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Twin ribozyme mediated removal of nucleotides from an internal RNA site

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Abstract

Over the past two decades, the structure and mechanism of catalytic RNA have been extensively studied; now ribozymes are understood well enough to turn them into useful tools. After we have demonstrated the twin ribozyme mediated insertion of additional nucleotides into a predefined position of a suitable substrate RNA, we here show that a similar type of twin ribozyme is also capable of mediating the opposite reaction: the site-specific removal of nucleotides. In particular, we have designed a twin ribozyme that supports the deletion of four uridine residues from a given RNA substrate. This reaction is a kind of RNA recombination that in the specific context of gene therapy mimics, at the level of RNA, the correction of insertion mutations. As a result of the twin ribozyme driven reaction, 17% of substrate are converted into the four nucleotides shorter product RNA.
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RNA engineering has been shown to be a potent strategy for the development of molecular tools that have potential for a variety of applications in molecular biology, gene therapy, and bioanalytics. For example, antisense RNA, small interfering RNA as well as a number of ribozymes have been used to silence undesired gene expression [1–4]. Furthermore, the combination of ribozymes with aptamers has led to aptazymes, which are catalytic RNA molecules, the activity of which can be regulated by external effectors [5]. This feature has allowed engineering reporter ribozymes that are capable of fast and reliable detection of small organic molecules, metal ions, oligonucleotides, peptides and protein domains [6,7].

Based on rational design strategies, we have developed a number of functional RNAs: twin ribozymes for RNA double cleavage [8], for site-specific RNA labeling and alteration of RNA sequence [9–12] as well as a redox-sensitive riboswitch [13]. Among these developed RNAs, twin ribozymes support the insertion of additional nucleotides into suitable RNA substrates [9–11]. We have extended this strategy and now present a twin ribozyme that mediates the site-specific removal of nucleotides from an arbitrarily chosen RNA substrate, thus demonstrating another type of a ribozyme mediated RNA processing reaction.

Twin ribozymes are derived from the hairpin ribozyme, a small naturally occurring RNA structure. The hairpin ribozyme is derived from the negative strand of the tobacco ringspot virus satellite RNA [14,15]. It catalyzes the reversible cleavage of a specific phosphodiester bond within a suitable RNA substrate, with the internal equilibrium of the hairpin ribozyme reaction shifted towards ligation [16]. While the cleavage reaction produces characteristic fragments with 5′-OH and 2′,3′-cyclic phosphate termini, respectively, ligation proceeds via ring opening of the cyclic phosphate, and thus is associated with a beneficial enthalpy. This enthalpy win is sufficient to compensate for the entropic cost of ligation, thus making ligation the preferential reaction in equilibrium. Equilibration in favor of ligation however, is only possible, if substrate/product fragments remain tightly bound to the ribozyme. If on the contrary, cleavage fragments can easily dissociate and if dissociation is faster then re-ligation, the gain of entropy becomes significant and the cleavage reaction is favored.

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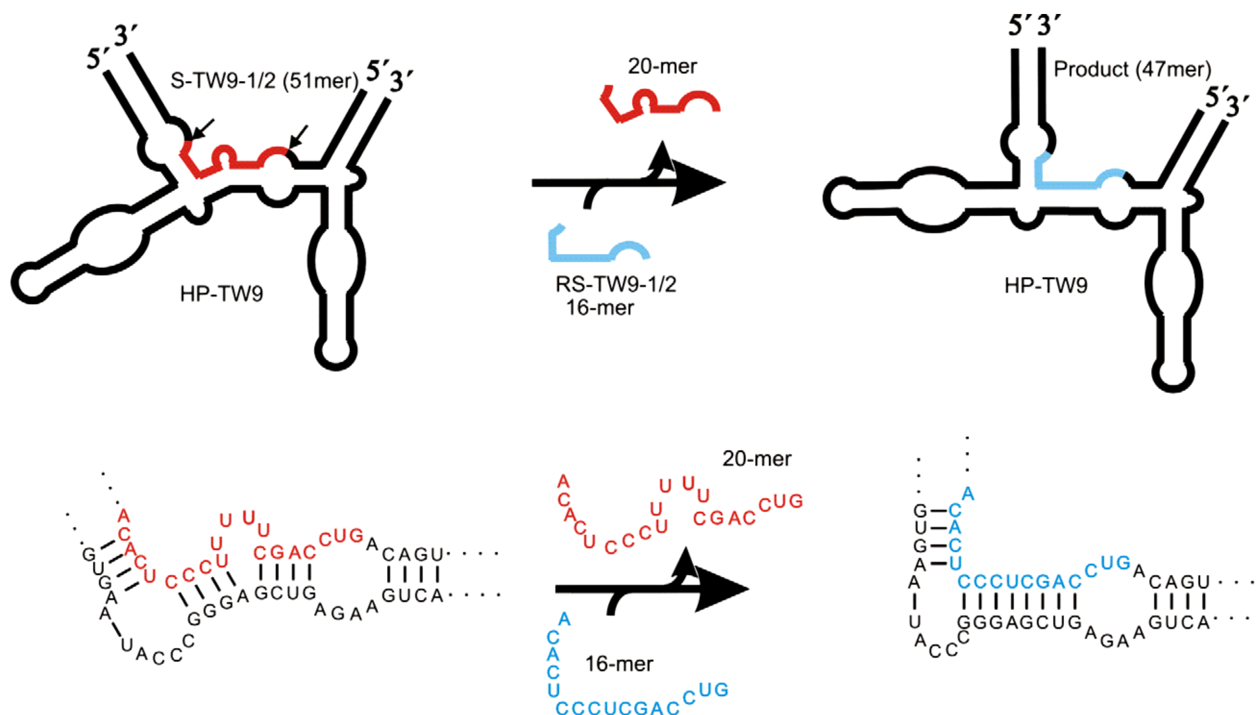

Fig. 1. Twin ribozyme mediated sequence exchange reaction. Substrate RNA S-TW9-1/2 is annealed to twin ribozyme HP-TW9 and cleaved at two different sites indicated by arrows. The fragment extending between the two cleavage sites (20-mer, red) is replaced on the ribozyme by the insertion oligonucleotide RS-TW9-1/2 (16-mer, blue), which subsequently becomes ligated to the flanking substrate fragments to form the ribozyme–product complex. In two parallel experiments, either a non-labeled substrate (S-TW9-1) was used in conjunction with a fluorescently labeled insertion oligonucleotide (RS-TW9-1) (compare Fig. 2), or the substrate RNA was labeled at both termini with a fluorescent dye (S-TW9-2) and reacted with a non-labeled insertion fragment (RS-TW9-2) (compare Fig. 3). The lower part of the figure shows the sequences of the central part of the twin ribozyme–substrate duplex and of the twin ribozyme–product duplex.

This characteristic feature allows modulating hairpin ribozyme activity by structural manipulation. Strikingly, hairpin ribozymes with substrates bound in a stable secondary and tertiary structure, favor ligation, while less stable ribozyme–substrate complexes preferentially undergo cleavage [16–18].

We have made use of this characteristic feature of the hairpin ribozyme for the design of an RNA repair ribozyme. Following a rational design strategy, we have combined two hairpin ribozymes into one molecule (dubbed twin ribozyme). The engineered twin ribozyme HP-TW9 (Fig. 1) supports two RNA cleavages and two ligation reactions in a strictly controlled fashion. The fragment resulting from cleavage in the first step of the reaction forms a distorted duplex with the ribozyme binding arm, and thus can easily dissociate. It is replaced at the ribozyme with a fragment that forms a more stable duplex, thus being preferentially ligated. Based on the specific design of this system, the final RNA product is shortened by four-specific nucleotides.

Materials and methods

Preparation of twin ribozyme HP-TW9. Twin ribozyme HP-TW9 was transcribed *in vitro* from a double stranded DNA template. To this end, two synthetic DNA primers (5′-CGA ATG TAA TAC GAC TCA CTA TAG GGA GAA AGA GAG AAG TGA ACC AGA GAA ACA CTG CGC TTC GGC GCA GGT ATA TTA CCT GGT ACC CGG GAT CTG-3′ and 5′-TAG CGC TGC AAG GGG TAG GTC GTA ATG TAC CAC GCG CGA ACG CGT GTG TTT CTG ACC TTG ACT TCT CAG ATC CCG GGT ACC AGG TAA TAT-3′, Purimex, Germany) overlapping at their 3′-ends by 24 complementary bases (underlined) were extended to double stranded DNA template using Klenow Fragment (MBI Fermentas, Germany) as described previously [8]. The DNA template was amplified by PCR using 0.04 U/ml Taq polymerase, 0.004 U/ml *Vent* polymerase (New England Biolabs, UK) and 100 pmol of each PCR primer (5′-CGA ATG TAA TAC GAC TCA-3′ and 5′-TAG CGC TGC AAG GGG-3′, Purimex). The DNA template was purified by electrophoresis on an 8% native polyacrylamide gel, eluted from the gel with 2 M $LiClO_4$ and precipitated from acetone. The ribozyme HP-TW9 was prepared by transcription of the DNA template with His-tagged T7 RNA polymerase as described previously [12].

Substrate and repair oligonucleotide synthesis. The amino-modified RNA substrates S-TW9-1 (5′-NH_2-C6-UAG CGC UGC AAG UGA CAG UCC AGC UUU UUC CCU CAC AGU CCU CUU UCU CCC-C3-NH_2-3′) and S-FR-TW9-2 (5′-GUC CAG C(NH_2-C6-dT)C CCU CAC AGU CCU CUU dT-3′) as well as the unmodified substrate S-FR-TW9-1 (5′-GUC CAG CUC CCU CAC AGU CCU CUU dT-3′) were prepared by solid phase synthesis on an automated synthesizer (Gene Assembler Special, Amersham Pharmacia Biotech) using standard PAC-phosphoramidites, TFA-amino-C6-CED phosphoramidite, thymidine succinyl polystyrene, 3′-amino-modifier and aminomodifier C6dT (Chemgenes Corporation, USA) as described previously [14]. For coupling of amino functions with ATTO680-NHS ester (ATTO-TEC GmbH, Germany), 200 μg dye in 50 μl dry DMF was added to 5 nmol oligonucleotide in 50 μl carbonate buffer (200 mM, pH 8.3). The reaction mixture was shaken at room temperature in the dark for 3 h. After ethanol

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