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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.

Keywords: Catalytic RNA; Gene therapy; Insertion mutation; Rational design; Ribozyme; RNA editing; RNA engineering; RNA repair

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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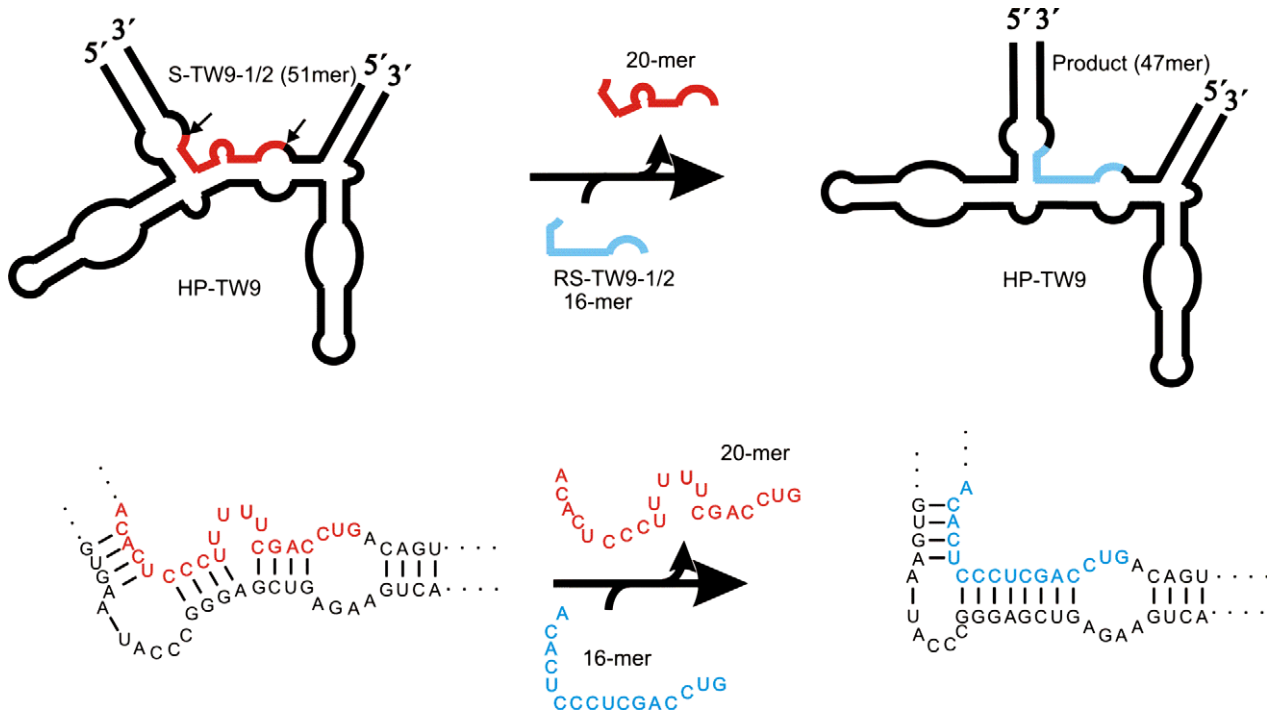

Fig. 1. Twin ribozyme mediated sequence exchange reaction. Substrate RNA S-TW9-1/2 is annealed to twin ribozyme HP-TW9 and cleaved at two different sites indicated by arrows. The fragment extending between the two cleavage sites (20-mer, red) is replaced on the ribozyme by the insertion oligonucleotide 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

precipitation, labeled oligonucleotides were purified by RP-HPLC on a 250-10 Nucleodur 100-5 C18 EC column (Machery-Nagel, Germany) using tetraethylammonium acetate elution buffer (0.1 M, pH 7.5) with acetonitrile gradient (5–30%). Product fractions were concentrated and desalted over a NAP column. Repair oligonucleotides RS-TW9-1 (5′-GUC CAG CUC CCU CAC A-3′) and RS-TW9-2 (5′-GUC CAG C(ATTO680-NH-C6-dT)C CCU CAC A-3′) containing a 2′,3′-cyclic phosphate group were obtained from cleavage of synthetic oligonucleotides S-FR-TW9-1 and S-FR-TW9-2 with a hairpin ribozyme derivative HP-WTL [19].

Twin ribozyme mediated RNA fragment exchange reaction. A solution of ribozyme HP-TW9 and substrate S-TW9-1 or S-TW9-2 in Tris–HCl, buffer, pH 7.5, was heated at 90 °C for 2 min followed by incubation at 37 °C for 15 min. After adding $MgCl_2$, the mixture was incubated for further 15 min at 37 °C. The exchange reaction was started by addition of the repair oligonucleotide. The final concentrations of the components in a 20 μl reaction volume were: 100 nM twin ribozyme, 100 nM substrate, 100 nM repair oligonucleotide, 10 mM $MgCl_2$, and 40 mM Tris–HCl, pH 7.5. The reaction was carried out at 37 °C for 8 h. One microliter aliquots were taken at suitable time intervals and added to 19 μl of a 7 M urea solution containing 50 mM EDTA to stop the reaction. Samples were analyzed on 15% polyacrylamide gels using a DNA Sequencer Long ReadIR 4200 (LI-COR Biosciences, USA); data were processed with the Gene ImagIR 4.05 software.

Results and discussion

Twin ribozyme HP-TW9 has been designed to bind a 51-mer RNA substrate, which in a two step reaction is converted into the 47-mer product. In the process, a fragment of 20 nucleotides of the substrate RNA needs to be replaced with a synthetic 16-mer fragment, carrying a 2′,3′-cyclic phosphate and being separately added to the reaction mixture.

As explained above, engineering of the twin ribozyme involved combination of two hairpin ribozymes into one molecule. The substrate S-TW9 is bound to the ribozyme, whereby the cleavage sites are defined as marked by arrows. In the central part of the substrate, four nucleotides (UUUU) are bulged out, weakening the binding of the red sequence patch. Thus, upon cleavage at both sites, the resulting fragment can easily dissociate from the ribozyme. Subsequently, the added 16-mer oligonucleotide (blue) can bind into the gap left behind by dissociation of the red sequence, now forming a contiguous duplex of eight base pairs. Even though the number of base pairs formed between the ribozyme and the added sequence patch (blue) is the same as between the ribozyme and the part of substrate that is cleaved out (red), the stabilization brought about by making the formerly distorted duplex (with the red sequence patch) now contiguous (with the blue sequence patch), should allow for preferential ligation. For experimental validation of this design, twin ribozyme HP- TW9 was prepared by run off transcription *in vitro* (see Materials and methods). The substrate S-TW9-1 and the repair oligonucleotide RS-TW9-2 were chemically synthesized (Materials and methods). RS-TW9-2 was internally labeled with a fluorescence dye (ATTO680) to allow for detection of the repair product as described before [11,12].

As can be seen from fragment length analysis (Fig. 2A), there is a clear conversion of the 16-mer repair oligonucleotide into three slower running species resulting from single site ligation to only one of the remaining substrate fragments (30-mer and 33-mer, respectively) as well as from ligation at both sites to give the full-length product RNA (47-mer). In the process, 17.2 ± 0.5% of the labeled repair oligonucleotide was incorporated into the RNA substrate (Fig. 2B). A similar result has been achieved using a substrate labeled with dyes at both termini and a non-labeled repair oligonucleotide (Fig. 3). With this approach, detection of the substrate (51-mer, Fig. 3A) as well as of the cleavage products (34-mer/17-mer, and 37-mer/14-mer, respectively) together with all ligation products is possible. As can be seen in Fig. 3A, again there is a clear conversion of the substrate RNA into slower running species, indicating the successful exchange of the 20-mer fragment (red in Fig. 1) against the 16-mer (blue in Fig. 1). As reproduced in

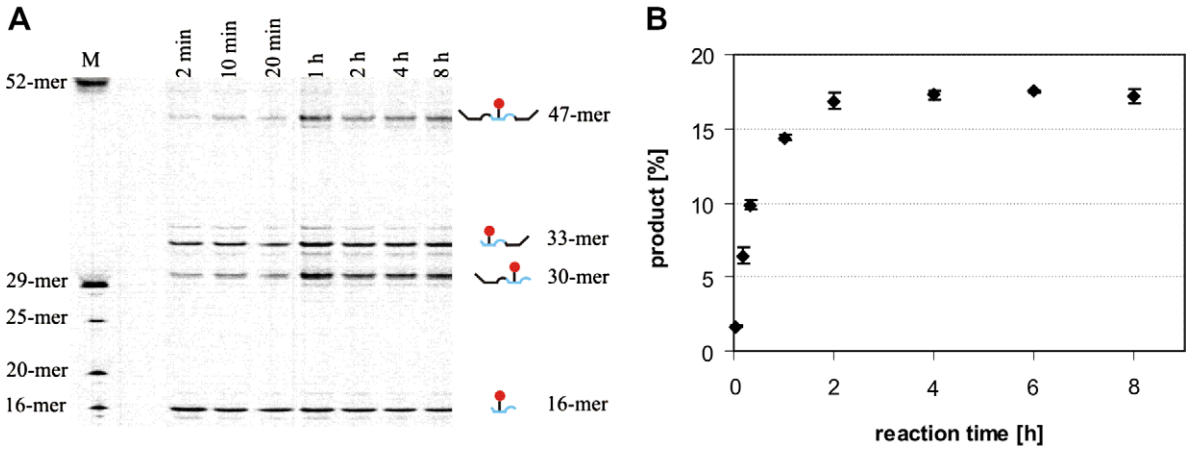

Fig. 2. (A) Monitoring of the sequence exchange reaction using a fluorescent-labeled insertion oligonucleotide (RS-TW9-1) and a non-labeled RNA substrate (S-TW9-1). Aliquots of the reaction were analyzed after time intervals indicated on top of each lane. M, length marker. Individual fragments were detected by excitation at 680 nm and emission of the dye at 700 nm using a LI-COR DNA Sequencer as described [12]. (B) Time course of the reaction (average yields of 47-mer product with standard deviations resulting from four independent measurements are shown).

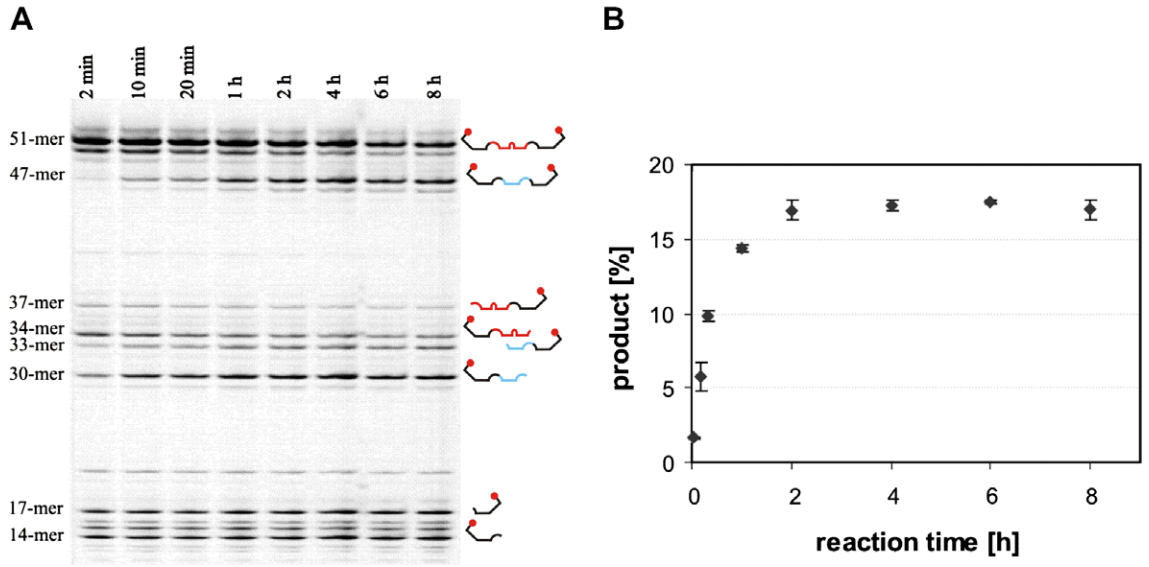

Fig. 3. (A) Monitoring of the sequence exchange reaction using a 5′,3′-end-labeled substrate (S-TW9-2) and a non-labeled insertion oligonucleotide (RS-TW9-2). Data were collected after time intervals indicated on top of each lane. Individual fragments were detected by excitation of ATTO680 at 680 nm and emission at 700 nm using a LI-COR DNA Sequencer as described [12]. (B) Time course of the reaction (average yields of 47-mer product with standard deviations resulting from three independent measurements are shown).

several experiments, there are two bands detected corresponding to substrate and two bands corresponding to product. We assume that the lower running species in each case results from a single end-labeled substrate, which could not be separated from the double end-labeled substrate by HPLC purification. In agreement with the results shown in Fig. 2, also this experiment shows $17.0 \pm 0.7\%$ conversion of the 51-mer substrate into the 47-mer product after 8 h (Fig. 3B).

Beyond its relevance for application in molecular biology and medicine, the described system demonstrates another type of RNA recombination reaction, the kind of which may have played a role in RNA world scenarios. There, RNA molecules being capable of supporting the insertion, removal or replacement of nucleotides within specific RNA sequence context may have contributed to extension of sequence space and thus to the enhancement of genetic diversity. In the context of medical application, the twin ribozyme mediated reaction mimics the repair, at the level of RNA, of small insertion mutations. The substrate binding sequence of the hairpin ribozyme can be easily adapted to other substrates [20] and thus offers the possibility of developing customized twin ribozymes. Alternatively, twin ribozymes that specifically recognize and process a given RNA may be selected from a partially randomized library.

Our results demonstrate that engineered twin ribozymes in addition to the repair of deletions and base replacements have the potential for site-directed repair of insertions. At present, the efficiency of the reaction is only 17%. The percentage of product yield strongly depends on the specific design of the system. The most crucial point is the number of canonical base pairs between the two cleavage/ligation sites. The size of the loop presumably is less important. It is required for duplex distortion, but has no influence on the stability of the newly formed duplex between the ribozyme and the fragment to be ligated. Thus, the dissociation capacity of the cleaved out fragment becomes most determining for the reaction efficiency. In previous studies [21] we have used a substrate that upon binding to the ribozyme formed a three uridine loop flanked by duplexes of 6 base pairs each (Table 1, 2 row). Under identical conditions as used here, the yield of the RNA product was only 5%. Thus, increasing the loop size (from three to four U) and most importantly, decreasing the duplex length (from 12 base pairs to eight) significantly increased the product yield. Obviously, dissociation of the cleaved out fragment in HP-TW9 is much more facilitated due to its weaker binding to the ribozyme. We have observed the same trend in systems of twin ribozyme mediated mismatch repair (Table 1, 3 and 4 row). While a substrate forming a single G–A mismatch with the ribozyme located between a 3 bp and a 8 bp duplex was converted into the product with only 2% yield, a substrate with two mismatches separated by 2 bp and flanked on each side by 3 bp duplexes was repaired with 20% yield [21,22]. Furthermore, we have previously shown that the fragment exchange can be supported by increasing the reaction temperature. Based on the thermal stability of duplexes between the ribozyme and the sequence patches to be exchanged, a carefully chosen temperature regime can support the dissociation of the cleaved out fragment, while still allowing formation of the duplex between ribozyme and sequence patch to be ligated in a sufficiently stable mode [12]. Since the study presented herein is focused on models for RNA repair, we have carried out

Table 1
Repair of substrates forming loops, mismatches and duplexes of distinct length with twin ribozymes

	Substrate–ribozyme duplex[a]	Product–ribozyme duplex[a]	Description of substrate–ribozyme duplex	Yield
1.[b]	UU U U -CCCU CGAC- GGGA——GCUG-	-CCCUCGAC- -GGGAGCUG-	4 base loop 4 bp + 4 bp	17%
2.	U U U -CCCUCU AAAGAC- GGGAGA——UUUCUG-	-CCCUCUAAAGAC- -GGGAGAUUUCUG-	3 base loop 6 bp + 6 bp	5% [21]
3.	G -CCC CUAAAGAC- -GGG GAUUUCUG- A	-CCCUCUAAAGAC- -GGGAGAUUUCUG-	1 mismatch 3 bp + 8 bp	2% [21]
4.	U C -AGA UC UAC- -UCU AG AUG- C C	-AGAGUCGUAC- -UCUCAGCAUG-	2 mismatches 3 bp + 2 bp + 3 bp	20% [22]

[a] The central part of the ribozyme–substrate or ribozyme–product duplex is shown.
[b] HP-TW9 with substrate S-TW9.

experiments with HP-TW9 at physiological temperature of 37 °C. Under these conditions exchange of the red against the blue sequence patch may be limited by insufficient dissociation of the cleaved out patch such that the maximal product yield to be achieved is around 17%. In general, the twin ribozyme mediated removal of nucleotides is much more difficult to achieve than the insertion of nucleotides. While in the latter case a distorted short duplex is converted into a contiguous and longer duplex [9], the twin ribozyme mediated removal of nucleotides profits only from conversion of a distorted into a contiguous duplex with unchanged length. Thus, there is a much smaller driving force and naturally the yield will be lower than observed for nucleotide insertion. For the system shown here, 17% is likely to be the maximal yield. However, dependent on the specific RNA substrate and the specific purpose, the efficiency of ribozyme mediated removal of nucleotides may be further improved for one by the specific design (number of base pairs, mismatches, loops) and second by variation of reaction conditions (temperature, salt). This should not only enhance the prospects of ribozymes for future gene therapy; it also paves the way to a number of applications in molecular biology, such as site-directed RNA editing or protein engineering.

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