (21,22). Yeast strain PJ69-4a (*MAT**a** trp1-901 leu2-3 112 ura3-52 his3-200 gal4D gal80D LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ*) (23) was cultured on supplemented minimal medium (SMM; synthetic dextrose minimal medium supplemented with adenine sulfate, uracil, L-tryptophan, L-histidine and L-methionine, each at 20 mg/l and L-leucine at 100 mg/l) (21). 5-Fluoro-orotic acid (5-FOA) (Sigma) was used at 1 g/l, and X-gal indicator plates, and β-galactosidase assays, were as described previously (15).

PJ69-4a was initially transformed with pCMV-GFP(URA3) and pMUT-GFP(URA3), and selected on –URA medium. The RzGVP constructs were then individually introduced, and selected on –URA –TRP medium. Single colonies were inoculated into –URA –TRP broth, and grown overnight at 30°C. The cultures were then diluted, and 1×10^4 cells of each strain were spotted on the selective media indicated in Figure 3.

RNA was isolated (15) from cells grown overnight in –HIS medium, and reverse transcribed with Superscript RT II (Life Technologies) using the manufacturer's instructions. *Trans*-spliced cDNA was PCR amplified using a 5′ primer corresponding to a sequence in the CMV target gene, TCCAAGGAGATATATAACAATGC, and a 3′ primer

corresponding to a sequence in GVP region of the ribozymes, ACGTCCTCGCCGTCTAAGTGGAG. Amplified cDNA was sequenced at the Cornell BioResource Center with the CMV target primer.

Genetic selection

Equal aliquots of the 13 *trans*-splicing RzGVP constructs (Fig. 2B) were mixed to create a RzGVP library and transformed en masse into PJ69-4a harboring either pCMV-GFP(URA3) or pMUT-GFP(URA3). Five percent of the transformation mixture (~1 × 10^6 cells) was plated to –URA –TRP to determine transformation efficiency, and the remainders were plated to –URA –TRP –HIS medium to select for effective splicing events. After 5 days of growth on –URA –TRP –HIS, 24 colonies were randomly selected, patched to –HIS medium, and grown for 2 days. Each was then patched to –HIS +5-FOA and assayed for target-independent growth after 5 days. 5-FOA is a toxic analog of a compound in the uracil biosynthetic pathway, and only cells that have lost the *URA3*-encoding target plasmid are able to grow in its presence.

To characterize the RzGVP genes that conferred prototrophy for histidine, yeast strains from –HIS plates were inoculated into –TRP broth and grown overnight. Total DNA was isolated, and transformed into *Escherichia coli* strain KC8 (*pyrF*, *leuB600*, *trpC*, *hisB463*) (15). KC8 cells transformed with RzGVP constructs were selected for tryptophan prototrophy on M9 minimal medium supplemented with uracil, L-histidine and L-leucine (40 mg/l each), and ampicillin (100 mg/l). Plasmid was isolated and analyzed by restriction digest from four KC8 colonies obtained from each yeast strain prototrophic for histidine.

Stringent test for specificity

The GVP 3′ exons in constructs pARzGVP, pBARzGVP, pCBARzGVP and pDCBARzGVP were replaced with the DTA 3′ exon from pcisRzDTA (11) as *Kpn*I–*Pst*I fragments to create constructs pARzDTA, pBARzDTA, pCBARzDTA and pDCBARzDTA. Growth rates of PJ69-4a cultures harboring these constructs, as well as constructs pADH424, p2ATcis-RzDTA, p2ATΔP5RzDTA, p2AT9RzDTA, p2AT54RzDTA and p2AT302RzDTA (see above), were measured by taking OD_{600} readings of –TRP broth cultures maintained in log phase for at least six generations.

RESULTS

Ribozyme design

The *trans*-splicing ribozymes used are based on the group I intron of the large ribosomal RNA subunit of *Tetrahymena thermophila*. This intron autocatalyzes its own excision and ligation of the exon sequences via two sequential transesterification reactions (6). The reaction mechanism of *trans*-splicing is assumed to be the same as for *cis*-splicing, with the exception that the 5′ exon is on a separate molecule from the intron and 3′ exon, necessitating an intermolecular association via base pairing (compare Fig. 1A and B). General principles for the design and construction of *trans*-splicing ribozymes, and details of the reaction mechanism, were described previously (7,11).

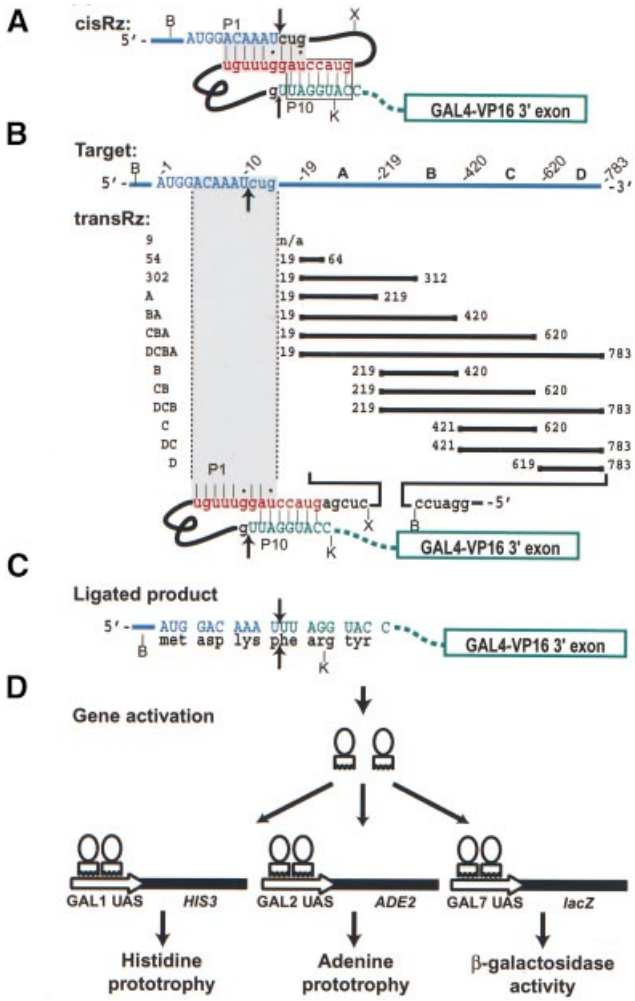

Figure 1. Ribozyme design and gene activation. (**A**) Schematic diagram of the *cis*-splicing control ribozyme in pcisRzGVP. 5′ exon sequences are represented in blue, 3′ exon sequences are indicated in green and intron sequences are black, except for the IGS which is indicated in red. The modified P1 helix is shaded, and the modified P10 helix is boxed. Watson–Crick base pairs are indicated by vertical dashes, and G:U wobble base pairs are indicated by dots. Arrows indicate the 5′ and 3′ splice sites. Restriction endonuclease sites: B, *Bam*HI; X, *Xho*I; K, *Kpn*I; P, *Pst*I. (**B**) Schematic diagram of the *trans*-splicing ribozymes. The horizontal blue line represents the target transcript. Sequences surrounding the 5′ splice site are indicated, with the 5′ exon in uppercase. Regions of the target complementary to the EAS regions of the ribozymes are indicated by A, B, C and D, with the limits of complementarity indicated by nucleotide number (relative to the AUG start codon). The *trans*-splicing ribozymes are represented below the target. The name of each construct is on the left, and the corresponding EAS is represented on the right as horizontal bars. The numbers flanking each EAS region correspond to the complementary sequences in the target. Other symbols are as described for (A). (**C**) Nucleotide and amino acid sequence at the anticipated splice junction (indicated by arrows). 5′ exon sequences are in blue and 3′ exon sequences are in green. (**D**) Activation of downstream genes after translation of the spliced mRNA. The GAL4-VP16-derived proteins bind to GAL4-recognized UASs (open arrows) and promote transcription of *HIS3* to confer histidine prototrophy, *ADE2* to confer adenine prototrophy and *lacZ* for detectable β-galactosidase activity (solid bars). The figures are not drawn to scale.

Ribozymes designed to splice *GAL4*-derived transcription activator sequences into the CP mRNA of CMV were created in both *cis*- and *trans*-splicing configurations (Fig. 1A and B). Ribozymes targeted to CMV sequences are desirable as CMV

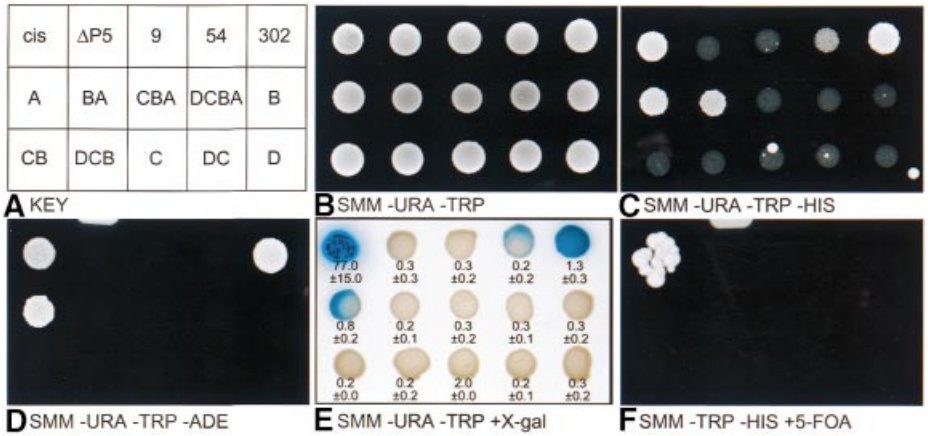

Figure 2. Ribozyme-mediated phenotype alteration on indicator media. (**A**) Legend for the position of each yeast strain harboring pCMV-GFP(URA3), and the indicated ribozyme construct. (**B**-**F**) Growth of yeast strains on the indicated plates. (**E**) Quantitative β-galactosidase values were obtained from four experiments, and variation is expressed as standard deviation. Plates were photographed after 5 days incubation at 30°C, except (E), which was photographed after an additional 2 weeks incubation at 4°C.

is a prevalent agricultural pathogen with a host range exceeding 800 species, and is recognized as the causal agent of numerous disease epidemics worldwide (13). To facilitate monitoring the target in the yeast assay, the CP sequences were fused in frame to sequences encoding the GFP (CMV-GFP; Fig. 1B). The sole sequence requirement in the target mRNA is a uridine that forms a wobble base pair with a guanosine in the internal guide sequence (IGS) of the ribozyme, thereby defining the 5′ splice site (24,25). A uridine 10 nt downstream of the CP AUG initiation codon was chosen as the 5′ splice site. Selecting a uridine close to the initiation codon minimizes the N-terminal fusion produced after splicing to the 3′ exon. Furthermore, selecting a splice site close to the 5′ terminus of the target transcript may facilitate helix formation between the target and the ribozyme (4). An inactive control target was also created to gauge ribozyme specificity by mutating this uridine to a guanosine, thereby preventing recognition of the 5′ splice site (MUT-GFP).

Sequences in the intron IGS were altered from wild type *Tetrahymena* sequences to allow formation of an artificial helix P1 with the CMV target while maintaining the essential U:G wobble base pair at the 5′ splice site (Fig. 1A and B). A splicing-deficient control ribozyme was created to monitor splicing-independent expression of the 3′ exon. This construct, pΔP5RzGVP, is identical to pcisRzGVP (Fig. 1A), except that the P5abc region of the intron core, which is essential for splicing avtivity (26), is deleted.

The 3′ exon encoding a GAL4-derived transcription activator was fused downstream of the ribozyme so that splicing would result in an in frame fusion to the target coding sequence (Fig. 2C). The *GAL4*-derived 3′ exon does not contain an AUG start codon which otherwise may contribute to spurious expression. Sequences at the 5′ end of the 3′ exon and in the IGS were adjusted to allow formation of a P10 duplex (Fig. 1A and B), which is hypothesized to facilitate alignment of the exons (27), and is demonstrated to improve *trans*-splicing efficiency (7).

Previous studies indicated that increasing the length of the EAS improves both *trans*-splicing efficiency and specificity (7,11). To test the universality of this finding, the EAS regions in this work ranged from 0 nt (p9RzGVP) to 764 nt (pDCBARzGVP) (Fig. 1B). Furthermore, previous *trans*-splicing ribozyme designs incorporated an unpaired region between helix P1 and the EAS/target duplex to potentially facilitate formation of helix P10 after the first transesterification reaction. To gauge the tolerated gap between helix P1 and the EAS/target duplex, the EASs of the ribozymes were designed to leave target sequences ranging from 5 to 605 nt unpaired, while the unpaired ribozymes sequences were maintained at 10 nt in all constructs containing an EAS (Fig. 1B).

Complementation of metabolic deficiencies

Yeast was selected as a eukaryotic model organism for developing and assaying the *trans*-splicing ribozymes since yeast is easy to manipulate, grows rapidly and well developed genetic tools are available. In addition, yeast shares many aspects of gene expression and cellular organization with higher eukaryotes that may affect ribozyme efficacy, but cannot be addressed in prokaryotic systems. The yeast strain used, PJ69-4a (23), has selectable marker genes downstream of GAL4-recognized UASs that are expressed only in the presence of a GAL4-derived transcription activator: the *GAL1-HIS3* gene to confer histidine prototrophy, the *GAL2-ADE2* gene to confer adenine prototrophy and the *GAL7-lacZ* gene to enable β-galactosidase assays (Fig. 1D).

PJ69-4a cells harboring either the target construct, pCMV-GFP(URA3), or mutant target construct, pMUT-GFP(URA3), were individually transformed with each of the 15 RzGVP plasmids, and selected on –URA –TRP. Individual colonies were inoculated into –URA –TRP broth, grown overnight and 1×10^4 cells of each were spotted onto selective media (Fig. 2). Growth of all strains was rapid on –URA –TRP medium, as expected since selection is for the presence of target and ribozyme plasmids only (Fig. 2B). The growth rate of strains harboring pcisRzGVP was reduced relative to the others (not shown), suggesting expression of the GAL4-VP16 transcription activator from a *cis*-splicing construct is strong enough to be mildly toxic. A reduction in growth rate resulting from high expression levels of GAL4-VP16 is documented (28).

On media indicative of GAL4 activity, cells harboring pcisRzGVP were prototrophic for histidine and adenine, and β-galactosidase activity was observed (Fig. 2C, D and E, respectively), demonstrating efficient activation of the *GAL1-HIS3*, *GAL2-ADE2* and *GAL7-lacZ* indicator genes. As expected for this *cis*-splicing construct, GAL4 activity was evident in the presence (Fig. 2C–E) and absence of target construct, pCMV-GFP(URA3), as evident by growth on indicator medium containing 5-FOA (Fig. 2F). Importantly, growth on media indicative of GAL4 activity was not observed with cells expressing the inactive, internally deleted ribozyme, ΔP5RzGVP, indicating that complementation of the metabolic deficiencies was strictly dependent on delivery of GAL4 activity via splicing. These results demonstrate that (i) the modifications made to sequences flanking the intron catalytic core do not prevent splicing activity, (ii) splicing yields a translatable chimeric mRNA encoding a GAL4-derived transcription activator and (iii) the transcription activator with a short CMV CP-derived peptide fusion is active in binding DNA at GAL4-recognized UASs, and is able to promote transcription of the downstream genes.

For cells harboring *trans*-splicing ribozymes, growth on media indicative of GAL4 activity was observed in conjunction with the proper target (Fig. 2C and D), but not with the mutant target [pMUT-GFP(URA3); not shown], nor in the absence of target (Fig. 2F). These results demonstrate a strict reliance on the target mRNA for expression of the *GAL4* sequences fused to the *trans*-splicing ribozymes. On –URA –TRP –HIS medium (Fig. 2C), the relative growth rates of strains exhibiting growth were p302RzGVP > pARzGVP > pBARzGVP > p54RzGVP. The EAS lengths in these constructs are 293, 200, 401 and 45 nt, respectively. No growth was evident in the other strains. On –URA –TRP –ADE medium (Fig. 2D), growth was only observed with the *trans*-splicing constructs p302RzGVP, and pARzGVP (p302RzGVP > pARzGVP). The *GAL2-ADE2* gene of PJ69-4a is a more stringent marker than the *GAL1-HIS3* gene (23), and it is thus not surprising that strains with limited growth on –URA –TRP –HIS medium were unable to propagate on –URA –TRP –ADE medium. Figure 2E demonstrates that delivery of the GAL4 activity via *trans*-splicing also activates the *lacZ* gene from the *GAL7* promoter. Values obtained from quantitative β-galactosidase assays supported the findings obtained by observing growth rates on –URA –TRP –ADE medium: p302RzGVP confers greater GAL4 activity in the presence of the target than the other *trans*-splicing constructs.

To establish the accuracy of *trans*-splicing, total RNA was isolated from cultures harboring the target construct pCMV-GFP(URA3), and either p302RzGVP or pARzGVP. RT–PCR was performed with a 5′ primer corresponding to a sequence in the 5′ exon of the target, and a 3′ primer corresponding to a sequence in the GVP 3′ exon of the ribozyme. The amplified cDNA products were sequenced, and found to contain the expected sequence at the splice junction (AUG GAC AAA U/UU AGG UAC C; Fig. 1C).

Genetic selection assay

Based on the finding that the ribozymes conferred target-dependent growth with different efficiencies, a genetic screen for selecting optimized *trans*-splicing constructs from a ribozyme library was established. It was predicted that colonies harboring the most efficient ribozyme constructs would predominate on selective media, relative to less efficient constructs. The 13 *trans*-splicing ribozyme constructs were mixed in equal proportions to create a ribozyme library. This library was then introduced to PJ69-4a expressing either the proper target or the mutant target, and the number of colonies growing on selective media was scored (Table 1).

Colony development on –URA –TRP indicated that 1.3×10^5 total transformants were obtained for strains harboring either the proper or mutant target. For the pMUT-GFP(URA3) mutant target strain, no colonies developed on –URA –TRP –HIS medium, as expected from the established specificity of the *trans*-splicing reaction. For the pCMV-GFP(URA3) target strain, 1140 colonies became established after 5 days of growth. Twenty-four of these were selected at random and patched to –HIS medium, where growth was rapid (Fig. 3A). Each was then transferred to –HIS +5-FOA medium to assay for the maintenance of histidine prototrophy after loss of the target plasmid (Fig. 3B). Twenty-three strains did not show growth after 5 days incubation, indicating a reliance on the *URA3* encoding target plasmid for complementation of the histidine deficiency. One strain became established on –HIS +5-FOA medium, indicating target-independent histidine prototrophy. Since each of the plasmids in the ribozyme library was individually demonstrated to confer histidine protrophy in a target-dependent fashion, prototrophy in this strain probably resulted from a rare DNA rearrangement.

Ribozyme-encoding plasmids were rescued from each of the 24 histidine prototrophs, and identified by restriction digest analysis. The 23 strains dependent on the target for histidine prototrophy contained the most efficient construct: p302RzGVP. This result demonstrates the effectiveness of phenotypic conversion to prototrophy for selecting the most efficient ribozymes from a library of constructs. The splice junction was characterized in two of the cultures that demonstrated target-dependent growth on –HIS medium by RT–PCR and sequencing. The amplified cDNA had the

Table 1. Colony formation of strains harboring either pMUT-GFP(URA3) or pCMV-GFP(URA3) target plasmids on selective media after introduction of the indicated ribozyme library

Plasmids introduced	Colony development on SMM media			Plasmids recovered[a]
	–URA –TRP	–URA –TRP –HIS	–HIS +5-FOA[b]	
pCMV-MUT(URA3) + *trans* Rz library	1.3×10^5	0	n.a.	n.a.
pCMV-GFP(URA3) + *trans* Rz library	1.3×10^5	1140	1/24	23/23 p302Rz-GVP
pCMV-GFP(URA3) + *cis/trans* Rz library	1.3×10^5	1200	4/6	2/2 p302Rz-GVP

n.a., not applicable.
[a]Proportion of Rz plasmids identified from strains demonstrating target-dependent growth on –HIS medium.
[b]Proportion of analyzed strains prototrophic for histidine independent of the *URA*-encoding target plasmid.

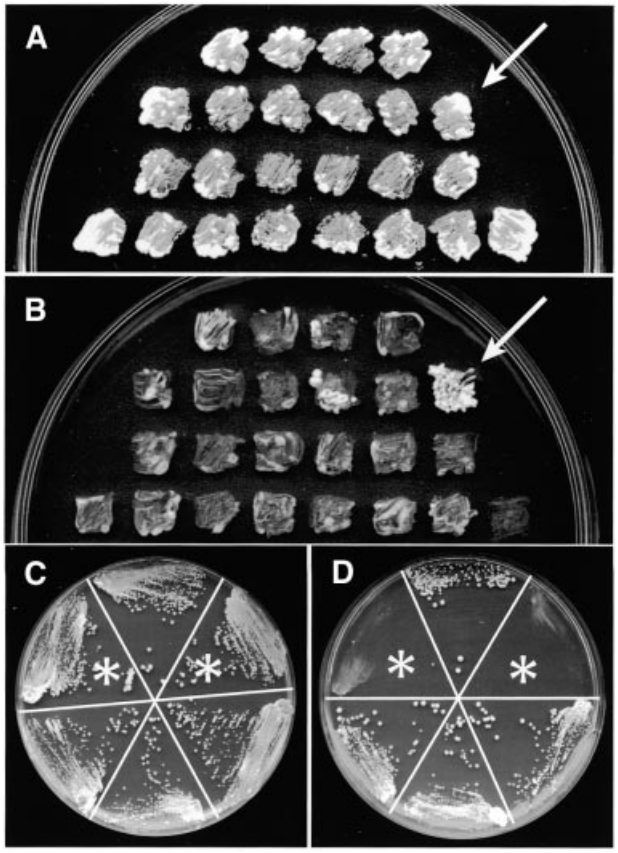

Figure 3. Test for target-independent growth on selective media. (**A**) Twenty-four strains transformed with pCMV-GFP(URA3) and the *trans*-splicing ribozyme library patched to –HIS medium. Strains were chosen randomly from the colonies that became established after introducing the ribozyme library to pCMV-GFP(URA3) cultures and selecting for histidine prototrophy on –URA –TRP –HIS medium. (**B**) Yeast stains from (A) patched to –HIS +5-FOA medium. One strain that exhibited target-independent growth (i.e. growth on medium containing 5-FOA) is indicated by the arrow. (**C**) Six strains transformed with pCMV-GFP(URA3) and the *cis/trans*-splicing ribozyme library streaked to –HIS medium. Strains were chosen randomly from those that became established after introducing the ribozyme library to pCMV-GFP(URA3) cultures and selecting for histidine prototrophy on –URA –TRP –HIS medium. (**D**) Yeast stains from (C) streaked to –HIS +5-FOA medium. The two strains that required the target plasmid for histidine prototrophy (i.e. failure to grow in the presense of 5-FOA) are indicated by asterisks.

expected sequence (AUG GAC AAA U/UU AGG UAC C; Fig. 1C).

Counter selection against indiscriminate splicing

The 13 *trans*-splicing ribozymes analyzed in this study did not complement the metabolic deficiencies of the yeast host strain in the absence of the intended target (Fig. 2F). Therefore, based on this criterion of phenotypic conversion to prototrophy, each demonstrated high specificity. However, in a library of greater complexity, target-independent expression of the 3′ exon might occur by either non-specific *cis*- or *trans*-splicing. For non-specific *cis*-splicing, at least three criteria must be met: (i) a region upstream of the ribozyme IGS (i.e. the EAS) must be able to fold back and base pair with the IGS to form a P1 helix; (ii) this unintended helix must contain a U wobble base paired with a G in the IGS at position 4, 5 or 6 of the P1 stem to define the 5′-splice site (25,29); and (iii) for translation of the spliced RNA, sequences upstream of the spurious 5′ splice site must contain a potential AUG start codon positioned such that it is in frame with the 3′ exon following splicing. Implicit in the third criterion is that there are no stop codons between the AUG and the 3′ exon-encoded open reading frame of the splice product. For non-specific *trans*-splicing to occur, an unintended helix P1 must be able to form between the ribozyme IGS and a separate RNA molecule, followed by criteria (ii) and (iii) discussed for *cis*-splicing. It is worth noting that available evidence suggests that the presence of an EAS minimizes indiscriminate interactions between the ribozyme IGS and other transcripts (11).

Nevertheless, since expression of the 3′ exon-encoded gene could theoretically occur via indiscriminate splicing, a practical genetic selection scheme for optimized *trans*-splicing constructs must be able to differentiate between target-specific and non-specific events. Therefore, the target gene was maintained on a plasmid encoding the *URA3* gene, and could be eliminated by plating to medium containing 5-FOA. Strains emerging from a ribozyme library on –HIS medium, which are also able to grow on –HIS +5-FOA medium, would harbor indiscriminate ribozymes, whereas those unable to grow on –HIS +5-FOA medium would contain ribozymes exhibiting high target specificity and efficiency.

To test whether counter selection on 5-FOA medium can effectively differentiate between desirable target-dependent ribozymes, and undesirable indiscriminate ones, the *trans*-splicing ribozyme library was 'spiked' with the self-splicing construct, pcisRzGVP, and the genetic selection assay was repeated (Table 1). Six colonies growing on –URA –TRP –HIS medium were streaked to –HIS media, and subsequently to –HIS +5-FOA medium (Fig. 3C and D). Of these six strains prototrophic for histidine, two did not grow on –HIS +5-FOA medium, indicating a dependence on the target plasmid for prototrophy. The ribozyme plasmid identified in these two was the most efficient *trans*-splicing construct, p302RzGVP. Therefore, target-dependent and -independent expression of the GAL4 encoding 3′ exon is readily differentiated by counter selection for the target plasmid.

Negative selection

Although the *trans*-splicing ribozymes described are specific enough to prevent target-independent growth on selective media (Fig. 2F), a more stringent assay for scrutinizing spurious expression of the 3′ exon may be desirable in certain applications. Previous work established that low levels of 3′ exon expression could be monitored by using a 3′ exon encoding the DTA chain polypeptide, and measuring growth rates in liquid media relative to appropriate controls (11). In this study, we used this growth-rate assay to establish the effect of the EAS on minimizing 3′ exon expression, since it is hypothesized that increasing EAS length improves specificity by 'shielding' the active site from non-specific transcripts (11). The *GVP* 3′ exons were therefore replaced with *DTA* sequences, and the growth rates of cultures harboring p2ATΔP5DTA, p2AT9RzDTA, p2AT54RzDTA, pARzDTA, p2AT302RzDTA, pBARzDTA, pCBARzDTA and pDCBARzDTA were established in the absence of target relative to a strain harboring the pADH424 parental plasmid (Fig. 4).

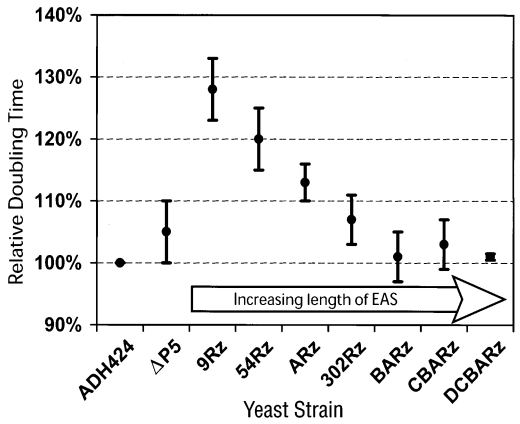

Figure 4. Relative growth rate of strains expressing ribozymes with DTA sequences as the 3′ exon. Relative doubling times of yeast strains harboring the indicated RzDTA constructs normalized to the parent construct, pADH424 (100%), in the absence of the intended target. The *trans*-splicing constructs are arranged by increasing length of EAS. Results are from three independent trials and variation is expressed as standard error of the mean.

The doubling time of cultures harboring p2ATΔP5DTA was 5% greater than those harboring pADH424. Ribozymes derived from the *T.thermophila* rRNA intron that lack the P5abc region are inactive (11,26). The increase in doubling time is therefore independent of splicing. Cultures expressing ribozymes fused to *GFP* sequences do not demonstrate growth reductions relative to controls, indicating that the ribozyme itself is not toxic (B. G. Ayre, unpublished). The growth rate reduction in cultures harboring p2ATΔP5DTA is thus attributed to spurious expression of the *DTA* sequences. Translation initiation at a ribozyme internal AUG sequence, in conjunction with frame shifting events, is the simplest explanation for 'leaky' expression of the 3′ exon.

For the *trans*-splicing ribozymes, an inverse trend was observed between EAS length and growth rate: shorter EAS sequences had longer doubling times (Fig. 4). Cultures expressing ribozymes with EAS lengths of 0 nt (p2ATRzDTA), 45 nt (p2AT54RzDTA) and 200 nt (pARzDTA) grew slowly relative to strains expressing the inactive ribozyme (p2ATΔP5DTA). These reductions in growth rates are thus ribozyme dependent, but target independent, and may result from indiscriminate splicing. Cultures expressing a ribozyme with 293 nt of EAS (p2AT302RzDTA) grew at a rate equivalent to p2ATΔP5DTA cultures, suggesting that indiscriminate splicing is minimized. Significantly, strains expressing ribozymes with 401, 601 and 764 nt of EAS (pBARzDTA, pCDARzDTA and pDCBARzDTA, respectively) grew faster than the inactive ribozyme control, suggesting that the EAS region not only limits non-specific splicing but may also buffer potential toxicity by minimizing read-through effects. These results demonstrate the utility of this growth-rate assay for monitoring subtle differences in 3′ exon expression among different constructs with high sensitivity.

DISCUSSION

This work arose from the question of whether or not *trans*-splicing ribozymes could be used as a general mechanism for delivering new gene activities dependent on the presence of a particular target transcript. We previously demonstrated that similar ribozymes could deliver sufficient cytotoxic gene activity to prevent colony formation in yeast, and could therefore potentially be used to specifically ablate virus-infected or malignant cells (11). For the work presented here, it was rationalized that transcription factors, like cytotoxins, are sensitive biological indicators, with a small number of molecules being sufficient to activate high levels of downstream gene expression. If *trans*-splicing ribozymes could deliver sufficient quantities of a transcription factor, the transcription factor could in turn drive the expression of virtually any gene of interest at biologically relevant levels.

To explore this possibility, ribozymes targeted against CMV CP sequences were created with 3′ exon sequences encoding a GAL4-derived transcription factor. Splicing was expected to result in a chimeric mRNA consisting of the transcription factor sequences fused in frame with the AUG start codon of the CMV CP target. The translation product of this mRNA was in turn expected to bind at GAL4-recognized UASs, and induce transcription of the downstream genes. The downstream genes of interest were *HIS3* to confer histidine prototrophy, *ADE2* to confer prototrophy for adenine and *lacZ* to confer β-galactosidase activity (Figs 1 and 2).

To establish the assay, and demonstrate its utility for rapidly assessing ribozyme function in eukaryotic cells, a series of ribozymes were constructed with EASs ranging from 0 nt (p9RzGVP) to 764 nt (pDCBARzGVP) (Fig. 1B). Previous work indicated that increasing the length of EAS improved both *trans*-splicing efficiency (7) and specificity (11). Consistent with previous findings, increasing the length of the EAS to 293 nt resulted in increased levels of gene delivery. However, beyond this length, efficiency dropped. *Trans*-splicing activity was observed with an EAS of 401 nt, but not with 601 nt or 764 nt (Fig. 2C).

From this result, we conclude that the length of antisense sequence does not contribute directly to ribozyme efficiency. More likely, the EAS provides one or more short stretches of solute-exposed nucleotides which are able to base pair with solute-exposed nucleotides in the target transcript. This initial pairing would anchor the ribozyme to the target, and duplex formation between the two molecules would then align the 5′ splice site of the target with the ribozyme active site to enable the splicing reaction. Similar mechanisms of helix formation across important regulatory sequences from an initial site of nucleation are well characterized in natural antisense interactions (30).

Based on this theory, we propose that the ribozyme with 293 nt of extended antisense sequence is the most efficient because it serendipitously contains nucleotides which are best able to interact with the target. Longer antisense sequences would contain these sequences, as might shorter sequences, however the nucleotides may not be available for interaction with the target due to unique secondary and tertiary structures within the transcripts. If this model is correct, more efficient ribozymes with shorter antisense sequences should be possible, providing that the antisense sequences are solute-exposed and complementary to solute-exposed target sequences. However, empirical design and *in vivo* testing of ribozymes is laborious, and although several computer-based and *in vitro* technologies have been applied to optimizing

ribozymes (14,31), they cannot predict the behavior of a transcript in the complex cellular environment.

A more efficient approach would be to create a population of constructs and use *in vivo* genetic selection to identify individuals with desirable characteristics from the pool. Phenotypic conversion from histidine auxotrophy to prototrophy in yeast was therefore used to select ribozymes with high affinity for the CMV CP-derived target from a library of constructs. Of the strains emerging from the genetic selection assay, 24 were analyzed in detail, and 23 harbored p302RzGVP. This result correlates well with those obtained when the constructs were tested individually: yeast strains harboring the target plasmid and p302RzGVP became established on selective media more rapidly than those harboring the other *trans*-splicing constructs (Fig. 2C). Selection for adenine prototrophs could similarly be used (Fig. 2D), and assaying β-galactosidase activity (Fig. 2E) provides a further measure of relative ribozyme activity.

The *trans*-splicing ribozyme library was created by directional cloning of PCR-amplified target gene segments, such that each ribozyme contained target sequences in the antisense orientation. This approach allowed precise analysis of defined constructs, but limited library complexity. Libraries of greater complexity may contain ribozymes with improved target affinity since each unique transcript is expected to have different solute-exposed regions due to variations in secondary and tertiary structures, or protein interactions. To create libraries of greater complexity, target sequences could be sheared, or digested with Dnase I, and cloned upstream of the ribozyme catalytic core. Alternatively, a random sequence library could be constructed from synthetic oligonucleotides. The same library could then be used to select efficient interactions with any target transcript of interest, and the interactions would not necessarily rely on strict Watson–Crick base pairings. Non-canonical interactions, such as tetra-loop interactions with the minor groove of double-stranded RNA (32,33), can have greater stability than standard Watson–Crick interactions. Furthermore, most naturally occurring antisense interactions 'display' antisense sequences in a loop structure terminating a duplexed stem (30,34). Displaying random sequences at the end of a stem–loop structure incorporated into the EAS region may similarly improve interaction between the ribozyme and the target. Random oligonucleotide libraries for selecting optimal target sites *in vitro* are described with 5×10^9 individual ribozyme genes (14). Libraries for *in vivo* screening could in principle be scaled to any size, but in practice would be subject to the same constraints that apply to yeast 'two-hybrid' protein interaction screens (23). Initial experiments with random sequence libraries are encouraging (B. G. Ayre, unpublished).

Although a number of strategies could be used to create a ribozyme library, some individuals within the library may exhibit target-independent *cis*- or *trans*-splicing, and result in false positives. A practical genetic selection scheme for identifying efficient *trans*-splicing constructs from a large pool must therefore readily differentiate target-dependent *trans*-splicing from non-specific events. For this reason, the CMV-GFP target gene was cloned into a yeast plasmid encoding the *URA3* gene, and could be selected against by plating cells on media containing 5-FOA. In an experiment to select efficient *trans*-splicing ribozymes from a library containing *cis*-splicing constructs, it was found that transferring colonies from –HIS medium to –HIS +5-FOA medium was effective in differentiating target-independent events from legitimate *trans*-splicing (Fig. 3). Yeast strains that were not able to grow on –HIS +5-FOA medium harbored the most efficient *trans*-splicing construct, p302RzGVP (Table 1).

Trans-splicing ribozymes incorporating *GAL4*-derived sequences as the 3′ exon did not confer growth to yeast on selective media in the absence of the specific target (Fig. 2F), and are therefore specific as defined by the criterion of phenotypic conversion to prototrophy. However, to assess non-specific expression of the 3′ exon with greater stringency, a very sensitive and straightforward assay was employed by replacing GAL4 3′ exon sequences with those encoding DTA chain, and measuring growth rates in liquid culture. In this assay, spurious expression of the 3′ exon was evident as increases in culture doubling time (Fig. 4). Some of this expression was independent of ribozyme activity, as indicated by the increased doubling time of strains containing the splicing-deficient construct, p2ATΔP5DTA. Ribozyme-independent expression may result from non-canonical initiation, stop codon read-through, or frame-shift translation of the 3′ exon (there is no in frame AUG).

With active *trans*-splicing RzDTA constructs, a general inverse trend was observed between the length of the EAS and spurious 3′ exon expression. Yeast harboring constructs with relatively short EAS regions (i.e. p2AT9RzDTA, p2AT54RzDTA and pARzDTA) demonstrated 3′ exon expression exceeding that of the splicing-deficient control, suggesting indiscriminate splicing between these ribozymes and non-target transcripts. Ribozymes with longer EAS regions demonstrated 3′ exon expression equivalent to, or less than, the splicing-deficient control, supporting the premise that increasing the length of the EAS minimizes ribozyme interaction with non-targeted sequences. It was previously argued that longer EAS regions may form fortuitous secondary and tertiary interactions that exclude non-specific mRNAs from entering the active site. In contrast, interaction between the intended target and the EAS would initiate formation of an extended duplex and resolve these structures. The 5′ splice site of the intended target would then enter the ribozyme active site, and accurate splicing would proceed (11). It is noteworthy that ribozymes with the longest EAS regions, pBARzDTA, pCBARzDTA and pDCBARzDTA, demonstrated 3′ exon expression levels less than that of the splicing-deficient control, indicating the EAS also minimizes splicing-independent effects.

To summarize, we demonstrate that *trans*-splicing ribozymes can be used to deliver new transcription factors to living cells dependent on the presence of an intended mRNA target. The transcription factor can in turn drive the expression of a chosen gene, or genes, at biologically relevant levels, and have profound effects on the host organism. The array of genetic tools available and ease of manipulation make yeast an attractive model for developing RNA-based therapeutics, yet yeast has proved recalcitrant to *trans*-acting ribozyme technologies (35). Our results demonstrate functionality in yeast, and the specific delivery of growth-promoting gene activities presents a practical approach to applying *in vivo* genetic selection to ribozyme development in a eukaryotic host. Optimized ribozyme designs could then be readily introduced

to higher eukaryotes for toxin-mediated ablation, or induction of gene activity by a *trans*-activator, dependent on the presence of a specific target mRNA. Work is currently underway to test the *trans*-splicing ribozyme in higher plants. We anticipate that this technology will have broad applications in diverse fields such as biotechnology, therapeutics and genomic studies.

Furthermore, the genetic screen, as presented, identifies efficient and specific associations between the target and the antisense sequences of the ribozyme. Interaction with an intended target is the first and probably rate-limiting step in *trans*-splicing and *trans*-cleaving ribozyme technologies, as well as in RNA- and DNA-based antisense technologies. *Trans*-splicing ribozymes for genetic selection of optimal target interactions could thus be adapted to these other applications. For example, a *trans*-splicing ribozyme could be converted to a cleaving ribozyme by incorporating a hammerhead domain into the extended antisense region, or the group I intron could simply be deleted to create a provocative antisense design. This technology may therefore provide an *in vivo* alternative to computer prediction and *in vitro* selection of 'optimized' target sites (14), which do not take into account the inherent structure of RNA in the cellular environment, nor the extent to which proteins prevent RNA–RNA interactions. Apart from optimizing target–ribozyme interactions, the screen could also be used as an alternative to biochemical optimization of the ribozyme itself (36).

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