How bacterial cells keep ribonucleases under control

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One sentence summary: Cells employ a variety of strategies to regulate their complement of ribonucleases to ensure sufficient activity to meet their metabolic requirements and, at the same time, to avoid indiscriminate destruction of important RNA molecules.

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ABSTRACT

Ribonucleases (RNases) play an essential role in essentially every aspect of RNA metabolism, but they also can be destructive enzymes that need to be regulated to avoid unwanted degradation of RNA molecules. As a consequence, cells have evolved multiple strategies to protect RNAs against RNase action. They also utilize a variety of mechanisms to regulate the RNases themselves. These include post-transcriptional regulation, post-translational modification, trans-acting inhibitors, cellular localization, as well as others that are less well studied. In this review, I will briefly discuss how RNA molecules are protected and then examine in detail our current understanding of the mechanisms known to regulate individual RNases.

Keywords: endoribonuclease; exoribonuclease; post-translational modification; cellular localization; RNA protection

INTRODUCTION

Ribonucleases (RNases) play a central role in bacterial RNA metabolism (Deutscher 2006; Lehnik-Habrink et al. 2012; Arraiano et al. 2013). They are essential for the processing reactions that convert stable RNA precursors to mature, functional RNAs and for the reactions that cleave certain polycistronic messages to differentially regulate their individual mRNAs. RNases are also required for the degradative processes that normally turn over mRNAs, that remove defective RNAs for quality control purposes and that digest normally stable RNAs under certain stress conditions. Finally, RNases are necessary to mediate the many reactions involving regulatory RNAs. As a consequence of their participation in essentially every aspect of RNA metabolism, RNases serve as prime regulators of gene expression, perhaps even surpassing the importance of transcription for some genes (Esquerre et al. 2014).

On the other hand, RNases also have a dark side. They have the potential to be extremely destructive enzymes (Deutscher 2006; Lehnik-Habrink et al. 2012; Arraiano et al. 2013), and cells must control the many RNase activities present to ensure that elimination of functionally important RNA molecules is avoided. This can be accomplished by protecting the RNAs such that they resist RNase action or by directly regulating the RNases to limit their activity. While mechanisms that protect RNAs are well known, the study of RNase regulation has until recently received relatively little attention. In this review, I will briefly summarize mechanisms that are known to protect RNA molecules, and then will discuss our current understanding of how the RNases themselves are regulated, examining the variety of processes that bacterial cells employ to carefully control the amount, specificity and cellular location of specific RNases.

Factors protecting RNA against degradation

In a cellular environment containing upwards of 20 RNases, RNA molecules would be in a precarious situation unless they were somehow protected since RNases generally will bind and attack any available substrate. Inasmuch as wholesale degradation does not occur, cells must have evolved strategies to achieve RNA stabilization and allow the RNAs to function (Arraiano et al. 2010). Interestingly, some of these strategies are
reversible enabling cells to turn on degradative processes as the need arises. This introduces a temporal aspect to RNA degradation that allows certain RNAs to be cleaved only at specific times. A variety of factors are known to protect RNA molecules against degradation. These are summarized in Table 1 and are discussed below.

**Intrinsic structure of RNA**

RNA molecules have a strong tendency to favor compact structures (Mortimer, Kidwell and Doudna 2014). Such structures could bury RNA termini preventing the action of exoribonucleases. Likewise, they may occlude residues that serve as cleavage sites for endoribonucleases. In addition, complementary sequences present in RNAs lead to a variety of stem-loop or other structures that act as barriers to exoribonucleases. When these structures are at or close to the terminus of an RNA molecule, they can abrogate the action of an exoribonuclease. Many exoribonucleases require single-stranded regions to bind RNA, so secondary structures at RNA termini may not block exoribonuclease movement, but even their association with a potential substrate since a structured RNA is often unable to access the catalytic channel of the RNase. The protective effect of RNA secondary and tertiary structure is so strong that tRNA and tRNA molecules, which are highly structured, are resistant to degradative endonucleases (Mortimer, Kidwell and Doudna 2014). Intrinsic structure of RNA is reversible enabling cells to turn on degradative processes as the need arises. This introduces a temporal aspect to RNA degradation that allows certain RNAs to be cleaved only at specific times. A variety of factors are known to protect RNA molecules against degradation. These are summarized in Table 1 and are discussed below.

**Example**

| RNA structure       | Compact structure of tRNA and rRNA |
|---------------------|------------------------------------|
| Blocking RNA termini| Aminoacylation of transfer RNA      |
| Association with protein | rRNA in ribosomes                   |
| Translation of mRNA  | Coupling of transcription-translation |
| Regulatory RNAs      | sRNA/Hfq stimulation of RNase E action on mRNAs |
| Cellular localization | Degradosome association with cell membrane |
| RNase specificity    | RNase BN/Z inability to remove –CCA from tRNA; weak activity of RNase E on RNA with 5′-PPP |

**Table 1. Factors that protect RNA from the action of RNases.**

**Presence of moieties on RNA termini**

Exoribonucleases initiate their action at the termini of RNA molecules and therefore are affected by terminal structures. In Gram-negative organisms, such as *Escherichia coli*, the known exoribonucleases act in the 3′–5′ direction, whereas Gram positives, such as *Bacillus subtilis*, also contain RNase J1, able to digest in the 5′–3′ direction (Deutscher 2006; Lehnik-Habrink et al. 2012; Arraiano et al. 2013). In addition, certain endoribonucleases, such as RNases E and Y, although cleaving internally, are influenced by the structure at the 5′ terminus of the RNA substrate. Consequently, the presence or addition of moieties to the 3′ or 5′ terminus of an RNA molecule can strongly affect whether that RNA will be a substrate for a particular RNase.

**Due to the mechanism of nucleotide polymerization by RNA polymerase, RNA molecules are synthesized with a triphosphate moiety at their 5′ terminus. Since both RNase E and RNase Y activities are inhibited by the presence of the 5′ triphosphate, it serves to protect some RNAs from cleavage by these enzymes (Lehnik-Habrink et al. 2012).**

**Likewise, B. subtilis RNase J1 prefers substrates with a 5′ monophosphate terminus suggesting that the triphosphate would protect against the action of this enzyme as well (Lehnik-Habrink et al. 2012).** The 5′-triphosphate can be removed by a pyrophosphohydrolase enzyme present in both Gram-positive and Gram-negative cells raising the potential for regulation of RNA stability through the action of this enzyme (Condron and Bechhofer 2011; Mackie 2013). Once the initial cleavage by RNase E or RNase Y occurs, the resulting downstream product is monophosphorylated, and any further 5′-dependent degradation presumably would not be affected.

**The 3′ terminus of an RNA transcript may also be protected by addition of a moiety to that end of the molecule. This is known to occur with tRNA molecules that are protected by the process of aminoacylation. Uncharged tRNAs are substrates for the exoribonuclease, RNase T, which removes the terminal AMP residue and renders the tRNAs inactive. The terminal AMP can be restored by the action of tRNA nucleotidyltransferase resulting in an end-turnover process known since the discovery of tRNA. This apparently futile cycle has no known physiological function and can be disrupted by mutations in RNase T or tRNA nucleotidyltransferase. Inhibition of aminoacylation by elevating the temperature in a temperature-sensitive aminoacyl-tRNA synthetase mutant strain leads to AMP removal from only those tRNA isoacceptors affected by the mutant synthetase, directly demonstrating that the amino acid at the 3′ terminus protects the tRNA (Deutscher 1990).**

**Polyadenylation at the 3′ terminus also affects RNA stability. One might have expected that poly(A) tails added by poly(A) polymerase would protect RNA molecules, but, in fact, they often lead to destabilization. Poly(A) tails act as single-strand...**

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binding sites to recruit exoribonucleases that could not otherwise bind to the 3’ end of a structured RNA (Cheng and Deutscher 2005). Addition of poly(A) tails has also been shown to tag-defective tRNA precursors in a quality control process which removes precursors that are poorly converted to their mature forms (Li et al. 2002). Based on this information, polyadenylation is most likely a destabilizing determinant in bacteria, but situations in which it stabilizes a transcript would not be surprising.

Association with protein
The vast majority of RNAs are associated with protein in vivo including rRNAs as part of ribosomes and tRNAs associated with components of the translation system. Such association may play an important role in the stabilization of these RNAs by masking their termini against exoribonuclease action or covering endoribonuclease cleavage sites. The presence of rRNAs in the context of the ribosome also can alter the specificity of RNase action compared to that on naked RNA. For example, the 3’ terminus of 5S and 23S rRNAs are matured by the exoribonuclease RNase T. When purified RNase T acts on naked 5S and 23S RNA in vitro, it digests past their mature 3’ termini. However, when the same reaction is carried out on RNAs within a ribosome subunit, RNase T stops at the correct 3’ residue (Li and Deutscher 1995; Li, Pandit and Deutscher 1999). Based on these results, the context of the ribosome subunit effectively blocks RNase action to promote correct RNA maturation, and the findings emphasize the role of RNA association with protein as a potentially important determinant of RNA stability.

Trans-acting protein factors may be particularly important in differential mRNA stability, but this area of research is in its early stages. For example, it is known that the RNA chaperone, Hfq, can affect mRNA polyadenylation and cleavage by RNase E as well as playing an essential role in riboregulation facilitating the interaction of sRNAs with mRNA (Vogel and Luisi 2011; Bandrya and Luisi 2013; Mackie 2013). The effect of Hfq on mRNA stability may be positive or negative, but it serves to show how protein association with mRNA may influence RNA stability. Other examples in which protein binding alters message stability have been identified, and these may use a variety of mechanisms to obtain their effect, but are beyond the scope of this review.

Translation of mRNA
The stability of an mRNA ultimately comes down to its accessibility to an RNase, and for mRNA, accessibility is directly affected by translation due to the protection afforded by translating ribosomes. In view of the enormity of factors that influence translation efficiency and the density of ribosomes on a given mRNA, the possibilities for ultimately affecting mRNA decay are almost limitless. In addition, for many genes, transcription and translation are coupled, and such messages are usually associated with ribosomes. Decoupling transcription and translation, which occurs when transcription is catalyzed by the rapidly moving T7 RNA polymerase, results in mRNAs that are devoid of ribosomes, and these messages are extremely unstable (Iost, Guillerez and Luisi 1992). As a general rule, conditions and factors that promote translation of a specific mRNA would be expected to lengthen the half-life of that message, whereas decreasing the amount of translation would destabilize the mRNA.

Small regulatory RNAs
Small RNAs are ubiquitous in bacterial systems, and many of their physiological properties are manifested through effects on RNA stability using all the mechanisms already described. Thus, small RNAs can affect substrate RNA secondary structure; they can affect accessibility of RNA ends; they work in conjunction with the RNA-binding protein, Hfq; and they can strongly influence translation. Depending on the system, small RNAs may stabilize or destabilize substrate RNAs, and it is becoming increasingly evident that these molecules participate in innumerable processes affecting RNA stability (Lalouna et al. 2013; Saramago et al. 2014).

Cellular localization
As noted, RNases can act only if the substrate RNA is accessible. So, if the enzyme or substrate is in some way compartmentalized, the RNA will be protected. Until recently, the primary example of this type of regulation involved the non-specific endoribonuclease, RNase I (Deutscher 2003). This enzyme is localized to the periplasmic space, and as a consequence, is unable to access cellular RNAs. However, under certain conditions in which the cell membrane is damaged, RNase I may enter the cell, leading to massive RNA degradation and cell death. Thus, sequestering this destructive RNase away from potential cellular RNA substrates is an essential survival mechanism.

It is now clear that other RNases are sequestered as well (Campos and Jacobs-Wagner 2013). RNase E in E. coli and RNase Y in B. subtilis are bound to the inner cell membrane. While such localization would presumably compartmentalize the RNases and their associated degradosome components away from potential substrates, at some point the RNases must interact with the RNAs in order to catalyze the turnover and processing reactions. How this might occur, and the details of how the initial separation of RNase and substrate eventually leads to a productive interaction, is not at all clear. Moreover, how such movement might be integrated with protein synthesis, ribosome assembly and tRNA aminoacylation are interesting questions for which almost nothing is known.

Very recent studies have expanded the list of compartmentalized RNases. Escherichia coli, RNase II, a non-specific exoribonuclease, is associated with the cytoplasmic membrane through its N-terminal amphipathic helix, and mutations that alter the association affect cell viability under conditions in which RNase II is essential (Lu and Taghbalout 2014). Additionally, other RNA processing and degradation components including oligoribonuclease, Hfq, poly(A) polymerase, RNase III and the pyrophosphohydrolase, RppH, have all been suggested to be present in extended structures that coil around the cell periphery (Taghbalout, Yang and Arluison 2014), although this claim is controversial (Swulius and Jensen 2012). Verification of this new information and an understanding of how these observations can be incorporated into mechanistic models of RNA metabolism will require additional studies.

RNase specificity
A final factor that may help to protect RNAs is the specificity of the RNases themselves. While certain RNases are relatively nonspecific, most display some specificity with regard to secondary structure or nucleotide sequence and others may be highly specific (Lehnig-Habrink et al. 2012; Arraiano et al. 2013). As a result, RNases are often unable to act on a substrate despite its accessibility. In fact, in some situations an RNase may bind a resistant RNA and thereby protect it from an RNase for which it is a substrate. RNase specificity also can be subtle. For example, the exoribonuclease RNase T cannot digest through adjacent C residues (Zuo and Deutscher 2002). This specificity protects uncharged tRNA molecules in that only the terminal AMP residue...
of the 3′-terminal CCA sequence can be removed, protecting the rest of the tRNA molecule from degradation and enabling it to be repaired by tRNA nucleotidy/transferase. Likewise, RNase BN/Z does not act on tRNA precursors that already contain a CCA sequence thereby avoiding removal of these essential residues (Pellegrini et al. 2012; Dutta, Malhotra and Deutscher 2013). How this is accomplished will be discussed below.

From the previous discussion, it is evident that cells have evolved multiple strategies for protecting RNA molecules against the potentially deleterious effects that could result from harboring a large repertoire of RNases. Many of these factors work in concert to minimize unwanted RNase action, and many can be reversed to allow RNases to act when needed. Superimposed on these factors, cells have also evolved mechanisms that act directly on the RNases themselves to keep their activity under control. Examples of these mechanisms will be discussed below.

Regulation of RNases

A variety of processes and mechanisms have been identified that either downregulate RNase activities or limit their action to only select substrates. These include transcriptional, translational and post-translational processes, as well as the built-in specificities of the RNases themselves and the influence of factors that modulate RNase activity (Table 2). While our understanding of RNase regulation is still in its early stages, the diversity of regulatory mechanisms already known indicates that cells have adopted a wide number of strategies for keeping RNases in check including some that are unique in bacterial systems. Here, I will present a catalog of processes that are currently known to affect RNase activity and specificity highlighting the breadth of strategies that have already been uncovered.

### Table 2. Bacterial RNases and their regulation.

| RNase       | Regulation known† | Protein modification | Trans-acting factor | Other§ |
|-------------|-------------------|----------------------|---------------------|--------|
| **Endoribonucleases** |                   |                      |                     |        |
| RNase I     | Yes               | –                    | –                   | +      |
| RNase III   | Yes               | +                    | +                   | +      |
| RNase P     | No                | –                    | –                   | –      |
| RNase E     | Yes               | –                    | +                   | –      |
| RNase G     | No                | –                    | –                   | –      |
| RNase H’s   | No                | –                    | –                   | –      |
| RNase BN/Z+ | Yes               | –                    | –                   | +      |
| RNase Y     | Yes               | –                    | –                   | +      |
| **Exoribonucleases** |                   |                      |                     |        |
| PNPase      | Yes               | +                    | –                   | –      |
| RNase II    | Yes               | –                    | –                   | –      |
| RNase D     | Yes               | –                    | –                   | –      |
| RNase T     | No                | –                    | –                   | –      |
| RNase PH    | No                | –                    | –                   | –      |
| RNase R     | Yes               | –                    | +                   | +      |
| RNase J1/J2+ | Yes              | –                    | +                   | +      |
| Oligo RNase | No                | –                    | –                   | –      |
| NanoRNases  | No                | –                    | –                   | –      |

† The information presented pertains to the E. coli member for each enzyme except for RNase Y, RNase J1/J2 and nanoRNases which are the B. subtilis enzymes.

†† Escherichia coli RNase BN/Z also has exoribonuclease activity. †‡ RNase J1 also has endoribonuclease activity, and it is listed as associated with RNase J2 to which it is frequently bound.§ Only clear examples of regulation have been considered. § Other may refer to cellular localization, high RNase specificity, translational effects that have not been well characterized. Toxin-antitoxin systems have not been considered.

**Ribonuclease E**

RNase E and its homologs are present in a large number of bacterial species (Mackie 2013). In E. coli, where it has been studied in most detail, RNase E participates in many pathways of RNA metabolism. It participates in the maturation of 16S and 5S rRNAs, in the processing of tRNAs and sRNAs, and in the initiation of degradation of mRNAs. Some recent evidence suggests that RNase E may also contribute to rRNA degradation (S. Sulthana, G.N. Basturea and M.P. Deutscher, unpublished observations). The involvement of RNase E in so many processes underscores a need to keep the level of this enzyme tightly regulated. Too much RNase E could lead to excessive RNA degradation, whereas too little could leave many RNA precursors unprocessed.

In view of the seemingly strict requirement for maintaining appropriate levels of RNase E, it is not surprising that this enzyme is subject to a potent autoregulation mechanism. When the amount of RNase E exceeds the cell’s needs, the enzyme attacks its own mRNA, repressing synthesis of more RNase E protein (Jain and Belasco 1995; Sousa, Marchand and Dreyfus 2001). This is an effective process which can modulate the half-life of RNase E message over a more than 10-fold range (<40 s to >8 min). This process also limits RNase E overexpression as a 21-fold increase in rne gene dosage leads to <3-fold increase in RNase E protein (Jain, Deana and Belasco 2002).

The mechanism of this autoregulatory process is mediated by the 5′ untranslated region (UTR) of the rne message (Diwa et al. 2000; Schuck, Diwa and Belasco 2009). Thus, fusing a 445-nt segment containing the 5′ UTR to lacZ brings β-galactosidase synthesis under the control of RNase E levels (Diwa et al. 2000). Analysis of the rne 5′ UTR revealed the presence of a stem-loop structure with a highly conserved internal loop that is primarily responsible for the feedback regulation (Schuck, Diwa and Belasco 2009) (Fig. 1). Exactly how this secondary structure element regulates rne mRNA stability is not completely understood.
RNase E also can be phosphorylated (Marchand, Nicholson and Dreyfus 2001). In an extremely interesting report, it was shown that expression of bacteriophage T7 gene 0.7 protein kinase in uninfected E. coli cells leads to extensive phosphorylation in the C-terminal half of RNase E, amounting to introduction of as many as 25 phosphate groups. Phosphorylation inhibits RNase E and stabilizes mRNAs synthesized by T7 RNA polymerase. On the other hand, cleavage by RNase E of the 55 precursor of 5S RNA is unaffected. Although this is an unusual experimental system, it does raise the possibility of RNase E regulation by covalent modification. Unfortunately, to date, no further work on this system has been carried out.

In a possible third mechanism of regulation, it has been reported that RNase E activity can be influenced by several proteins. These putative regulators include RraA, RraB and ribosomal protein L4 (Gao et al. 2006; Singh et al. 2009). Each of these proteins binds to the C-terminal half of RNase E leading to inhibition of RNase E activity. However, it is not yet clear whether these proteins actually regulate RNase E activity in vivo. RraA and RraB must be overexpressed in order to exert their inhibitory effect (Lee et al. 2003), and protein L4 is normally bound to ribosomes, though it is possible it could be released under certain conditions (Singh et al. 2009). Moreover, these proteins themselves would have to be regulated in response to some growth condition that requires reduction in RNase E activity, and they would have to be present in sufficient amounts that would significantly affect RNase E activity. It is not presently known what cellular conditions might necessitate such a response. Thus, we will have to await additional information before the physiological role of these proteins can be evaluated. The RNA chaperone Hfq may also be considered a protein regulator of RNase E activity since it is known to bind and occlude potential RNase E cleavage sites (Mackie 2013).

**Ribonuclease III**

RNase III and its homologs are double-strand-specific endoribonucleases that play critical cellular roles in stable RNA maturation, mRNA turnover and in gene regulation (Arraiano et al. 2013; Nicholson 2014). The enzyme also participates in phage RNA and plasmid RNA metabolism. As so many RNA-dependent processes are affected by cleavage of double-stranded RNA, control of RNase III amounts and activity can be of prime importance. At present, several types of regulatory mechanisms that affect RNase III are known, although many details of these processes still remain to be elucidated.

In E. coli, RNase III expression is maintained within limits by a post-transcriptional autoregulatory mechanism (Bardwell et al. 1989; Matsunaga et al. 1996; Matsunaga, Simons and Simons 1996). The rnc gene-encoding RNase III is part of the rnc-era-recO operon. The 5’ UTR of the message from this operon contains a double-stranded region that is a substrate for cleavage by RNase III. This region normally stabilizes the rnc message and can be transferred to other mRNAs to stabilize them as well. RNase III cleaves at a single site in the double-stranded stem of the centrally located, largest of three stem-loop structures within the first 215 nt of the rnc leader. Once cleaved, the now single stranded 5’ end of the rnc message becomes a substrate for rapid decay by RNase E. Similar mechanisms of autoregulation have also been found in Salmonella typhimurium (Anderson et al. 1996) and Streptomyces coelicolor although in the latter case one cleavage occurs in the RNase III-coding region (Xu, Huang and Cohen 2008). This autoregulatory mechanism would decrease the amount of RNase III when the enzyme is present in excess over its needs for action on other substrates, but would lead to message stabilization and increased expression of RNase III when its levels are too low for the amount of substrates available.

RNase III is also regulated by post-translational modification (Mayer and Schweiger 1983). Upon infection of E. coli with bacteriophage T7, the activity of RNase III increases 3- to 4-fold. The elevation of activity is due to the synthesis of an early T7 protein, the phage protein kinase which phosphorylates RNase III on a serine residue. Inasmuch as RNase III is required for
cleavage of the early region polycistronic mRNA of phage T7. Upregulation of RNase III activity by the virus would increase functional T7 messages. Moreover, as noted above (Mayer and Schweiger 1983), the T7 protein kinase inhibits RNase E, thereby stabilizing T7 messages. Together the action of T7 protein kinase on both RNase III and RNase E serves as an efficient mechanism to enhance T7 infection. Whether post-translational modification might also regulate these RNase activities in uninfected cells remains unknown.

Escherichia coli RNase III is also regulated by a low molecular weight protein, YmdB (Kim, Manasherob and Cohen 2008). The existence of an RNase III inhibitor in extracts was first recognized during in vitro processing of tmRNA. Re-examination of this initial finding identified YmdB, a conserved 18.8 kDa protein. YmdB inhibits RNase III in vivo and in vitro by binding to a region required for catalysis, and perhaps, for dimerization of RNase III monomers. YmdB levels increase during stationary phase, but do not correlate with reduced RNase III activity throughout this period. On the other hand, downregulation of RNase III activity during cold shock was affected by YmdB. Interestingly, YmdB inhibition of RNase III activity in vitro was affected only in the presence of Mn$^{2+}$, not Mg$^{2+}$, a finding that may be related to the region of RNase III that binds YmdB. Very recently, a detailed structural and computational analysis of the YmdB–RNase III interaction revealed that conserved residue R40 in YmdB interacts with residues of the subunit interface of RNase III (Paudyal et al. 2015). During the screening that identified YmdB, other trans-acting factors that affect RNase III also were detected, but were not examined further (Kim, Manasherob and Cohen 2008).

**Polynucleotide phosphorylase**

Polynucleotide phosphorylase (PNPase) is a processive, 3′–5′ exoribonuclease that uses phosphate as the nucleophile, releasing nucleoside diphosphate as a product (Arraiano et al. 2013). PNPase is widespread in bacteria where its primary role is in RNA degradation, although it may also participate in RNA maturation. In some organisms, the synthetic activity of PNPase is responsible for addition of poly(A) or poly(A)-like tails to existing RNAs (Mohanty and Kushner 2003). Structurally, PNPase is complex for a nuclease, existing as a homotrimer of ~85 kDa chains. A portion of PNPase is present in association with other proteins (RNase E, enolase and RhlB) in a structure termed the degradosome that participates in RNA degradation (Bandyra et al. 2013). Additional PNPase is found associated with two molecules of a 48–50 kDa protein in a five-subunit form of the enzyme. Some controversy surrounds the identity of this protein as it was originally reported to be enolase (Py et al. 1996) and later RhlB, an RNA helicase (Lin and Lin-Chao 2005). Moreover, the function of this form of PNPase remains unclear.

As was discussed in relation to RNase E and RNase III, PNPase expression is also subject to autoregulation by a mechanism that requires an initial cleavage of the pnp message by RNase III (Robert-Le Meur and Portier 1992, 1994). Endonucleolytic cleavage by RNase III occurs in a stem-loop structure in the 5′ UTR of pnp mRNA releasing a product that is rapidly degraded by RNase E in a process dependent on PNPase action (Jarrige, Mathy and Portier 2001). The exact role of PNPase in this mechanism has been somewhat uncertain, but the most recent evidence has coalesced into a single model (Carzaniga et al. 2009). Cleavage by RNase III in the stem-loop structure leaves a 37 nt RNA associated with the 5′region of the processed pnp message. This initial RNase III double-stranded product is insensitive to RNase E, and only upon digestion of the 37 nt fragment by PNPase, is a single-stranded 5′ region exposed that is accessible to RNase E action (Fig. 2). Support for this model comes from evidence that autoregulation is dependent on PNPase activity and on the KH and S1 RNA-binding domains of PNPase, and that it correlates with RNA-binding by PNPase (Matus-Ortega et al. 2007; Wong et al. 2013). Thus, when active PNPase is present in excess, it can act on its own message and repress further PNPase synthesis.

PNPase is essential for growth at low temperatures, and the level of the enzyme increases as much as 3-fold under these conditions (Marchi et al. 2007). Interestingly, the increase in PNPase expression at low temperatures is due to multiple post-transcriptional events including reversal of autoregulation (Beran and Simons 2001; Marchi et al. 2007). All the processes require an intact 5′ UTR on pnp mRNA, and there is a transient increase in pnp mRNA during the adaptation phase due to its stabilization (Mohanty and Kushner 2002). In addition, transcription termination is also transiently suppressed leading to pnp messages that extend into downstream genes. However, details of how these processes contribute to elevation of PNPase are still unclear.

An additional mechanism of PNPase regulation has recently been reported (Nurmoahmed et al. 2011). It was found that citrate, a Krebs cycle intermediate, can bind to and modulate PNPase activity. The effect can be either inhibitory or stimulatory depending on the site of binding. Most importantly, the citrate effect operates in vivo, as growth is suppressed in an E. coli strain dependent on PNPase for viability when citrate levels are increased. These findings open the possibility of a link between central metabolism and RNA degradative pathways, but additional studies will be necessary to unravel the details of any regulatory connection.

**Ribonuclease II**

RNase II is a processive 3′–5′ exoribonuclease that is the predominant activity against poly(A) in E. coli extracts (Arraiano et al. 2003). However, the enzyme is absent from B. subtilis (Deutscher and Reuven 1991). The primary role of RNase II is in RNA degradation, although it can function in stable RNA maturation as well. Little is known about the regulation of RNase II, but one report identified a gene, termed gmr, downstream of rnb, that affects the amount of RNase II present (Cairrho et al. 2001). In a deletion strain, lacking gmr, RNase II protein and activity are

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**Figure 2. Model for regulation of PNPase expression.** Autoregulation of PNPase initiates with an RNase III cleavage in the stem of a stem-loop structure in the 5′ UTR of the pnp message (step 1). The 37-nt 5′ fragment that results from this cleavage is digested by PNPase (step 2). The truncated, 5′ phosphorylated pnp mRNA is subsequently digested by RNase E (step 3).
elevated 3-fold, whereas there is no change in another 3′–5′ exoribonuclease, PNPase. The increase in RNase II in the gmr deletion strain is not due to an increase in rnb message, but rather to stabilization of RNase II protein. It was also found that RNase II levels vary in different growth media, but that this regulation disappeared in the gmr deletion strain. While extremely interesting, the mechanism of RNase II regulation by Gmr is completely unknown. Interestingly, Gmr is a cyclic-di-GMP phosphodiesterase, raising the possibility of RNase II regulation by cyclic di-GMP (Weber et al. 2006). Further work on this potential regulatory system is clearly warranted.

It has been reported (Zilhao et al. 1996) that RNase II levels can vary over a 5-fold range depending on the amount of PNPase present in the cell. Cells deficient in PNPase have an increased amount of RNase II, and cells overproducing PNPase decrease rnb mRNA and RNase II. This regulation appears to be reciprocal since removal of RNase II leads to elevation of PNPase. The mechanism of this reciprocal regulatory process is not clear, but it suggests that E. coli somehow coordinates the amount of these two processive nucleases so that their total level remains constant. The relation between these two enzymes is further emphasized by the fact that cells lacking both nucleases are inviable (Donovan and Kushner 1986), indicating an overlap in their functions.

Recently, RNase II was shown to be associated with the cytoplasmic membrane through its NH2-terminal amphipathic helix, and that RNase II is present as an organized cell structure that coils around the cell periphery (Lu and Taghbalout 2014). Most importantly, this assembly of RNase II appears to be physiologically important since in the absence of PNPase, a condition which renders RNase II essential, viability was affected when exponential-phase RNase R acetylated, while the stationary-phase enzyme is unmodified? The acetylation reaction is catalyzed by the product of the yfiQ gene, now called pka (Liang, Malhotra and Deutscher 2011). No other modification was found on either protein suggesting that the difference in binding of tmRNA–SmpB by the two forms of RNase R is entirely due to the single acetyl modification. This conclusion was supported by mutational analysis of Lys544. Conversion of the Lys residue to Arg stabilized the exponential-phase enzyme, whereas conversion to Ala also destabilized the stationary-phase enzyme. Thus, a positive charge at position 544 (Lys or Arg) results in RNase R stabilization, while no charge at this position (Acetyl-Lys or Ala) leads to RNase R decay. Based on these data, a model was proposed (Fig. 3) which interaction of the C-terminal region of RNase R with Lys544 prevents tmRNA–SmpB binding, but when Lys544 is acetylated the interaction does not occur leaving the C-terminal region exposed and able to bind tmRNA–SmpB (Liang, Malhotra and Deutscher 2011). Mutation of acidic residues in the C-terminal region, which also prevent the interaction, support this conclusion. Recent structural information on RNase R also is supportive of the model (Venkataraman et al. 2014).

Ribonuclease R

RNase R is a third, processive, 3′–5′ exoribonuclease present in E. coli, and it is widespread in other organisms as well (Arraiano et al. 2013). Like RNase II, RNase R is a member of the RNB family of exoribonucleases. However, it differs from the former enzyme in that it can digest structured RNA (Cheng and Deutscher 2005). RNase R participates in degradation of defective stable RNAs during quality control processes and of tRNA during starvation (Cheng and Deutscher 2003; Zundel, Basturea and Deutscher 2009; Basturea, Zundel and Deutscher 2011). It is also involved in mRNA decay, particularly of those transcripts that contain structured regions, such as REP (repetitive extragenic palindromic) sequences (Cheng and Deutscher 2005). RNase R is a component of the trans-translation machinery where it acts to remove the defective, pre-existing message (Richards, Mehta and Karzai 2006). RNase R also participates in 3′ processing reactions including those of 16S rRNA in E. coli (Sulthana and Deutscher 2013), tRNA in Mycoplasma genitalium (Alluri and Li 2012) and 16S rRNA in Pseudomonas syringae under cold shock conditions (Purusharth, Madhuri and Ray 2007). In the latter two situations, RNase R appears to be essential for the maturation process.

The regulation of RNase R has been studied extensively in E. coli revealing a complex, multi-faceted process that is so far unique among bacterial proteins. RNase R protein and activity increase dramatically under a variety of stress conditions including stationary phase, cold shock and elevated temperature (Cairrao et al. 2003; Chen and Deutscher 2005). This increase, amounting to 3- to 10-fold depending on the genetic background, is due to stabilization of RNase R. In contrast to most other E. coli proteins which are stable, RNase R is extremely unstable in growing cells with an average half-life of ∼10 min. However, under stress conditions the enzyme becomes stable, and as a consequence, its level increases (Chen and Deutscher 2010). Examination of the factors responsible for RNase R instability revealed that it was due to association of the exponential-phase protein with two components of the trans-translation machinery, tmRNA and its associated protein cofactor, SmpB (Liang and Deutscher 2010). Deletion of either of these components increased the half-life of RNase R 3-6-fold and greatly increased the regulatory system is clearly warranted. This in turn over. As a consequence, no modified RNase R remains in stationary-phase cells. It was also found that RNase R in cold-shocked cells does not contain the acetyl modification due to the absence of Pka, explaining why RNase R is stable under this
Model to explain proteolysis of exponential-phase RNase R. In exponential-phase cells, acetylation of Lys544 eliminates its positive charge and thereby disrupts the interaction between the lysine residue and the C-terminal region of RNase R. This enables tight binding between the exposed C-terminal region and the tmRNA–SmpB complex. The proteases, Lon or HslUV, bind to the N-terminal region of RNase R and this binding is stabilized by a direct interaction between the protease and SmpB. Since SmpB is not bound to stationary-phase RNase R (Fig. 3), protease binding is weak and RNase R is stable. In fact, addition of proteases to stationary-phase RNase R in the presence of tmRNA–SmpB in vitro has little effect for periods as long as 60 min (Liang and Deutscher 2012b). (Fig. 3)

Unbelievably, there is an additional layer of regulation superimposed on this already complex system (Liang and Deutscher 2013). RNase R is largely (80%) bound to ribosomes in growing cells, whereas it is free in stationary-phase cells. RNase R binding to ribosomes is dependent on tmRNA–SmpB, non-stop mRNA and the modified form of ribosomal protein S12. Bound RNase R is part of the trans-translation machinery; its role is to degrade the non-stop mRNA that leads to ribosome stalling. Interestingly, the ribosome-bound form of RNase R is completely stable, while the free form is extremely unstable, turning over with a half-life of 2 min. Although degradation of the free form of RNase R also is dependent on tmRNA–SmpB, the process is independent of ribosomes, indicating that the turnover of RNase R in exponential-phase cells is not a consequence of its role in trans-translation. Free RNase R is deleterious to growing cells. Mutations that decrease ribosome binding increase the cell’s doubling time and lead to inappropriate degradation of RNA, including stable RNAs. Thus, it appears that the primary role of RNase R is on ribosomes, and that the previously described regulatory process that degrades RNase R is designed to rapidly eliminate any free enzyme before it can attack cellular RNA. Clearly, this is not a problem in stationary-phase cells or cells under other stress conditions as RNase R is unbound and completely stable in such cells. As a side note, since RNase R is not bound to ribosomes in stationary-phase cells, these findings suggest that trans-translation does not occur under these conditions.

Figure 3. Model to explain differences in stability between exponential- and stationary-phase RNase R. In stationary-phase cells, Lys544 is unmodified, maintaining a positive charge on this residue, resulting in interaction between the Lys residue and a negative charge in the C-terminal region of RNase R. This interaction prevents effective binding of tmRNA–SmpB and the enzyme is stable. As cells enter exponential phase, acetylation of residue 544 eliminates the positive charge on the lysine and, as a consequence, disrupts the interaction between the lysine residue and the C-terminal region of RNase R. In the absence of an interaction, the C-terminal region of RNase R is exposed enabling the tight binding of the tmRNA–SmpB complex. This binding renders RNase R susceptible to subsequent attack by a protease.
Ribonuclease D

RNase D is a distributive 3′-5′ exoribonuclease that is present in both bacterial and eukaryotic systems (Arraiano et al. 2013). The enzyme was originally identified in E. coli based on its action on denatured tRNA. Subsequently, it was shown to act in tRNA processing and in maturation of other small, stable RNAs. However, it is not as effective as other RNases in these processes, suggesting that its primary function may not yet have been discovered. Elevated levels of RNase D are deleterious, particularly in cells lacking tRNA nucleotidyltransferase (Zhang and Deutscher 1988a,b), indicating that RNase can digest mature tRNAs in vivo when its levels are too high; there is no evidence that RNase D acts on mature tRNAs when it is present at normal levels.

While essentially no information is available regarding the regulation of RNase D in response to environmental changes, it is known that the constitutive level of the RNase is regulated at the translational level (Zhang and Deutscher 1989). The RNase D-coding region begins with a UUG initiation codon, found in only ~1% of E. coli genes. Changing the initiation codon to the more common AUG increased RNase D activity 10-fold and dramatically increased the amount of RNase D protein, based on immunoblotting. It is not known whether the UUG codon serves any other role in addition to lowering the potential expression level of RNase D.

Another interesting feature of the upstream region of E. coli rmd gene is the presence of a GC-rich hairpin structure followed by eight T residues in the region between the promoter and the Shine-Dalgarno sequence (Zhang and Deutscher 1992). Although removal of the complete hairpin structure had little effect on mRNA levels, it greatly decreased translation of the rmd message. The decrease was due entirely to removal of the eight U residues in the mRNA. In fact, conversion of just two U residues to A decreased translation 70%. Mutant transcripts bind 30S ribosome subunits with much lower affinity than wild-type mRNAs suggesting that the U-rich region serves as an enhancer of translation by facilitating ribosome binding. However, it is not known whether this sequence serves any direct regulatory role.

One hint that RNase D expression may, in fact, respond to changes in environmental conditions comes from recent studies in our laboratory indicating that rmd message and RNase D are undetectable in stationary-phase cells (T. Dutta, C. Taylor and M.P. Deutscher, unpublished observations). These findings suggest that RNase D functions primarily in exponential-phase cells, and that its substrates may become elevated as RNase D disappears.

Ribonuclease BN/Z

The RNase Z family of endoribonucleases is widespread from bacteria to eukaryotes. The enzyme functions in 3′ processing of tRNA precursors, but only those that lack an encoded CCA sequence (Lehnik-Habrink et al. 2012; Arraiano et al. 2013). RNase Z cleaves after the discriminator nucleotide, generating a product that is a substrate for subsequent CCA addition by tRNA nucleotidyltransferase. Interestingly, E. coli also contains an RNase Z family member, termed RNase BN, despite the fact that tRNA precursors in this organism already contain an encoded CCA sequence, raising the question of what its function might be in these cells. In contrast to other members of the RNase Z family, RNase BN has both exoribonuclease and endoribonuclease activity (Dutta and Deutscher 2009). RNase BN can function in E. coli tRNA processing, but it does so relatively poorly compared to other RNases present in the cell; its activity only becomes evident when other maturation nucleases are absent (Li and Deutscher 1995). When acting on the cell’s CCA-containing tRNA precursors, RNase BN can utilize either of its two activities, but it cleaves after the CCA sequence when acting as an endoribonuclease or trims up to the CCA sequence using its exoribonuclease activity (Dutta, Malhotra and Deutscher 2012). It does not remove the CCA sequence (Dutta and Deutscher 2010). RNase BN also functions in maturation of four of the eight tRNAs encoded by bacteriophage T4, but in this situation it acts like other RNase Z’s, cleaving after the discriminator nucleotide in precursors that lack a CCA (Seidman et al. 1975). Likewise, in B. subtilis, RNase Z is essential for maturation of some tRNA precursors, but only those that do not contain a CCA sequence. Precursors that have a CCA are processed like those in E. coli, using an exoribonuclease that trims up to, but not through, the CCA sequence (Pellegrini et al. 2003).

Inasmuch as RNase Z cleaves after the discriminator nucleotide, a major question for understanding regulation is how it avoids acting on those tRNA precursors that already contain a CCA sequence, and more importantly, how it avoids acting on mature tRNAs which would lead to a futile cycle of removal and re-addition of the CCA residues (Dutta, Malhotra and Deutscher 2013). Using RNase BN, it was found that discrimination against CCA-containing substrates is due to two factors—the adjacent C residues of the CCA sequence and residue Arg274, which is present in a conserved sequence motif located in a channel that leads to the enzyme’s catalytic site (Dutta, Malhotra and Deutscher 2013). When both the CC sequence and Arg274 are present, substrates are unable to move from the channel into the catalytic site, as shown by the ability of the RNase to continue to act on the small, chromogenic substrate, bis-(p-nitrophenyl phosphate). However, substitution of either C residue with an A or U or conversion of Arg274 to Ala leads to the inhibition of action against the small substrate, indicating that the catalytic site is now occupied by the substrate RNA. Thus, CCA-containing RNAs cannot access the catalytic site, whereas those lacking a CCA can. These findings explain why RNase BN, and presumably other RNase Z family members, is unable to act on CCA-containing substrates. Thus, the built-in specificity of this RNase prevents its action on mature tRNAs.

Toxin–antitoxins

Although a large field in itself, and beyond the scope of this review, I briefly mention toxin–antitoxin systems as another example of RNase regulation. Many toxins are RNases that target mRNAs based on sequence specificity. Normally, these RNase toxins are kept inactive due to the presence of an antitoxin. Many different examples have been elucidated in recent years that use a variety of mechanisms. For a detailed review of this topic, see Cook et al. (2013).

CONCLUSIONS

Studies of RNase regulation and protection of RNAs are at an early stage, but it is already clear that cells have evolved many strategies for keeping their RNA complement safe from the unwanted action of the many RNases present. From the examples highlighted in the foregoing discussion, it is evident that many interesting regulatory processes have already been identified, some which are so far unique. Considering the large number of RNases that still remain to be studied, it is likely that there will be many more unexpected findings in this emerging field.
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