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Evidence for recycling of external guide sequences during cleavage of bipartite substrates *in vitro* by reconstituted archaeal RNase P

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Abstract

RNA-mediated RNA cleavage events are being increasingly exploited to disrupt RNA function, an important objective in post-genomic biology. RNase P, a ribonucleoprotein enzyme, which catalyzes the removal of 5′-leaders from precursor tRNAs (pre-tRNAs), has previously been utilized for targeted degradation of cellular RNAs. In one of these strategies, borne out in bacterial and mammalian cell culture, an external guide sequence (EGS) RNA when base-paired to a target RNA makes the latter a substrate for endogenous RNase P by rendering the bipartite substrate-EGS complex a pre-tRNA structural mimic. In this study, we first obtained evidence that four different mesophilic and thermophilic archaeal RNase P holoenzymes, reconstituted *in vitro* using their respective constituent RNA and protein subunits, recognize and cleave such substrate-EGS complexes. We further demonstrate that these EGSs engage in multiple rounds of substrate recognition while assisting archaeal RNase P-mediated cleavage of a target RNA *in vitro*. Taken together, the EGS-based approach merits consideration as a gene knock-down tool in archaea.

Keywords

Targeted RNA degradation; turnover

Artificial non-coding (nc) RNAs, either chemically synthesized or genetically expressed, have found wide utility *in vivo* and *in vitro* for a variety of applications.[1–8] These strategies mimic natural RNA-guided RNA cleavage (or antisense) events, which in turn encompass mechanisms to disrupt the normal cellular function of a target RNA. To illustrate, the discovery of RNAi in eukaryotes motivated the development of artificial small interference RNAs (siRNAs) and micro RNAs (miRNAs) to knock-down gene expression *in vivo*.[3,6–8] Various ribozymes have also been tailored either to cleave *in trans* target RNAs or *in cis* to generate precise termini in RNA samples used for high-resolution structural studies.[9–11] Regardless of whether the RNA-mediated RNA cleavages are catalyzed by RNAs, proteins or ribonucleoproteins (RNPs), the efficiency of sequence-specific targeting depends on

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turnover/recycling of the RNA agent that degrades the target RNA. For instance, at the heart of the RNAi machinery is the RNA-induced silencing complex (RISC). RISC loaded with miRNA/siRNA *in vitro* directs multiple rounds of target mRNA cleavage;[12,13] while the high efficiency of RNAi *in vivo* might result from such turnover, it is also likely to exploit other factors (e.g., helicases) which aid product release. In this study, we demonstrate robust turnover in an RNA degradation strategy that exploits an artificial RNA in conjunction with RNase P,[14] a ubiquitous RNP involved in tRNA maturation (Fig. 1A).

RNase P is composed of a catalytic RNA moiety and a varying number of protein subunits: one in bacteria, and at least four and nine in archaea and eukarya, respectively.[15–17] Since RNase P catalyzes the removal of the 5′-leader of all precursor tRNAs (pre-tRNAs), several studies focused on identifying the common sequence/structure features in all pre-tRNAs that permit their recognition and effective cleavage by RNase P.[18–21] While dissecting the substrate-recognition properties of bacterial and human RNase P, it was discovered that a substrate could be made up of two RNA molecules if they (through non-covalent interactions) generate a pre-tRNA-like structure (Fig. 1B).[5,22–24] This observation provided the underpinning for the idea that any cellular RNA can be made into a substrate for RNase P if it is bound by an artificial, external guide sequence (EGS) to form a pre-tRNA-like structure (Fig. 1C). The EGS is an RNA consisting of a tRNA-derived T, variable and anticodon stem-loops, which are flanked by single-stranded regions complementary to a desired target RNA (Fig. 1C).

EGSs, expressed from transgenes, have indeed been used successfully to down-regulate target gene expression in bacteria as well as plant and mammalian cell cultures (including successful inhibition of viral replication);[25–29] comparative studies have also highlighted some similarities and differences in the efficacy of EGSs and siRNAs in tissue culture.[30,31] Use of EGSs and cellular RNase P as functional genomics tools or therapeutic drugs, however, requires a complete understanding of their mechanism of action, the central focus of this study. For example, although the cleavage specificity of EGSs is well established, recycling of EGSs has not been investigated quantitatively. In previous studies using partially purified human RNase P *in vitro*, only three or fewer turns of the EGS were reported when [S] ≫[EGS].[24,26,32] We have now investigated the issue of robust EGS recycling using *in vitro* reconstituted archaeal RNase P[33–37] to explore the potential of the EGS method in archaea where reliable gene knock-down methods are currently not available.

Figure 2 is a scheme representing the various steps involved in EGS/RNase P-mediated target RNA degradation. A binding reaction (characterized by K_{D1}) between the target RNA shown as substrate S and the EGS generates S_{BS}, the bipartite substrate (Fig. 2). RNase P reversibly binds S_{BS} to form an E-S_{BS} complex (exemplified by K_S). In the presence of Mg^{2+}, an essential cofactor for RNase P-mediated hydrolysis, E-S_{BS} undergoes an irreversible chemical cleavage (at rate k_c) to cleave the scissile phosphodiester linkage in the target RNA. For simplicity, we depict initial departure of the 5′-leader (product P1) upon RNase P cleavage leaving behind E-P_{BP}, a non-covalent complex of the enzyme and the bipartite product P_{BP} (i.e., EGS bound to product P2). P_{BP} must then be released from RNase P (K_P) to free the enzyme for another round of catalysis. Dissociation of the EGS from P_{BP} (K_{D2}) completes the cycle and permits EGS recycling. If the substrate S is present in excess, it can compete with P2 and help displace P2 from P_{BP} thus accelerating EGS recycling.

Cleavage of bipartite substrates by archaeal RNase P

Using recombinant RNase P RNA (RPR) and protein (RPP) subunits, we recently reconstituted in vitro Methanococcus maripaludis (Mma), Methanothermobacter thermautotrophicus (Mth), *Methanocaldococcus jannaschii* (*Mja*) and *Pyrococcus furiosus* (*Pfu*) RNase P and showed that they can efficiently cleave pre-tRNA^{Tyr}.[34–37] For this study, we first examined if these *in vitro* assembled RNase P holoenzymes can also cleave bipartite substrates. As demonstrated earlier with partially purified plant RNase P,[38] our bipartite model is made up of two parts of a cyanobacterial pre-tRNA^{Gln}. While *in vitro* transcription was used to generate the EGS part of pre-tRNA^{Gln}, the 26-nt target RNA substrate was chemically synthesized and radiolabeled at its 5′-end (Fig. 1B). When these two molecules anneal together, they should form a pre-tRNA-like structure (albeit with a nick in the D-loop; Fig. 1B) and be recognized and cleaved by archaeal RNase P. Indeed, *Pfu* and *Mma* RNase P cleaved the bipartite substrate at the expected position (Fig. 3, lanes 6 and 10, respectively). Control reactions demonstrated that cleavage of the substrate was dependent on the presence of the EGS, and was not attributable to the remote possibility of a nuclease contamination in the recombinant RPR or RPP preparations (Fig. 3, lanes 3 to 5 and 7 to 9). We further confirmed that *in vitro* reconstituted *Mja* and *Mth* RNase P holoenzymes also cleave this bipartite substrate accurately (Fig. S1).

EGS can direct multiple rounds of substrate RNA cleavage

Using a 10-fold excess of the model target RNA substrate (500 nM) relative to EGS (50 nM), we inquired if EGS recycling did occur. The extent of substrate cleaved by RNase P depends on the dissociation of the EGS from P_{BP} (Fig. 2) and its subsequent re-annealing to another substrate molecule. If there is no EGS recycling, then the maximum product formed will equal the EGS concentration (i.e., one-tenth of the input substrate). In fact, since 90% of the target RNA substrate was cleaved in 20 min by *in vitro* assembled *Pfu* RNase P, the EGS molecules recycled at least eight times in 20 min under the assay conditions used (Fig. 4A). As the assays were performed at 55°C, shown to be optimal for cleavage of pre-tRNA^{Tyr} by *in vitro* assembled *Pfu* RNase P,[35] the possibility existed that this high assay temperature somehow facilitated EGS recycling (Fig. 2). However, when we tested RNase P from *Mma*, a mesophilic archaeon, we observed robust EGS recycling even at 37°C, although assaying the same enzyme at 50°C resulted in more rapid and near-complete cleavage of substrate (Fig. 4B). Incidentally, these results mirrored those obtained with partially-purified, native *Mma* RNase P, which supported EGS recycling both at 37°C and 45°C, albeit to a much greater extent at the latter (Fig. S2). (*Note*: While *Mma* is a mesophile, we showed previously that both *in vitro* assembled and partially-purified, native *Mma* RNase P exhibit optimal pre-tRNA cleavage between 45 to 50°C.[37])

To establish the ability of *Mma* and *Pfu* RNase P to sustain several cycles of target RNA substrate recognition and cleavage, we used a 40-fold excess of substrate over EGS. In both instances, we observed 20 to 25 rapid cycles that showed a linear relationship with time and a slowing in EGS recycling thereafter (Fig. 4C). Although this later plateau could be attributed to loss of catalytic activity resulting form prolonged incubation of the enzyme at high temperatures, it is more likely due to a build-up of P2 and the ensuing competition between S and P2 (Fig. 2), an aspect which we decided to investigate further.

Is the binding affinity of S or P2 to EGS similar?

Although the number and identity of complementary base pairs is identical in both S_{BS} (S:EGS) and P_{BP} (P2:EGS; Figs. 1 and 2), there is reason to expect dissimilar binding stabilities for these two bipartite complexes. Bases immediately external to a RNA duplex (i.e, those dangling and abutting the helical structure) do stack with adjacent base pairs and

influence overall stability.[39,40] Therefore, the substrate (S) RNA that contains a single-stranded 5′-leader, which is absent in P2, might bind to the EGS with a lower K_D than P2 (i.e., $K_{D1} < K_{D2}$, Fig. 2). The 5′-leader has dangling nucleotides that can stack with the acceptor-stem equivalent in S_{BS}. However, since 3′ dangling nucleotides, which are present in both S and P2, are energetically favored over their 5′ counterparts,[39] the 5′-leader's contribution might be modest. In fact, gel-shift assays revealed K_D values of 86 ± 11 nM for the binding of S to EGS and 135 ± 20 nM for the binding of P2 to EGS (Fig. S3); this 1.6-fold difference in binding reactions performed at 55°C increases to 2.5-fold if the binding is performed at 37°C (52 ± 13 nM and 130 ± 25 nM, respectively; data not shown). Although difficult to measure, it is conceivable that the stacking interactions of these dangling nucleotides might be further stabilized in the active site of RNase P, and could possibly magnify the difference in K_D values observed for the binding of EGS to S versus P2. These findings would also be complemented by studies that assess the kinetics of displacement when S is added to P_{BP} and P2 is added to S_{BS}.[41]

Concluding remarks

In the EGS methodology, while RNase P is a true enzyme, it was vital to establish that the EGSs could recycle. We have demonstrated that the EGS, a small RNA which renders a given cellular target RNA a substrate for endogenous RNase P, indeed recycles several times as assessed by *in vitro* assays with a model bipartite substrate and different archaeal RNase P holoenzymes (Fig. 4). The EGS/RNase P-based method certainly merits consideration as a gene knock-down approach in archaea both for functional genomics and metabolic engineering; however, optimization of EGS design will be critical prior to its use in archaeal environs, especially since some variants of the original EGS are more effective in cultured cells.[5,32]

Studies undertaken to elucidate the kinetics by which an oilgonucleotide annealed to a single-stranded RNA/DNA is replaced by another similar oligonucleotide revealed that the overall replacement rate is the sum of both the dissociative and sequential displacement pathways (Fig. S4).[42] Insights from such studies are directly applicable to the displacement of P2 in P_{BP} by S, the target RNA substrate (Fig. 2). To illustrate, the dissociative route involves dissociation of P_{BP} into EGS and P2 prior to annealing of the released EGS to S, while the sequential pathway entails strand invasion of S causing partial melting of P_{BP} before complete displacement of P2 (Fig. S4). Experiments with model oligonucleotides revealed that at moderate (< 40°C) temperatures and physiological ionic conditions, the overall replacement rate is a linear function of the concentration of the displacing oligonucleotide and is dictated largely by the sequential displacement route. If this were to hold in the EGS approach, it is possible that rather than the K_D values for assembly of S_{BS} and P_{BP}, the likely higher affinity of RNase P for the bipartite substrate (S_{BS}) compared to the bipartite product (P_{BP}) might prove a more critical determinant in ensuring EGS recycling. Such an expectation for preferential partitioning (i.e., $K_S \ll K_P$, Fig. 2) is supported by the observation that interactions between the bacterial RPP and pre-tRNA leader[43,44] selectively increase the binding affinity of bacterial RNase P for the pre-tRNA substrate over mature-tRNA product.[45–47] Although such recognition elements have not yet been identified in archaeal/eukaryal RNase P, their absence would be surprising.

The proven ability of EGSs in inhibiting viral replication in cell cultures[26,27] suggests that there might be additional favorable factors (yet determined) in the cellular environment which further enable and potentiate the EGS method. For example, what is the fate of the products from RNase P-mediated cleavage of a cellular RNA complexed with an appropriate EGS? Are these products degraded by cellular endo-/exo-nucleases that play a critical role in RNA turnover (as part of non-sense mediated decay and RNAi mechanisms)?[48] If so, the

expected competition between P2 and S for the EGS (Fig. 2) might not be relevant during targeted degradation of a cellular RNA, even though this manifests as a plateauing effect during later cycles of EGS turnover *in vitro* (Fig. 4). Also, if single-stranded RNA-binding proteins promote the rate of association of the EGS with the target RNA substrate,[49] annealing kinetics *in vivo* might override thermodynamic stability considerations[41,50] given that the bipartite substrate once formed might be rapidly bound and cleaved by RNase P. In fact, our observations that higher assay temperatures enhance EGS recycling might be a reflection of faster annealing of S and EGS, and even possibly selective enhancement of the enzyme's affinity for the substrate over the product. Given the dependence of EGS recycling on temperature, it is conceivable that EGS/RNase P-mediated knock-down of gene expression might be a viable approach in archaea.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS USED

EGS	external guide sequence
pre-tRNA	precursor tRNA
RPR	RNase P RNA
RPP	RNase P protein

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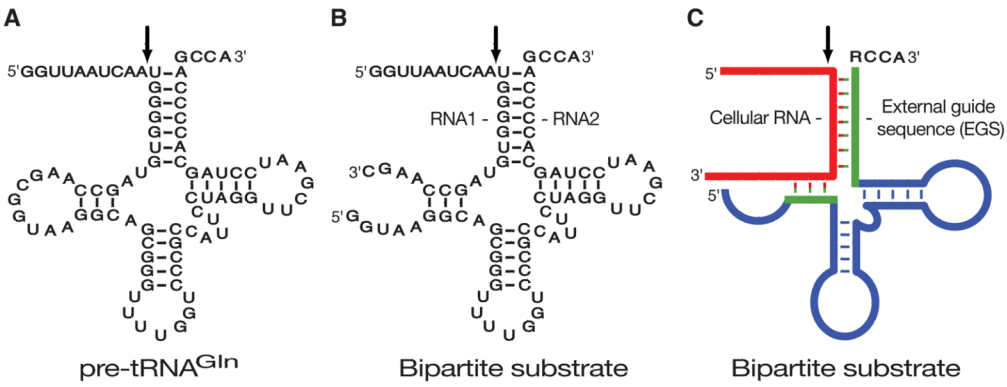

Figure 1.

Rationale underlying use of RNase P for targeted degradation of cellular RNAs. (A) Secondary structure of a *Synechocystis* sp. PCC 6803 pre-tRNA^{Gln}, a natural substrate of RNase P. (B) A non-covalent complex of two RNAs that (despite a discontinuity) form a pre-tRNA-like structure, which is recognized and cleaved by RNase P. (C) Any target RNA when specifically annealed to a customized external guide sequence (EGS) can be rendered a substrate for RNase P. Arrow indicates site of cleavage by RNase P.

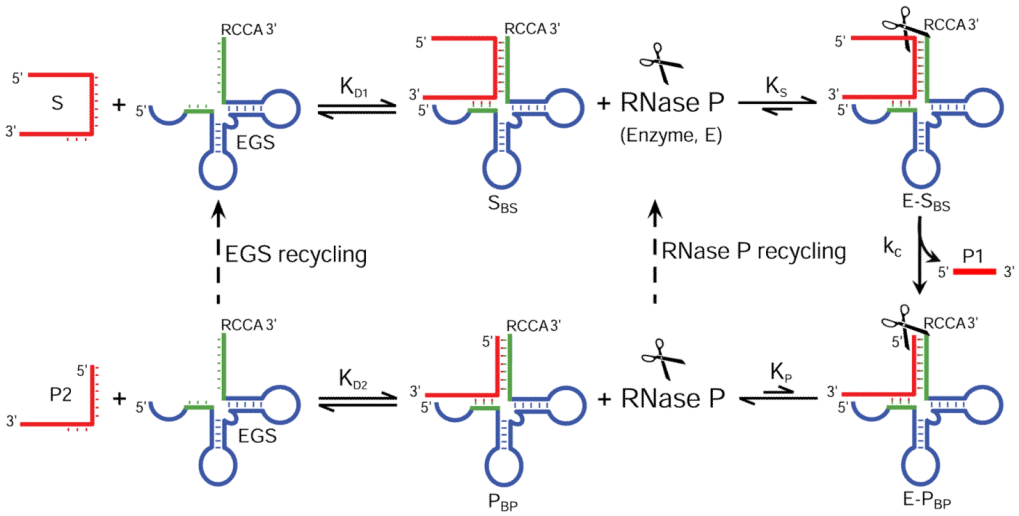

Figure 2.

Schematic of the various steps involved in the EGS/RNase P-mediated target RNA degradation, including generation and cleavage of the bipartite substrate and EGS recycling.

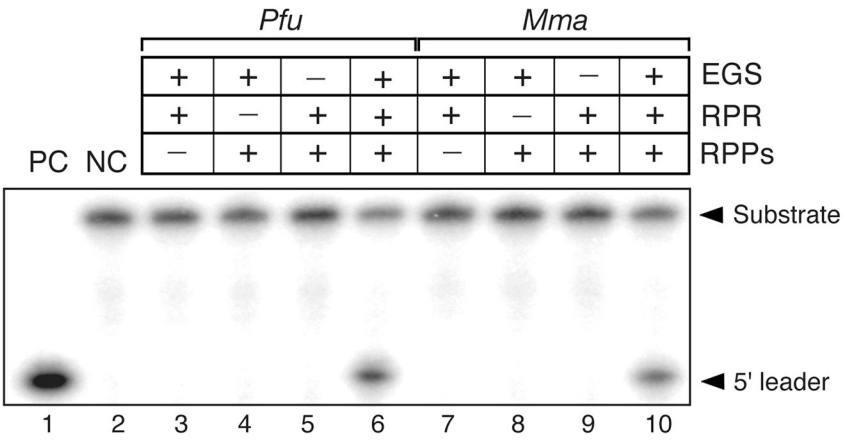

Figure 3.

Cleavage of a pre-tRNA^{Gln}-based bipartite substrate by archaeal RNase P. The model target substrate (RNA1, Fig. 1B) was chemically synthesized (Integrated DNA Technologies, Coralville, IA) and 5′-labeled using T4 polynucleotide kinase and γ-[^{32}P]-ATP. The EGS (RNA2, Fig. 1B) was generated using T7 RNA polymerase-mediated *in vitro* transcription. Fifty nM of substrate RNA (10,000 dpm) and 50 nM of EGS were first mixed together, and then incubated at 55°C with *in vitro* assembled *Pfu* RNase P (lanes 3 to 6) in 50 mM Tris-HCl (pH 8, 25°C), 30 mM $MgCl_2$ and 800 mM NH_4OAc. With *Mma* RNase P, assays were performed at 37°C in 50 mM Tris-HCl (pH 8, 25°C), 7.5 mM $MgCl_2$ and 500 mM NH_4OAc. The procedures used for reconstitution of *Pfu* and *Mma* RNase P holoenzymes are described elsewhere.[35,37] The assays (final volume, 10 μl) were performed in a thermal cycler. At the end of the 5-min incubation, the reactions were terminated by addition of 10 μl urea-phenol dye [8 M urea, 0.04% (w/v) bromophenol blue, 0.04% (w/v) xylene cyanol, 0.8 mM EDTA, 20% (v/v) phenol] and separated on a 15% (w/v) polyacrylamide gel containing 8 M urea. The reaction products were visualized by phosphorimaging on the Typhoon (GE Healthcare). The control reactions that lacked either EGS or archaeal RPR/RPPs (lanes 3 to 5 and 7 to 9) underwent similar treatment. The 5′-leader (10 nt) generated from digestion of 5′-labeled pre-tRNA^{Gln} by *Pfu* RNase P was used as the reference size marker for the product (corresponds to P1 in Fig. 2).

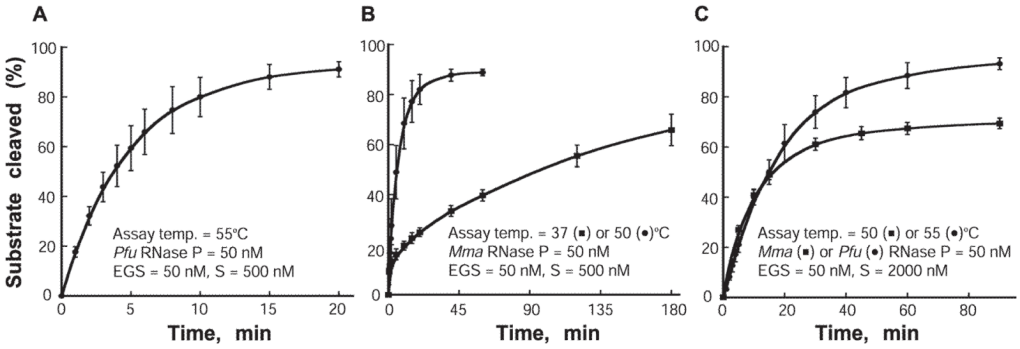

Figure 4.

EGS recycling during cleavage of bipartite substrates by archaeal RNase P. Each panel is a time-course of either *Pfu* or *Mma* RNase P-mediated cleavages of the bipartite substrate at the indicated temperature and concentrations of enzyme, S and EGS RNAs. Reactions were performed as described in legend to Figure 3. After phosphorimaging, the resulting bands were quantitated by ImageQuant (GE Healthcare) to assess the extent of substrate cleaved. Values reported are the mean and standard deviation from three independent experiments.