Minireview

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RNase P: Variations and Uses*

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This essay will bring to date a picture of the properties of RNase P from several organisms and a summary of how this enzyme can be used to decrease specific gene expression. Current details of how the enzyme works and other features governing its reaction are reviewed elsewhere (1–3).

RNase P is responsible for generating the mature 5′-end of tRNAs by a single endonucleolytic cleavage of their precursors. It is an essential, ubiquitous enzyme present in all cells and cellular compartments that synthesize tRNA: bacterial cells, eukaryotic nuclei, mitochondria, and chloroplasts. The essential function *in vivo* of RNase P has been demonstrated in those systems amenable to genetic analysis such as bacteria (4) and yeast nuclei (5) and mitochondria (6, 7). All known RNase P enzymes are ribonucleoproteins and contain an RNA subunit essential for catalysis with the possible exception of RNase P in some plant chloroplasts and trypanosome mitochondria (8, 9).

The chemical mechanism of RNase P involves essential divalent metal ions (2) and is thought to be an in-line S_N2 displacement reaction (1). The endonucleolytic cleavage generates 5′-phosphate and 3′-hydroxyl end groups in the products. For our purposes, the way in which the enzyme recognizes substrates is an important feature of its ability to lower the amount of any particular RNA and expression inside cells. Natural substrates can be reduced to two oligonucleotides, which when hydrogen bonded together (Fig. 1) resemble sufficiently the essential features of a substrate so that one of the oligonucleotides, the target RNA (*i.e.* any RNA inside the cell), is cleaved efficiently by the enzyme and inactivated. This important aspect of RNase P revolves entirely around its substrate recognition mechanism and does not depend on the fact that there is an RNA subunit in the enzyme.

The RNA Subunit of RNase P

The RNA component of RNase P from bacteria is encoded by the *rnpB* gene and varies in length between about 350 and 450 nucleotides (10). There is little sequence similarity among the 300 or so bacterial sequences except for a few short segments. Phylogenetic covariation analysis of the large data set has allowed the precise definition of the secondary structure and the identification of several tertiary interactions (11–15). This RNA from bacteria can be divided into two distinct structural classes: type A, represented by *Escherichia coli*, which is the ancestral type found in most bacteria (the RNA subunit of the enzyme from *E. coli* is called M1 RNA and is referred to by that name herein), and type B, represented by *Bacillus subtilis*, which is found in the low GC content Gram-positive bacteria (16). An intermediate structure (type C) is found in green non-sulfur bacteria (12). Despite differences in the secondary structure organization of type A and type B RNAs, both RNAs can be modeled into a similar three-dimensional structure with the evolutionarily conserved nucleotides placed in nearly identical positions (16). These computer-aided modeling efforts illustrate a common phenomenon in RNA architecture wherein different, nonhomologous sequence elements are used for long range structural interactions that result in functionally equivalent structures. The ultimate proof of the three-dimensional structure and chemical mechanism of the bacterial RNase P RNA will await the publication of results from crystallographic studies.

RNase P RNA from Archaea can also be divided into two structural classes, type A, the most common and similar to the ancestral bacterial type A structure, and type M, a derived structure found only in two species (17) so far. Similarly, the eukaryotic RNase P sequences can also be fit to a minimal consensus secondary structure reminiscent of the bacterial RNase P RNA structure (18). Despite the low sequence conservation, it has been possible to identify several conserved regions and helical elements present in all these RNAs (17, 18). Two of the conserved sequence elements correspond to helix P4 and adjacent sequences, which is an essential part of the catalytic center of the bacterial enzyme. Another case of RNase P RNA variation in sequence length is found in yeast mitochondria, where there are large differences in nucleotide size (between 227 and 490) in different yeast species (19), although they can be drawn into a two-dimensional structure similar to the other RNase P RNAs.

The Protein Subunit(s) of RNase P

In bacteria, RNase P contains a single protein subunit of about 120 amino acid residues. The sequences of this protein are poorly conserved (20). There is, however, a short, conserved basic sequence motif essentially shared by all of them. This conserved sequence is called the RNR motif and can be defined as $KX_{4-5}AX_2RNX_2(K/R)RX_2(R/K)$. Other conserved residues are a few aromatic amino acids close to the amino terminus of the protein. Despite the low conservation in primary sequence, the three-dimensional structure of these proteins is probably similar because in most cases they are functionally interchangeable, and heterologous reconstitution of RNase P using an RNA of one source and protein from a different source is in general feasible (21–23).

The protein composition of nuclear RNase P has been studied mainly in yeast and humans. Compared with the simplicity of the bacterial RNase P protein complement, there is a significant increase in complexity of the nuclear enzyme. Nine proteins have been identified as subunits of yeast nuclear RNase P, ranging in size from 16 to 100 kDa (24). For the human nuclear enzyme, 10 proteins have been identified ranging in size from 14 to 115 kDa (25, 26). Because the methods of total fractionation have not yielded pure enzyme, there still is considerable difficulty in establishing all the proteins that make up an intact holoenzyme complex. In any case, at least four of the human proteins are homologs of proteins from yeast RNase P, but none has clear homology to the bacterial protein subunit. Most of the nuclear RNase P protein subunits are shared with the related endonuclease MRP (mitochondrial RNA processing), which also contains an RNA subunit. To date no eukaryotic RNase P holoenzyme has been reconstituted from purified RNA and protein subunits. The development of such a system *in vitro* would be an important step forward in the characterization of eukaryotic RNase P.

Despite the presence of a bacteria-like RNA, archaeal RNase P possess an eukaryotic RNase P-like protein set (68). In some cases the archaeal RNA can reconstitute a functional holoenzyme with the bacterial protein subunit (27, 28).

Mitochondria also exhibit a large diversity of RNase P architecture. We have already mentioned the large variation in RNase P RNA subunit size within yeasts. The mitochondria from some prim-

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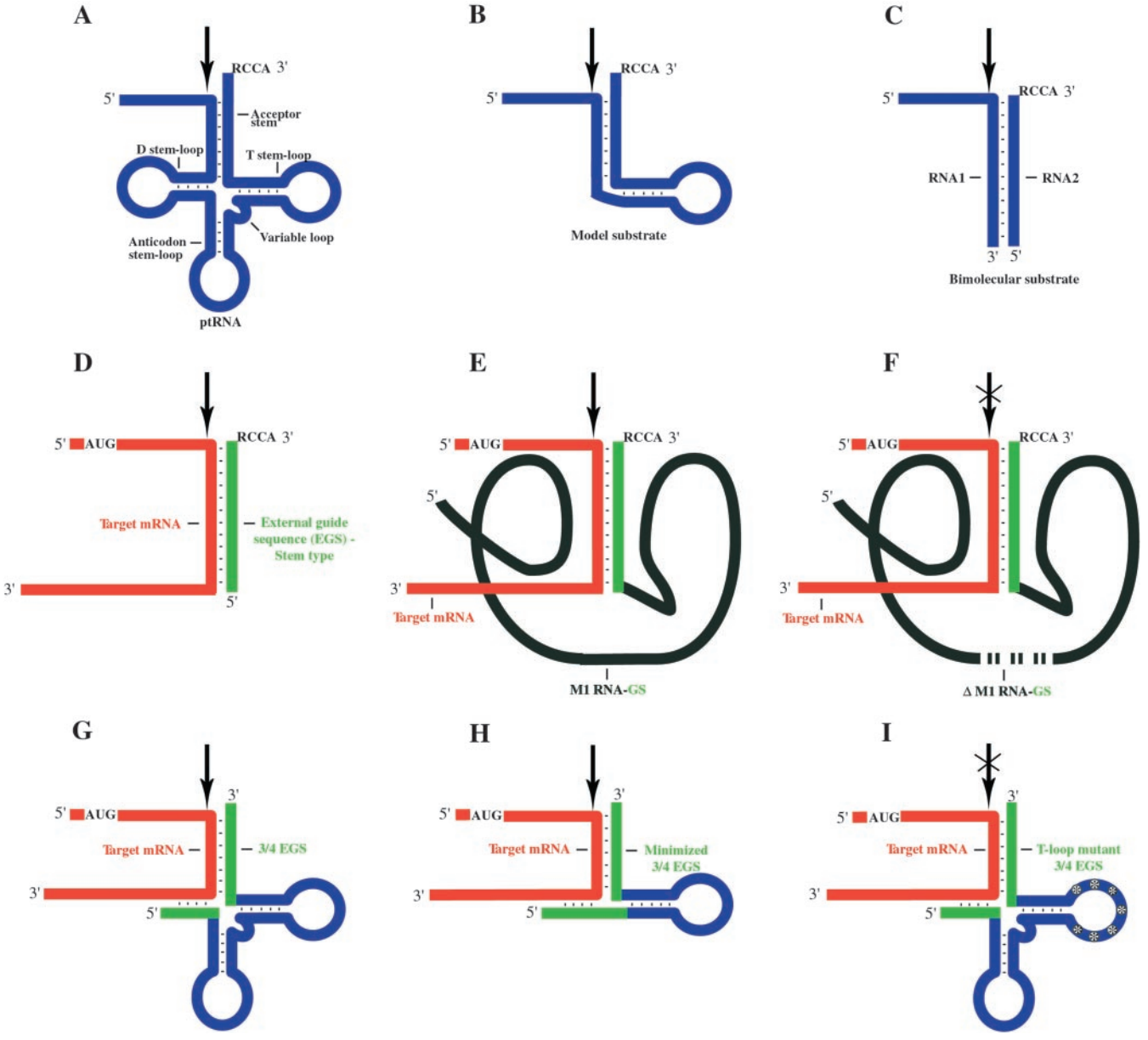

FIG. 1. **Substrates for RNase P cleavage.** *Arrow* indicates the site of cleavage by RNase P. *A*, cleavage of an *E. coli* natural precursor tRNA. *B*, cleavage of a modified substrate. *C*, a bimolecular substrate for *E. coli* RNase P. *D*, a target RNA-stem EGS complex that is cleaved by bacterial RNase P. *E*, M1 RNA in which the 3′-end is covalently attached to the 5′-end of the EGS to facilitate rapid cleavage of the target RNA binding to the EGS. *F*, ΔM1 RNA, a catalytically inactive ribozyme, covalently attached to the 5′-end of the EGS to serve as a negative control for antisense effects. *G*, a target RNA-¾ EGS complex that is recognized by eukaryotic RNase P. *H*, a minimized ¾ EGS. *I*, failure of human RNase P to cleave a bipartite substrate made up of a target RNA and a ¾ EGS with mutations in the T loop.

itive eukaryotes such as *Reclinomonas americana* contain a bacteria-like gene for the RNase P RNA (29). The only protein component characterized for a mitochondrial RNase P is a 105-kDa protein in *Saccharomyces cerevisiae* with no homology to any other protein subunit of RNase P (6, 30). In contrast, highly purified *Aspergillus nidulans* mitochondrial RNase P contains seven polypeptides ranging in size from 16 to 55 kDa (31). A most peculiar case is the RNase P from human mitochondria, whose RNase P contains an RNA subunit identical to the nuclear RNA subunit (32). The protein composition of human mitochondrial RNase P has not been characterized.

In certain chloroplast and trypanosome mitochondria the RNase P activities appear to lack an RNA subunit. The spinach chloroplast enzyme is not well characterized in terms of composition and structure, but there is evidence that suggests that RNase P is composed solely of protein in plant chloroplasts (8). The catalytic mechanism of the plant chloroplast RNase P is different from the RNA-based mechanism of bacterial and nuclear RNase P (33). This fact has prompted the suggestion that the higher plant chloroplast RNase P might be evolutionarily unrelated to all the others (34). The genome of the chloroplasts of some algae codes for a bacteria-like RNase P RNA gene (35–37). However, in the only case studied (*Cyanophora*), the coded RNA is not active in the absence of protein (38, 39), but it was possible to reconstitute a functional holoenzyme with a protein subunit from cyanobacteria (39) and the plastid-encoded RNA.

In *Leishmania* and *Trypanosoma* mitochondria, there is evidence that all tRNAs are imported from the cytoplasm as mature tRNAs (40). Nevertheless, there is a report of a 5′ tRNA processing activity (protein alone in RNase P) in *Trypanosoma* mitochondria (9).

Ribozymes and RNase P

To characterize the efficacy of RNase P in lowering gene expression, a brief description of some similarities with other ribozymes is profitable. Ribozymes and antisense molecules carry out their inactivation of specific gene expression by hydrogen bonding between the target RNA in question and part of the ribozyme or the anti-

sense oligonucleotide (41, 42). This mechanism dictates the particular gene specificity of these reactions. Hammerhead, hairpin molecules, and δ RNA are so-called because of a two-dimensional structure that can be drawn from their schematic diagrams (41–45) (for reviews, see Refs. 46–50). We note that the efficiency of most of the hammerhead methods for target inactivation work is better than 60% for a single enzyme directed against a single target and that a helicase receptor unit attached to a hammerhead solves the problem of finding a suitable site on the target RNA (51). We shall focus here generally on the action of external guide sequences (EGSs)[1] and RNase P.

External Guide Sequences and RNase P

To understand the mode of action of EGSs and RNase P, one has to examine the fundamental reactions carried out by the enzyme, that is the cleavage of tRNA precursors. The cleavages of a tRNA precursor and of a minimal model substrate are illustrated in Fig. 1. Forster and Altman (52, 53) have shown that the model substrate could be further simplified in that two complementary RNAs form a stem that can be recognized and cleaved by *E. coli* RNase P (Fig. 1*C*). This finding led to the postulate that any (m)RNA could be targeted for degradation by *E. coli* RNase P in the presence of a complementary EGS that forms a sequence-specific complex with the (m)RNA and thereby renders that RNA a non-natural substrate for RNase P (Fig. 1*D*). The sequence RCCA is included in the 3′-end of the EGS to mimic the 3′-end of precursor tRNAs, the natural substrates for *E. coli* RNase P. The success of the RNase P-mediated approach *in vivo* depends on (i) stable expression of the EGSs (using either constitutive or regulated promoters), (ii) colocalization of the target mRNA substrate (the EGS and RNase P within the same subcellular compartment), and (iii) accessibility of the target mRNA to binding by the EGS.

When the EGS molecule is in a complex with the target RNA, a stemlike structure (typically with 13–16 bp) is generated. EGSs of this type are referred to as "stem" EGSs. An example of this kind of EGS is provided by looking at chloramphenicol drug resistance in *E. coli*. When *E. coli* harboring a chloramphenicol resistance gene (chloramphenicol acetyltransferase, *cat*) was transformed with a plasmid encoding a stem EGS specific for the *cat* gene, the *cat* mRNA was selectively destroyed by endogenous RNase P, and consequently expression of chloramphenicol acetyltransferase was decreased (54). These cells were therefore rendered sensitive to chloramphenicol and resulted in phenotypic conversion of drug-resistant bacteria to drug sensitivity. An independent study demonstrated that microbial viability can be decreased to less than 10% of the wild type strain if the EGS-mediated approach is employed to reduce the level of expression of essential proteins such as gyrase A and the protein subunit of RNase P (55). The latter investigation also showed additivity of combined use of EGSs and that a three-nucleotide mismatch between target and EGS can be tolerated with no loss in efficiency. However, an EGS specific for the gyrase A mRNA in *Salmonella typhimurium* was ineffective in targeted cleavage of the gyrase A mRNA in *E. coli* because there were six nucleotides (out of 16 nucleotides that are complementary in the EGS) that differed in sequence between the two homologous mRNAs.

Another agent used by this technology has the EGS covalently linked to M1 RNA, the catalytic RNA subunit of *E. coli* RNase P (Fig. 1*E*). This works well in bacteria (56) and in the nucleus of mammalian tissue culture cells (57). The complex formed when the GS binds to its target mRNA, would be immediately bound and cleaved by the ribozyme (M1 RNA) that is covalently bound to the GS. The utility of such an approach has been borne out in several studies (see below).

By attaching M1 RNA to a GS specific for the thymidine kinase mRNA (from herpes simplex virus 1, HSV-1), the specific cleavage of thymidine kinase (TK) mRNA both *in vitro* and *in vivo* was demonstrated, and TK protein was reduced by 80% (57). Moreover, when HSV-1 mRNAs encoding essential viral proteins were targeted by the appropriate M1 RNA-GSs, a 1000-fold decrease in viral replication was observed (58). Similarly, when human U373MG cells expressing M1-GSIE were infected with human cytomegalovirus, it resulted in reduced expression of both IE1 and IE2, two transcriptional activators, and thereby effected a 150-fold decrease in viral titer (59).

Murine hematopoietic cells stably transformed with the *BCR-ABL* gene display BCR-ABL-induced tumorigenicity and offer a suitable model system for studying chronic myelogenous leukemia. Cobaleda and Sanchez-Garcia (60) introduced into these cells a gene encoding M1 RNA fused to a guide sequence that is complementary to the unique nucleotide sequence present only at the translocation site and in the chimeric BCR-ABL mRNA. This sequence-specific ribozyme was able to specifically cleave the chimeric transcript, decrease expression of the BCR-ABL chimeric protein, and cause cell death.

In the experiments that involve M1 RNA linked to a guide sequence (M1 RNA-GS), coupling the GS to a catalytically inactive version of M1 RNA, ΔM1 RNA, provides a suitable control to determine the degree to which disruption of gene expression is not mediated by RNase P (Fig. 1*F*). For example, only 12% inhibition of TK expression was observed when mouse cells expressing the ΔM1 RNA-GStk were infected with HSV-1 (57). Clearly, the RNase P-mediated inhibition of gene expression (~80%) far exceeded that attributable to antisense effects.

In contrast to bacterial RNase P, eukaryotic RNase P is unable to cleave the simple complex shown in Fig. 1*D*. Initial studies on recognition of bipartite substrates by human RNase P revealed that cleavage does occur when three-fourths of the tRNA molecule is presented as the EGS (Fig. 1*G*) (62). These EGSs, termed ¾ EGSs, form a sequence- and structure-specific complex with the corresponding target mRNA (Fig. 1*G*). Subsequent SELEX (systematic evolution of ligands by exponential enrichment) studies and deletion analysis have demonstrated that the ¾ EGS can be minimized further without drastically altering recognition and cleavage of the bimolecular substrate by human RNase P (61, 62). In this modified design (Fig. 1*H*), the EGS is about 30 nucleotides in length and is complementary to 11 nucleotides in the target mRNA. Unlike the natural tRNAs, the acceptor and D-stem equivalent in the bimolecular substrate contain seven and (about) four base pairs in the target RNA, respectively (Fig. 1*H*), generally interrupted by two unpaired bases, one of them being U8, a conserved unpaired nucleotide in natural tRNAs. This is the essence of substrate specificity of eukaryotic RNase P. In fact, most studies carried out with eukaryotic RNase P use the ¾ EGS (Fig. 1*G*) or one missing the anticodon loop and stem (Fig. 1*H*). The active agent is only the structure of the EGS and the target RNA.

Knowledge of the sequences of the single-stranded and accessible sites in an mRNA permits design of either the stem or ¾ EGS against a particular target mRNA. A regulatable T7 RNA polymerase promoter has been utilized to ensure both inducible and high levels of expression of EGSs in *E. coli* BL21(DE3) cells (54–56). In eukaryotic cells, EGSs have been constitutively produced by employing retroviral long terminal repeats or polymerase III promoters (such as the U6 small nuclear RNA promoter). Moreover, because U6 small nuclear RNA predominantly resides in the nucleus, the EGSs synthesized by the U6 promoter are expected to colocalize in the nucleus along with the target mRNA and RNase P.

The replication of influenza virus in cell culture could be prevented by RNase P-mediated degradation of viral mRNAs (63). C127 mouse cells were stably transfected with synthetic genes that constitutively expressed EGSs directed against the polymerase subunit 2 (PB2) and nucleocapsid (NP) genes, whose expression is vital for replication of the influenza virus. When challenged with the influenza virus, C127 cells expressing EGSNP and EGSPB2 were able to inhibit both flu protein synthesis and viral particle production by 90–100%.

To prove that RNase P is the effective cleavage agent, a control experiment was performed in which the T-loop sequence of the EGS was changed. Normally, mutations in the T-loop make the mRNA-EGS complex resistant to RNase P cleavage (Fig. 1, *I*) (63). Mouse cells were stably transfected with synthetic genes encoding T-loop sequence mutants of EGSNP and EGSPB2. These mutants were unable to inhibit viral replication (63).

In all the studies described above, the EGSs were expressed either transiently from a plasmid or stably after integration of the

[1] The abbreviations used are: EGS, external guide sequence; GS, guide sequence; HSV, herpes simplex virus; TK, thymidine kinase.

respective gene into the genome of the host cell. However, EGSs also have been delivered as an oligonucleotide (with a cationic lipid as a carrier) into the cell (64). When EGSs directed against the protein kinase C (EGSPKC-α) mRNA were delivered to T24 human bladder carcinoma cells using transfection agents (like Lipofectin), down-regulation of PKC-α was observed. Although the EGSPKC-α possessed nine nucleotides that were complementary to nine contiguous nucleotides in PKC-ζ mRNA, the expression of PKC-ζ was unaffected.

Minimizing the EGSs to about 30 nucleotides in length has permitted chemical synthesis and the introduction of various chemical modifications such as 2′-*O*-methyl substitution, phosphorothioate backbone modification, and a 3′–3′ inverted thymidine at the 3′-end, etc. These modifications (with the exception of those introduced in the T-loop) did not inhibit cleavage by human RNase P and yet afforded remarkable protection of the EGS from degradation by nucleases in the human serum (65).

Kalb and his collaborators (66) have also shown that a recombinant HSV that contains an EGS directed to the mRNA of an *N*-methyl-D-aspartate receptor can infect primary cultures of neuronal cells and reverse some aspects of the cytotoxic effect of *N*-methyl-D-aspartate receptor ligands.

Substrate recognition studies *in vitro* using maize and rice RNase P have established that plant RNase P can also cleave bimolecular substrates similar to those recognized by human RNase P (67). Because substrate recognition by RNase P is conserved in both plant and animal kingdoms, the EGS-based methodology is broadly applicable.

Conclusion

The successful function of the EGS technology has been very well illustrated for the decrease of gene expression in bacteria and mammalian tissue culture cells. What remains is a test of the function of EGSs in animal and plant systems.

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