Ribonuclease Multiplicity, Diversity, and Complexity*

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Probably every RNA molecule undergoes some reaction that requires the action of a ribonuclease (RNase). As the details and complexity of RNA metabolism have emerged in recent years, encompassing many types of RNA molecules and many diverse reactions, there has been a concomitant increase in interest in the enzymes that carry out these events. Consequently, this has led to a rapidly expanding catalogue of RNases and has engendered a much greater appreciation for their multiplicity and diversity. The new RNases are generally highly specific and structurally diverse, and quite different from the small, nonspecific, classical RNases that frequently have been used for protein structural studies. In fact, some of the new RNases are not proteins!

Although considerable work has gone into identifying and characterizing RNases and many new ones have been isolated, this field is still in its early stages (1, 2). Nevertheless, several points are already clear: many more distinct RNases exist than might have been imagined just a few years ago (3); many RNases have overlapping specificities in vitro; both RNase activity and RNase expression may be regulated; and RNase action can be as important as transcription for determining levels of gene expression. A number of the RNases have now been extensively purified and studied, and mutants have been obtained, particularly in prokaryotes, that have helped to ascertain their metabolic functions. In addition, RNase genes have been cloned and sequenced. All of this information has greatly expanded our knowledge about RNases and their physiological roles in cells despite many RNA metabolic events for which no RNase has yet been identified (4).

The profusion of RNases has, at the same time, resulted in some confusion and nomenclature problems. This has come about because of the inherent difficulty of devising an absolutely specific assay for a particular RNase and the absence, in many cases, of mutants that eliminate an RNase activity. As a consequence, some RNases have been rediscovered with a different substrate and given a new name. In other cases, incompletely purified enzymes have been named, although their properties are now known to be amalgams of several RNases. In the discussion here the emphasis will be on RNases for which sufficient information (biochemical or genetic) is available to ensure that they represent a single entity. Because of space limitations it will not be possible to discuss all the known RNases. Rather, the focus will be on those that illustrate certain general principles.

RNase Involvement in RNA Metabolism

The reactions that make up RNA metabolism and for which RNase involvement appears necessary can be divided into three categories based on the structure and function of the product molecules. Examples from each category are presented in Table I.

<table>
<thead>
<tr>
<th>Category</th>
<th>Example</th>
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<tbody>
<tr>
<td>Transcription</td>
<td>tRNA synthesis</td>
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<tr>
<td>Translation</td>
<td>Protein synthesis</td>
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<tr>
<td>Degradation</td>
<td>RNA degradation</td>
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Processing reactions convert one RNA molecule into another, which is either a functional RNA or an intermediate on the pathway to a functional RNA. Included in this group are reactions that mature the 5' and 3' termini of RNA precursor molecules, those that separate individual RNAs from polycistronic transcripts (i.e., mRNA-mRNA, tRNA-tRNA, rRNA-rRNA, tRNA-mRNA, and tRNA-rRNA), and those that lead to removal of introns. Based on what is already known about RNA processing, this group encompasses a large number of very different types of reactions. Accordingly, it might be expected that many different RNases with very different specificities would be necessary to catalyze all of these reactions.

Turnover reactions alter RNA in some manner, but the RNA is subsequently restored to its original, or to an almost original, functional form. Examples of this group of reactions include the end turnover of the C-C-A sequence of tRNA (1), the breakage and rejoining of the anticodon loop of Escherichia coli tRNA by Hg2+ (10) or elevated temperature (11) also is associated with degradation of stable RNAs. In these situations it is likely that regulatory switches are necessary to turn on the degradative machinery because under usual growth conditions "stable" RNA is unaffected. In fact, bacterial mutants are known, which display increased degradation of stable RNA under certain conditions (12, 13). Presumably, the normal shutoff controls have been affected in these mutants.

Another large class of RNA molecules that are subject to degradation are the by-products of the many RNA processing reactions that occur in cells. Likewise, denatured, damaged, and incorrectly synthesized RNA molecules need to be eliminated. These latter classes of RNAs potentially could compete with functional RNAs.

Cells also have specific mechanisms for degrading the usually stable RNA species, rRNA and tRNA, in response to physiological stresses. Thus, starvation conditions promote degradation of stable RNAs to supply nucleotides for cell survival (9). Exposure of cells to Hg2+ (10) or elevated temperature (11) also is associated with degradation of stable RNAs. In these situations it is likely that regulatory switches are necessary to turn on the degradative machinery because under usual growth conditions "stable" RNA is unaffected. In fact, bacterial mutants are known, which display increased degradation of stable RNA under certain conditions (12, 13). Presumably, the normal shutoff controls have been affected in these mutants.

The total number of RNases that might be involved in the various degradative reactions is not yet known. A small number of nonspecific RNases could accomplish the task if the mechanism of degradation simply involved attack on any RNA molecule that was not in some way protected. However, it is more likely that all the degradative reactions are highly regulated and that at least the initiation of the degradative processes is carried out by specific RNases responding to a variety of signals and catalyzing very specific cleavages.

A Single Cell Contains Many Distinct RNases

Probably the most unexpected new information that has emerged from all the increased attention given to RNases in recent years is the multiplicity of these enzymes that can be present in a single cell. Much of what we know regarding this point has come from studies with E. coli (2, 3). Not only have many distinct RNases been identified and purified from this organism, but the availability of mutants that eliminate a single enzyme has been invaluable in distinguishing among those that are closely related. The combination of biochemical and genetic analysis has already revealed the existence of close to 20 distinct RNases in E. coli (Table II); undoubtedly, more will be found because there are many metabolic events for which no RNase has yet been identified.

Inspection of Table II indicates the wide variety of RNases already known and their diverse substrate specificities and functions. About 10 different endoribonucleases have been identified, and this number may be even larger, as several of the enzymes that have been grouped together because of the possibility that they might be different manifestations of the same protein could actually turn out to be distinct. Of the eight exoribonucleases listed in
**TABLE I**

<table>
<thead>
<tr>
<th>Type of reaction</th>
<th>Specific process</th>
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<tr>
<td>Processing</td>
<td>Maturation of 5′ and 3′ termini of RNA precursors</td>
</tr>
<tr>
<td>Turnover</td>
<td>End turnover of 5′-CCA sequence of tRNA</td>
</tr>
<tr>
<td>Degradation</td>
<td>Turnover of mRNAs</td>
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The table, first seven are clearly separate entities both biochemically and genetically (3). However, oligoribonuclease, which was identified and characterized biochemically early on (14), may yet turn out to be an additional activity of one of the other enzymes.

Table II also points up the growing nomenclature problem. We rapidly running out of alphabet to name new RNases, and even more seriously, two separate enzymes, one an endo and one an exon, have both been designated RNase R (3, 15). Moreover, the currently used nomenclature gives no indication of an enzyme’s specificity or function. When this is coupled with the naming of RNases from other organisms, in which the same letter designation has been used for a completely different enzyme, it is clear that a state of confusion is approaching.

Examination of the substrate specificities and functions of the RNases listed in Table II reveals a certain amount of overlap in their action, particularly for the exoribonuclease. Moreover, while mutations affecting the endoribonucleases, III, P, or HI, can have minor consequences for cell function (1-2), those eliminating individual exoribonucleases have essentially no deleterious effects (16). Such findings indicate that whereas alternate RNases can back up the functions of the exoribonucleases, this is much less effective with the endoribonucleases.

The degree of functional overlap among the *E. coli* exoribonucleases was shown most dramatically by mutations eliminating these enzymes. Cells lacking RNases II, D, BN, and T grow only slightly more slowly than wild type (17). However, introduction of an *rph* mutation, affecting RNase PH, into the aforementioned strain leads to inviability (16). Interestingly, the presence of any one of the five exoribonucleases is sufficient to support *E. coli* growth (18). The data indicate that there is such a high degree of functional overlap among these five enzymes in *vivo* that any one of them can take over the functions of all the others to some level. Some combinations of exoribonuclease mutations do have profound consequences (19). In the most dramatic example, elimination of both RNase II and polynucleotide phosphorylase (PNPase) results in inviability (20), most likely because they are the primary two enzymes that remove the RNA fragments generated by endonucleolytic cleavage of mRNA (20). Likewise, removal of the two known phosphorylating nucleases, PNPase and RNase PH, leads to very slow growth (16), although the metabolic step affected is not known.

**RNases Can Exist in Different Forms**

Several examples are now known of RNases that are found in more than one form. The physiological significance of multiple RNase forms is generally not understood, and in some instances, may simply represent artificial modifications or associations caused by the isolation procedure. Nevertheless, the multiple forms are of interest since they may represent a means of regulating RNase specificity or activity.

Two active forms of PNPase are known (21). One, form A, consists of three α subunits of 85 kDa each, whereas form B contains, in addition, two β subunits of 48 kDa each (22). Although both PNPases are active, their catalytic properties have not been examined in any detail that would explain the role of the β subunit. A similar situation was observed with *E. coli* RNase J upon infection of cells with bacteriophage T4 (23). In this case, RNase D increased from 40 to 65 kDa due to its association with a phage-specific low molecular mass protein. The catalytic properties of RNase D were not altered by this association, so that its physiological significance, if any, is not understood.

*E. coli* RNase I is a nonspecific endonuclease that hydrolyzes RNA in the absence of divalent cations to release 3′-phosphoryl-terminated mono- and oligoribonucleotides. Several nonnuclear endonucleases have been purified, which display some similarities and some differences when compared with RNase I. Thus, RNase T is a spherothix enzyme (25), whereas RNase I is found in the periplasmic space (26). Both enzymes are encoded by the *rnt* gene, and their specificity and stability differences may be a consequence of differences in free sulphydryl groups (25). RNase M is another closely related enzyme (27). It has an identical molecular mass and similar tryptic pattern to RNase I (28). Yet, it can be separated from RNase I and shows a different substrate specificity (27). Most importantly, RNase M appears to be present in strains with an interrupted *rnt* gene, although quantitative measurements were not reported (15). A fourth enzyme, RNase R, was also found in this RNase I negative strain. It can be separated from RNase M but has very low activity (15). Whether the two enzymes are themselves distinct or two forms of the same enzyme is not clear. Moreover, whether there is any relation of the latter two enzymes to RNase I remains to be determined.

Another interesting situation that may reflect two forms of the same protein involves *E. coli* RNases E and K. RNase E was originally identified as an endonuclease that converts 9 S RNA to p5S RNA (29). It was subsequently shown to play an important role in the initiation of mRNA degradation (30, 31). RNase E is thought to be a product of the *rne*/*ams*/*hmp* gene because a mutation in this gene leads to accumulation of 9 S RNA *in vivo* and a thermosensitive RNase E (32); however, this point has not been proven conclusively. RNase E was originally reported to be a protein of 70 kDa (33), but recent sequencing of the *rne* gene (after several apparently erroneous reports) indicates that it encodes a protein of 70 kDa (34). Several overlapping questions are particularly intriguing: that another enzyme, RNase K, which has been implicated in the degradation of ompA and bla mRNAs, is also affected by a mutation in the *rne* gene *in vivo*, although its activity is not altered in *vitrō* (31). RNase K, however, purifies as a protein of 55–60 kDa (35). RNase E and RNase K activities are also inversely affected by shifts in growth rates (36). Whether the second enzyme is a fragment of the *rne* gene or a separate gene, remains to be determined (37). Surprisingly, a *groEL* mutation has now been shown to affect 9 S RNA processing (RNase E activity), but not *ompA* mRNA cleavage (RNase K activity) (38). Moreover, RNase E activity is precipitated by a GroEL antibody, and highly purified GroEL contains RNase E, but not RNase K activity. The conclusion from this study was that RNase E associates with GroEL, although the catalytically active peptide could not be identified (38). The explanation for
all of these observations is not yet apparent. It is possible that RNase E and RNase K share a catalytic subunit encoded by nce or that RNase K represents an active, physiologically relevant proteolytic fragment of RNase E.

The foregoing discussion indicates that multiple forms of RNases may arise by association with additional subunits, sulfhydryls under physiological conditions, it is now clear that there are two RNase H activities in a variety of species, including E. coli, but these are encoded by separate genes (59). Multiple forms could also arise by covalent modification of RNases (see below).

**Mode of Action and Specificity**

Several different categories of RNases can be distinguished based on their mode of action on RNA chains. Endoribonucleases cleave RNA molecules internally, whereas exoribonucleases act at the end of RNA chains. So far, the exoribonucleases that have been identified release mononucleoside triphosphates, but it is possible that an enzyme could recognize the end of an RNA chain and yet cleave off oligonucleotides (40). Exoribonucleases are known, which cleave on either side of the phosphodiester bond. In general, the low molecular weight, degradative endoribonucleases cleave RNA in such a manner as to generate 3'-phosphoryl-terminated products; the 3'-end of RNA is sometimes cleaved off (40). Endoribonucleases are known, which cleave on either side of the phosphodiester bond. In general, the low molecular weight, degradative endoribonucleases cleave RNA in such a manner as to generate 3'-phosphoryl-terminated products subsequent to the formation of a 2', 3'-cyclic intermediate (3, 41, 42). These enzymes function in the absence of divalent cations; contrast, the high-specificity endoribonucleases which participate in RNA processing and turnover usually generate 3'-hydroxy termini and require a divalent cation for catalysis (3). Exoribonucleases are known, which can initiate attack at either the 3' or 5' terminus of an RNA chain. All the identified bacterial endoribonucleases hydrolyze RNA in the 3' to 5' direction, releasing 5'-mononucleotides (3, 43). These enzymes invariably require a divalent cation for activity. Eukaryotic cells contain, in addition, exoribonucleases whose mode of action is 5' to 3' (44, 45). These enzymes also require a divalent cation and release 5'-mononucleotides. One exception to the aforementioned groupings is spleen phosphodiesterase, which acts in the 5' to 3' direction and releases 3'-mononucleotides (46).

There has been considerable revision in our thinking about RNase action and recognition of the complexity of the problem. As a consequence, many of the early assumptions about the specificity of the enzymes have been questioned: (a) energy recapture upon RNA degradation and (b) energy input for RNA degradation. With respect to the first, two RNases are known, RNase P and RNase PH, that degrade RNA without generating nucleoside diphosphates (57, 58). RNase P, in this manner recaptures the energy present in the phosphodiester bond. Thus, both enzymes can synthesize RNA chains from nucleoside diphosphates and have equilibrium constants close to one (57, 59). It is not clear how extensive phospho-lytic degradation of RNA may be, but it is known that it is much more prevalent in Bacillus subtilis than in E. coli (60) and that this is due to differences in the ratio of phospho- and hydroxyl bonds in the substrate.

**Regulation of RNase Activity and Expression**

One of the more interesting questions about RNases is whether these enzymes are themselves regulated. If so, this would provide a mechanism for controlling the activity of the enzymes as a response to the kinetic pathways in which the RNases participate. Several strategies can be envisaged for influencing RNase activity: activation or inhibition by effector molecules, covalent modification of the RNase resulting in inactivation of cellular RNA (10, 66).

Energetic Aspects of RNase Action

Energetic considerations have received relatively little attention with regard to RNases. Two aspects of the problem invite examination: (a) energy recapture upon RNA degradation and (b) energy input for RNA degradation. With respect to the first, two RNases are known, RNase P and RNase PH, that degrade RNA phospho-lytically, generating nucleoside diphosphates (57, 58). RNase degradation in this manner recaptures the energy present in the phosphodiester bond. Thus, both enzymes can synthesize RNA chains from nucleoside diphosphates and have equilibrium constants close to one (57, 59). It is not clear how extensive phospho-lytic degradation of RNA may be, but it is known that it is much more prevalent in Bacillus subtilis than in E. coli (60) and that this is due to the ratio of phospho- and hydroxyl bonds in the substrate.
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been reported to influence RNAse activity. Thus, a Bacillus exoribonuclease is strongly inhibited by GMP (67), and a Bacillus endoribonuclease is sensitive to low concentrations of ATP (68). It has recently been reported that an endoribonuclease (RNase D) from mouse cells is dependent both on spermidine and TRNA for its activity (69). Further work on this system is awaited. Probably the clearest response of an RNAse to a small effector molecule is the 2-5A-dependent RNase (also known as RNase L or RNase F) of mammalian cells. This protein is completely dependent on 2-5A-oxyadenylates for activity (70).

Little is known about the covalent modification of RNases. It has been reported that E. coli RNase III is activated and phosphorylated by T7 protein kinase upon phage infection (71). Recent evidence indicates that RNase III can exist in two forms, one of which is dependent on the presence of Era protein, with which it is translationally coupled. This protein is suggested to be a proenzyme involved in phosphorylation of RNase III, but this remains to be proven. ADP-ribosylation of an RNase has also been observed. HeLa cell nuclei contain a double-strand RNase, which can be modified with NAD resulting in inhibition of the enzyme. Inhibition of ADP-ribose polymerase by 3-aminobenzamide prevents the RNase inhibition (73).

RNase activity may also be regulated by expression and turnover. It is known that E. coli RNase III regulates its own expression (74). A stem-loop structure upstream of the coding region in rnc mRNA is cleaved by RNase III leading to degradation of the message. Uncut messages have a 6-fold greater half-life and steady-state level. Thus, cells deficient in RNase III accumulate more of the enzyme. A similar situation was observed for pnp mRNA encoding PNP. RNase III also cleaves the pnp message in its 5-untranslated region so that in the absence of RNase III, PNPase mRNA is elevated and PNPase increases close to 10-fold (75). An added wrinkle in this system is that PNPase itself negatively autoregulates its expression at the translational level, but this is dependent on the prior cleavage by RNase III (76). An additional enzyme, E. coli RNase D, is apparently regulated at the translational level (77,78). The aforementioned examples make it extremely likely that RNase regulation will be the norm, rather than the exception, and that many types of mechanisms will be utilized to modulate RNAse activity and expression.

Future Directions

The RNase field is currently in an exponential phase as an ever increasing number of these enzymes are being discovered and studied. New work will define all the RNases involved in RNA metabolism. This will require continued identification and purification of the proteins (or RNAs), examination of their mechanism of action and substrate specificity determinants, and proof of their biological function. The latter information will only come from isolation of mutants defective in specific metabolic steps and/or RNases. At present, there is a particular absence of eukaryotic mutants such that functions cannot be assigned to most of the known RNases. Finally, much more work is needed on RNase regulation. We can look forward to many interesting and unexpected developments in this area in the coming years.