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REVIEW

Emerging features of mRNA decay in bacteria

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ABSTRACT

The problem of mRNA decay in *E. coli* has recently seen exciting progress, with the discoveries that key degradation enzymes are associated together in a high molecular weight degradosome and that polyadenylation promotes decay. Recent advances make it clear that mRNA decay in bacteria is far more interesting enzymatically than might have been predicted. In-depth study of specific mRNAs has revealed multiple pathways for degradation. Which pathway a given mRNA follows appears to depend in large part on the location of the initiating endonucleolytic cleavage within the mRNA. During the steps of mRNA decay, stable RNA structures pose formidable barriers to the 3′ → 5′ exonucleases. However, polyadenylation is now emerging as a process that plays an important role in maintaining the momentum of exonucleolytic degradation by adding single-stranded extensions to the 3′ ends of mRNAs and their decay intermediates, thereby facilitating further exonuclease digestion.

Keywords: degradosome; endoribonucleases; exoribonucleases; polyadenylation; RNA structural barriers

INTRODUCTION

When messenger RNA was discovered nearly 40 years ago (Brenner et al., 1961; Gros et al., 1961), its defining property was instability. At any one time mRNA represents a substantial fraction of the transcripts being made by RNA polymerase, but it is degraded quickly. The instability of mRNA has proven an important parameter that determines levels of gene expression and permits rapid responses to regulatory signals. The instability also immediately raised the question of how mRNA is broken down enzymatically to recycle the nucleoside monophosphate units to NTPs. At least three enzyme activities from *Escherichia coli* would be identified within a few years of the discovery of mRNA: polynucleotide phosphorylase (Littauer & Kornberg, 1957), poly(A) polymerase I (August et al., 1962), and RNase II (Spahr, 1964). However, because linking these or any other activities to the mRNA degradation process required characterizing mutants deficient in each activity, progress in understanding the mechanism of mRNA decay was made very slowly over the ensuing years only as such mutants were obtained.

This article reviews the advances that have made the problem of mRNA decay in bacteria a lively area of current research. It discusses the *E. coli* enzymes shown thus far to function in mRNA decay. Considerable emphasis is placed on the two exciting findings of the 1990s that have most stimulated increased interest in mRNA decay mechanisms in bacteria. First, key decay enzymes have been found associated in the degradosome, a multiprotein complex with the capacity to coordinate steps of turnover, and conceivably also to regulate degradation in response to available metabolites. Second, polyadenylation of bacterial mRNAs, a phenomenon first observed over 20 years ago but discounted as having little significance, has been rediscovered. Addition of a poly(A) tail to an mRNA has now been found to provide a way to clear the barriers to exonucleolytic degradation posed by stable RNA secondary structures. A final issue that is considered is the extent to which data obtained from studying several mRNA decay pathways in detail permits molecular models to be formulated for the degradation of an mRNA. For excellent recent reviews of this topic, see a comprehensive article on mRNA degradation in *E. coli* (Coburn & Mackie, 1999) and a minireview summarizing advances for a broader array of organisms (Carpousis et al., 1999).

ESCHERICHIA COLI RNases IN mRNA DECAY

In bacteria, mRNA degradation appears to be initiated by one or more endoribonucleolytic cleavages, followed by digestion from the 3′ ends of the products by 3′ → 5′ exoribonucleases. Of the over 20 RNases identified in *E. coli* (Deutscher, 1993a, 1993b), only six are currently known or implicated to function in mRNA de-

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cay. The remaining RNases are dedicated to rRNA and tRNA biosynthesis, although some of these enzymes act on specific mRNA substrates or show activity against poly(A) in cell extracts. Whether any of these enzymes accounts for nuclease activity on mRNA observed in mutant strains deficient in the major decay enzymes remains to be determined.

Three of the RNases of mRNA decay are site-specific endoribonucleases, and three are 3′ → 5′ exoribonucleases (Table 1). A third class of enzymes, 5′ → 3′ exoribonucleases, is not detected in bacteria but plays an important role in mRNA decay in eukaryotes. Of the endonucleases, RNase E (Rne protein) is the major enzyme of mRNA decay. It has in addition a biosynthetic role processing the 9S precursor to 5S rRNA and functions in the degradation of rRNA (Bessarab et al., 1998). For an RNase, it is a large protein (1,061 amino acids) with several functional domains. RNase E is essential for viability: mutations at codons 66 or 68 in the N-terminal segment that harbors catalytic activity confer temperature sensitivity (McDowall et al., 1993). Only the N-terminal 60% of the Rne protein is needed for cell survival (Kido et al., 1996). RNase E cleaves at specific sites in single-stranded regions of mRNA that are typically AU-rich but do not show strong sequence specificity. Although stem-loop structures are frequently present near sites of RNase E cleavage, the growing view is that they function to bias local structure in favor of leaving the cleavage site single stranded (Coburn & Mackie, 1999). The most unprecedented property of RNase E as an endonuclease is that it prefers a 5′ end for activity (Mackie, 1998). Circularized RNAs bearing well-characterized RNase E cleavage sites are resistant to cleavage by the enzyme in vitro, as are linear forms of the same RNAs with the 5′ end protected by an annealed oligonucleotide. Monophosphorylated 5′ ends are preferred over triphosphorylated 5′ ends (Lin-Chao & Cohen, 1991; Mackie, 1998). These properties of RNase E rationalize a large body of work indicating that determinants at the 5′ ends of many mRNAs govern an mRNA's susceptibility to decay and therefore have significant effects on mRNA half-lives. A second gene with extensive sequence similarity to the N-terminal half of RNase E (McDowall et al., 1993) is present in the *E. coli* genome. Mutations in this gene, originally denoted *cafA*, lower the temperature at which the temperature-sensitive *rne-1* mutant can grow (Wachi et al., 1997) and block maturation of 16S rRNA (Li et al., 1999). These findings and initial characterization of the cleavages made on the 16S rRNA precursor by the CafA protein indicate that it is a ribonuclease. Now renamed RNase G, this endonuclease appears to have a specificity that overlaps but is distinct from RNase E (Li et al., 1999; Tock et al., 2000), possibly explaining some of the cleavages observed on various mRNAs after inactivation of thermolabile RNase E. A third rRNA biosynthetic enzyme, RNase III, makes cleavages in double-stranded regions of certain mRNA transcripts. Although it clearly initiates decay of these (Portier et al., 1987; Bardwell et al., 1989; Régnier & Grunberg-Manago, 1989) and other specific mRNAs, the fact that null mutants in the gene show only a mild phenotype (Babitzke et al., 1993) suggests that RNase III does not have a major role in mRNA degradation.

The exonucleases with functions in mRNA decay include RNase II, a hydrolytic enzyme that removes nucleoside 5′ monophosphates from an RNA 3′ end. In the gram-negative bacterial strain *E. coli*, hydrolysis by RNase II probably accounts for 90% of the exonucleolytic activity on mRNA, whereas in the gram-positive strain *Bacillus subtilis*, hydrolytic degradation is only a minor component of the exonucleolytic activity (Deutscher & Reuven, 1991). The second major exonuclease of mRNA decay is polynucleotide phosphorylase, a phosphorolytic enzyme that uses inorganic phosphate to remove nucleotides from RNA 3′ ends, yielding nucleoside 5′ diphosphates. Inactivating either of these enzymes leaves *E. coli* strains viable, but inactivating both is lethal (Donovan & Kushner, 1986). Both enzymes degrade RNA processively, but have difficulty

TABLE 1. *E. coli* RNases in mRNA decay.

Enzyme	Gene	Monomer size (kDa)	Electrophoretic mobility (kDa) on SDS-PAGE	Subunit structure	Reference
A. Endoribonucleases					
RNase E	*rne*	118	180	dimer ?	Coburn et al., 1999
RNase G	*rng* (*cafA/mre*)	55	55	?	
RNase III	*rnc*	25	25	α2 dimer	Dunn, 1976
B. 3′ → 5′ Exoribonucleases					
RNase II	*rnb*	72.5	72.5	monomer	Gupta et al., 1977
Polynucleotide phosphorylase	*pnp*	77	85	α3 trimer	Portier, 1975; Soreq & Littauer, 1977
Oligoribonuclease	*orn*	20.7	20	α2 dimer	Ghosh & Deutscher, 1999; Zhang et al., 1998

establishing and maintaining a processive cleavage mode on structured RNA 3′ ends with fewer than 6–10 unpaired nucleotides beyond a stable stem-loop (Littauer & Soreq, 1982; Pepe et al., 1994; Coburn & Mackie, 1996a). The extent to which RNase II and PNPase are impeded by RNA structure has always been puzzling in light of the RNA 3′ ends they frequently encounter on phage, plasmid, and bacterial mRNAs. Rho-independent termination (Mott et al., 1985), REP (repetitive extragenic palindrome) sequences (Gilson et al., 1984; Stern et al., 1984), and processing by RNase III (reviewed in Court, 1993) leave stem-loops, many of which are thermodynamically quite stable. Because RNase II and PNPase are the principal exonucleases available to degrade mRNA, it is not surprising that additional mechanisms have evolved to remove RNA structural barriers or otherwise facilitate access of the two enzymes to structured mRNA 3′ ends (see below). It is of interest that the *E. coli* genome and many of the sequenced genomes contain a homolog of the gene for RNase II (Cheng et al., 1998). The *E. coli* gene encodes the exonuclease RNase R, which is responsible for the residual hydrolytic activity that occurs on mRNA substrates in the absence of RNase II (Deutscher, 1993b). These findings, and the fact that inactivating RNase R and PNPase is lethal (Cheng et al., 1998), suggest that RNase R may play a role in mRNA decay. The final RNase currently implicated in decay is oligoribonuclease. This enzyme has recently been shown to be required in the terminal steps of degradation to break down the remaining small mRNA fragments 2–5 nt long to mononucleotides (Ghosh & Deutscher, 1999). The gene encoding oligoribonuclease is essential and has orthologs across a wide range of organisms.

The known RNases shown to be involved in mRNA decay in *E. coli* thus define a small group of genes, most of which are essential individually or in combination. The phenotypes of mutant alleles in the genes, as they are combined sequentially, are the accumulation of larger and larger mRNA fragments and inviability (Donovan & Kushner, 1986; Arraiano et al., 1988; Ghosh & Deutscher, 1999). These results are clear evidence that a bacterial cell's inability to break down mRNA transcripts to nucleotide units is incompatible with continued growth and cell division.

THE DEGRADOSOME, A MULTIPROTEIN COMPLEX IN THE DEGRADATION OF mRNA

One of the most exciting findings of the past decade has been that key mRNA decay RNases in *E. coli* are associated with other proteins in a high-molecular-weight complex that has come to be termed the degradosome. The existence of a multiprotein machine that mediates mRNA decay is in fact not turning out to be an isolated example. During the 1990s several complexes with functions in mRNA decay and rRNA processing have been discovered. A complex related to the bacterial degradosome is observed in spinach chloroplast (Hayes et al., 1996). In yeast, a complex in the mitochondrion (mtEXO) and an exosome found in both the cytoplasm and nucleus contain putative helicases and exonucleases related to bacterial RNases (Margossian et al., 1996; Mitchell et al., 1997; Jacobs Anderson & Parker, 1998). The human homolog of one of the yeast exosome proteins has also been observed in a large complex (Mitchell et al., 1997). Thus, assemblies of RNases with helicases and other proteins may prove to be a common feature in RNA processing and decay (for a review, see Carpousis et al., 1999).

In the case of *E. coli*, the first indications of a protein complex came from two lines of inquiry. During the course of a persistent effort to purify RNase E activity to homogeneity, it was noted that RNase E copurified with PNPase, and that the fractions containing these activities included additional proteins (Carpousis et al., 1994). A complementary result was obtained during purification of PNPase on the basis of its binding activity toward an RNA substrate bearing a REP sequence (Py et al., 1994). Amino-terminal sequencing of the additional proteins identified the glycolytic enzyme enolase and the DEAD-box, ATP-dependent RNA helicase RhlB as components of the complex (Py et al., 1996). The same proteins were also found via a distinct approach, purification of epitope-tagged RNase E on a monoclonal antibody-conjugated agarose column (Miczak et al., 1996). Finding RNase E, PNPase, and RhlB associated in a complex suggested that the complex might coordinate endo- and exonucleolytic digestion and the unwinding of RNA structure. In support of this, degradation of the REP-containing RNA substrate and the highly structured 3′ end of ribosomal protein S20 mRNA is stimulated in the degradosome under conditions that activate the helicase (Py et al., 1996; Blum et al., 1999; Coburn et al., 1999). As yet, a function for the 5–10% of cellular enolase that is present in the degradosome (Py et al., 1996) has not emerged. Several other proteins have been observed to purify with the degradosome complex in smaller amounts. These include polyphosphate kinase, which reversibly synthesizes ATP from ADP and polyphosphate (Kornberg, 1995), DnaK, and GroEL. Functions for these proteins in mRNA decay have not been established, although the fact that GroEL is detected in degradosomes containing a temperature-sensitive RNase E but not wild-type RNase E suggests possible roles for the chaperonins in degradosome assembly or refolding (Miczak et al., 1996). The degradosome has been shown to harbor RNAs and RNA fragments, but as these RNAs include known substrates for RNase E but not tRNAs lacking RNase E cleavage sites, the presence of RNA presumably just reflects the degradosome at work degrading mRNA, processing 9S to 5S rRNA, and de-

grading rRNA (Bessarab et al., 1998). It should be noted that two other key enzymes of decay, RNase II and poly(A) polymerase I (PAP I), have thus far not been detected in degradosome preparations. However, PAP I can associate with RNase E (Raynal & Carpousis, 1999), and the specificity of RNase E on certain substrates is altered in a PAP I mutant strain (Xu & Cohen, 1995). Thus it will not be surprising if additional proteins are found to make interactions with the degradosome as it carries out reactions on various RNA substrates.

Characterization of the protein interactions in the degradosome has clarified to some extent why Rne is such a large protein. While the N-terminal half of the protein contains the endonuclease activity, cleavage specificity, and a putative S1 RNA binding domain, it now appears that the C-terminal half has a major role as a platform that organizes interactions with other proteins. As determined by Far Western blotting, the yeast two-hybrid assay, and coimmunopurification experiments with full-length or deletion variants of RNase E (Vanzo et al., 1998), the C-terminal half includes binding sites for RhlB (amino acids 734–738 and possibly flanking residues), enolase (amino acids 739–845), and PNPase (amino acids 844–1045). These experiments have also provided evidence for interaction of RNase E with itself and have identified a region of the Rne protein that stimulates the helicase activity of RhlB. Purification of the C-terminal half bearing an epitope tag revealed that this segment of RNase E is sufficient for recruiting the other degradosome proteins into the complex (Kaberdin et al., 1998). In view of the important role the C-terminal half of RNase E has in assembly of the degradosome, it is provocative that this part of the protein is significantly less conserved across bacterial species than the N-terminal endonuclease domain (Kaberdin et al., 1998). This finding raises the possibility that there will be differences in the interactions RNase E homologs have with other decay components.

Clearly there are more exciting developments on the horizon. One critical issue to resolve is the stoichiometry of the degradosome constituents, which at present is not certain (reviewed in Coburn & Mackie, 1999). Degradosome preparations thus far show significant size heterogeneity. Estimates for the molecular weight of the complex range from $1.5–2.4 \times 10^6$ Da. Evidence for interaction of RNase E with itself (Vanzo et al., 1998) and analysis of RNase E cleavage on ribosomal protein S20 mRNA substrates (Mackie et al., 1997) predict that at least a dimer of the Rne protein is present. Based on the molar ratios of degradosome proteins estimated from various preparations (Carpousis et al., 1994; Miczak et al., 1996; Coburn et al., 1999), the degradosome could contain at least one trimer of PNPase, two dimers of enolase, and a dimer of RhlB. The first efforts to reconstitute the degradosome from purified recombinant components has recently yielded a minimal degradosome that assembles spontaneously from Rne, PNPase, and RhlB in amounts consistent with these predictions (Coburn et al., 1999). The reconstituted minimal degradosome shows ATP-activated degradation of a REP-containing substrate that is indistinguishable from the activity observed for purified degradosome preparations. The activation of RhlB requires the Rne protein but not its endonuclease activity. A second unresolved issue that is critical to formulating models for mRNA decay is how much of the total amount of each degradosome component is localized in the degradosome relative to the amount distributed elsewhere in the bacterial cell. Equally important will be information from both in vivo and in vitro experiments that can shed light on whether the degradosome is a uniform, static structure or a dynamic complex that disassembles and reassembles, perhaps with variable components, on the various RNA substrates for processing or decay. The subcellular localization of the degradosome may in addition prove interesting, particularly in light of early reports of processing complexes associated with membrane fractions (Miczak et al., 1991) and the more recent isolation of mutants showing altered mRNA decay that map in an *E. coli* gene encoding the membrane-associated and ATP-dependent protease HflB/FtsH (Granger et al., 1998; Wang et al., 1998).

POLYADENYLATION MAINTAINS THE MOMENTUM OF mRNA DECAY

Within a year of the discovery of mRNA, a poly(A) polymerase from *E. coli* was described (August et al., 1962). This was well before eukaryotic mRNAs were found to bear poly(A) tails, but ironically, it would be 30 years before identification of the *E. coli* gene encoding poly(A) polymerase would set into motion developments that have clarified the function of polyadenylation in eubacteria. During this period poly(A) or oligo(A) tracts of variable lengths were repeatedly detected on phage and bacterial mRNAs isolated immediately after pulse labeling (reviewed in Sarkar, 1997). Also, more than one investigator was probably surprised when bacterial RNA used as a negative control for polyadenylated RNA showed higher than background levels of adenylated material. However, as it became evident over time that eukaryotic mRNAs were polyadenylated at high efficiency with poly(A) tails 80–200 nt long, whereas only a small fraction of prokaryotic mRNAs bore much shorter poly(A) tails, the prospects that polyadenylation in bacteria had a significant general function during the short lifetime of an mRNA seemed to dim.

Identification of the gene encoding PAP I using amino acid sequence information from the purified protein finally paved the way for significant advances (Cao & Sarkar, 1992b). The gene proved to be a known locus,

pcnB. Mutations in *pcnB* were first obtained during a genetic selection for decreased copy number of ColE1 plasmids (Lopilato et al., 1986). The fact that these mutants and subsequent deletion mutants (Liu & Parkinson, 1991) are viable indicates that the gene for PAP I is not essential. PAP I is a monomer of approximately 53 kDa that has the characteristic features of other members of the nucleotidyl superfamily (Martin & Keller, 1996), in particular a subfamily that includes eubacterial and eukaryotic CCA-adding enzymes and eubacterial poly(A) polymerases (Yue et al., 1996). A second poly(A) polymerase activity in *E. coli* has been reported (Kalapos et al., 1994). A putative gene for this activity was identified (Cao et al., 1996), but it has now been reported that the product of this gene is not a poly(A) polymerase (Mohanty & Kushner, 1999b). Therefore what enzyme activity accounts for the residual level of polyadenylation (~10%) observed in the absence of PAP I (O'Hara et al., 1995) remains to be determined. Current information continues to indicate that the poly(A) tails on mRNAs in bacterial strains are short and that the extent of polyadenylation is low. In wild-type strains, the tails are 10–40 nt long (O'Hara et al., 1995). Measurements of the extent of polyadenylation of specific mRNAs range from 0.01% (Goodrich & Steege, 1999) to a few percent (reviewed in Sarkar, 1997). These levels are increased if PAP I is overproduced and are decreased in the presence of excess PNPase (Mohanty & Kushner, 2000). It should be noted that polyadenylation is not restricted to mRNAs. Polyadenylation of precursor and mature forms of rRNAs and small RNAs has been observed (Li et al., 1998; Mohanty & Kushner, 2000), but the function of polyadenylation in the metabolism of these RNAs, if any, is not known.

Critical clues to the function of polyadenylation in bacteria first came from determining the mechanism by which mutations in *pcnB* decreased the copy number of ColE1 plasmids (He et al., 1993; Xu et al., 1993). In the absence of the *pcnB* product, the small RNA (RNA I) that represses plasmid DNA replication was found to be stabilized ~10-fold, thus increasing the steady-state concentration of RNA I molecules capable of repressing replication. The form of RNA I that accumulated in the *pcnB* mutant had undergone the RNase E cleavage that normally leads to its rapid decay, but was lacking the poly(A) tail present in *pcnB*$^+$ strains. These results suggested that polyadenylation is required for the rapid decay of RNA I. Subsequent studies of mRNAs gave parallel results (reviewed in Cohen, 1995; Sarkar, 1997). Poly(A) tails have been found on a variety of mRNAs (reviewed in Sarkar, 1997) and on the upstream products of endonucleolytic cleavage (Haugel-Nielsen et al., 1996). In poly(A)-deficient strains, the mRNAs for ribosomal proteins S15 (Hajnsdorf et al., 1995) and S20 (Coburn & Mackie, 1998) and mRNA in general (O'Hara et al., 1995) are stabilized. In agreement with these results, polyadenylation of RNA I and S20 mRNA in vitro stimulates their degradation by exonuclease digestion (Xu & Cohen, 1995; Coburn & Mackie, 1996b). Addition of an oligo(A) tail to the REP-containing RNA substrate promotes its decay by degradosome preparations as well, even when as few as 5 nt are added (Blum et al., 1999). These experiments, considered with strong evidence that RNA secondary structure impedes RNase II and PNPase, have been rationalized by the proposal that poly(A) tails stimulate mRNA decay by functioning as single-stranded extensions to facilitate 3′ → 5′ exonucleolytic degradation. Results from many laboratories have since affirmed this hypothesis. It is also now known from overexpressing PAP I under conditions that have no effects on cell viability or growth rate (Mohanty & Kushner, 1999a) that mRNA half-lives generally decrease with increased cellular poly(A) levels. Certain transcripts are stabilized, however, including the mRNAs for the decay enzymes PNPase and RNase E, but not RNase II and RNase III. The stabilization of specific transcripts encoding decay enzymes raises the possibility that regulatory controls exist to balance the mRNA decay and polyadenylation pathways.

A question left unanswered by the early studies of poly(A)-promoted degradation is when polyadenylation takes place during an mRNA's lifetime. Possibly because such a small fraction of specific mRNAs seemed to be polyadenylated at any given time, the view developed that polyadenylation initiates the decay process by marking an mRNA for immediate and complete degradation (Kushner, 1996). Long polycistronic mRNAs have never fit this hypothesis particularly well, however, because they begin to decay before their 3′ ends are even synthesized (Chow & Dennis, 1994; Nierlich & Murakawa, 1996). As it has become possible to study the degradation of individual mRNAs in more detail, the initiation model of polyadenylation as a general one for most mRNAs has undergone revision. One indication that polyadenylation would not necessarily prove to be an initiating step came from studies of ribosomal protein S15 mRNA, which established that endonucleolytic cleavage by RNase E between the coding sequence and the transcriptional terminator was the first and rate limiting step of decay (Régnier & Hajnsdorf, 1991). When poly(A) tails were later found on S15 mRNA, they were mapped to multiple locations, not only on the nascent terminated 3′ end, but also on decay intermediates produced by endonucleolytic and exonucleolytic digestion (Haugel-Nielsen et al., 1996).

Recent studies of the filamentous phage f1 mRNAs have permitted the timing of polyadenylation to be determined relative to other steps in their processing and decay pathways (Goodrich & Steege, 1999). These mRNAs (Fig. 1) are subject to both RNase E-independent (RNAs C, C*) and RNase E-dependent (RNAs D–G, T) cleavage (Kokoska et al., 1990; Goodrich & Steege, 1999). Following transcription and rho-

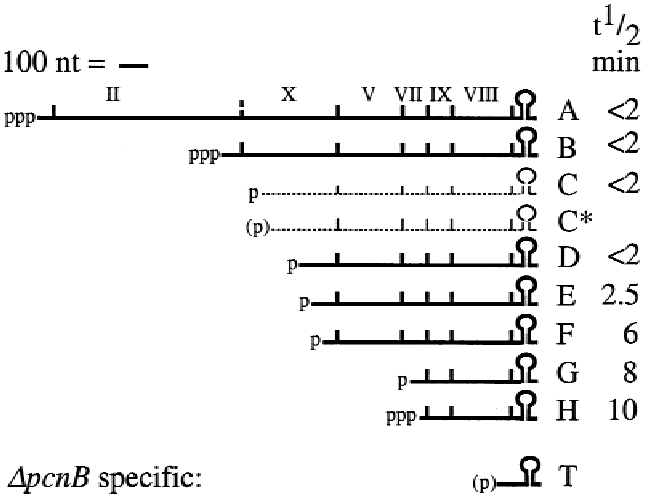

FIGURE 1. The major filamentous phage f1 mRNA species observed in infected hosts. The RNAs seen in *pcnB*$^+$ hosts range in size from 370 to 2,000 nt, and bear a stable stem-loop at their common 3′ ends generated by rho-independent termination. The diagram is based on work from many investigators (reviewed in Model & Russel, 1988). The half-lives for an RNase E$^+$ host are based on data for pulse-labeled RNA (Cashman et al., 1980). Gene numbers are indicated above the RNAs, and coding regions are marked by vertical lines. A dashed line marks the beginning of the smaller gene within an in-frame overlapping pair (genes II and X). The known or probable (parentheses) phosphorylation status of the 5′ ends is indicated for the primary transcripts and processed RNAs. The smallest RNA fragment, RNA T, accumulates to significant levels only in a *pcnB* deletion mutant host (Goodrich & Steege, 1999).

independent termination at a common site, a series of progressively longer-lived processed mRNA species appear in a 5′ → 3′ sequence. The primary transcripts are detected at 2 min after phage infection, RNAs D, E, and F by 5 min, and RNA G, not until 10 min (Blumer & Steege, 1984). The 3′ ends of the RNAs can thus be followed over time, because the end on RNA G is many minutes older than those present on the nascent primary transcripts. In a PAP I deletion mutant, no increase is observed in the amounts of the large primary transcripts or the products of RNase E-independent cleavage, suggesting that they undergo processing steps before being affected by the absence of polyadenylation. By contrast, the later-appearing processed RNAs are stabilized. RNA G, which bears the longest-lived 3′ end from transcription, is stabilized most markedly. A small RNase E cleavage fragment from the 3′ end of the RNAs (RNA T) that is detectable only at trace levels in wild-type strains is also greatly stabilized. This RNA does not appear until 15–20 min after infection, primarily because ribosomes on the heavily translated coding region for the major coat protein (gene VIII) protect the 3′-most distal cleavage site from RNase E. These results clearly place polyadenylation at a later rather than an initiating step of decay and suggest that PAP I only slowly accesses the 3′ end of the mRNAs. A recent study following the appearance of an adenylated form of RNA I concurs with this conclusion (Binnie et al., 1999). These findings may also suggest that early measurements of poly(A) levels using mRNAs isolated immediately after pulse labeling may have underestimated the extent of polyadenylation on bacterial, phage, and plasmid mRNAs.

A second feature of early proposals that has been addressed by more detailed analysis of individual mRNAs is the suggestion that addition of a poly(A) tail guarantees rapid and complete decay of the mRNA. Although there are certainly examples of mRNAs that appear to decay with all-or-none kinetics, increasing evidence shows overwhelmingly that stable RNA secondary structures pose formidable barriers to decay that are not necessarily eliminated in a single step. One telling reflection of RNA secondary structure is seen in the patterns of polyadenylation sites in several mRNAs. Unlike polyadenylation in eukaryotes, which occurs downstream from the specific signal AAUAAA, sites of poly(A) tail addition by PAP I show little or no sequence specificity. In the ribosomal protein S15 mRNA, for example, poly(A) addition sites map to 45 positions within a 185-nt region of the mRNA (Haugel-Nielsen et al., 1996). However, the positions of poly(A) addition are often not evenly distributed throughout an mRNA, and clusters of heterogeneous sites are observed (Cao & Sarkar, 1992a; Xu et al., 1993; Haugel-Nielsen et al., 1996; Sarkar, 1997; Goodrich & Steege, 1999). These findings raised the possibility that the progress of 3′ → 5′ exonucleolytic degradation through an mRNA is not uniform. A large body of in vivo evidence that secondary structures increase mRNA stability provides indirect support for this interpretation, and in vitro studies have demonstrated that stable hairpins impede PNPase and RNase II (Mott et al., 1985; McLaren et al., 1991). However, it has only recently been possible to define the actual events that occur as the exonucleases encounter structural barriers at the 3′ ends and internal positions within an mRNA. When Coburn and Mackie (1998) reconstituted the degradation of ribosomal protein S20 mRNA in vitro with purified enzymes, they found that complete degradation of the highly structured 3′ one-third of the mRNA required PAP I, PNPase, ATP, and inorganic phosphate. RNase II could not substitute for PNPase. Moreover, unless PAP I and ATP were present continuously, incomplete decay intermediates persisted. This result argues strongly that polyadenylation is a dynamic process in which repeated steps of poly(A)-tail addition and exonuclease digestion are needed to overcome the resistance of structured RNAs to exonucleolytic decay. In light of these in vitro results, the clustering of poly(A) addition sites observed in vivo for the *lpp mRNA* (Cao & Sarkar, 1992a), RNA I (Xu et al., 1993), ribosomal S15 mRNA (Haugel-Nielsen et al., 1996), f1 mRNAs (Goodrich & Steege, 1999), and others can be probably interpreted as good evidence for reiterative events at RNA structural barriers.

The picture of bacterial polyadenylation emerging is one of a process that has an important role in maintaining the momentum of mRNA decay. The substrate preferences displayed by PNPase and RNase II and the existence of stable structures within and at the ends of mRNAs combine to make degradation a stuttering process as the enzymes encounter structural barriers. At these barriers, the exonucleases compete with PAP I for the RNA 3′ ends. Addition of a poly(A) tail by PAP I promotes further decay, but even if a poly(A) tail is present, exonuclease digestion may or may not clear the barrier. RNase II, which tends to resect RNA ends bearing poly(A) extensions back to ≤10 nt from the base of a stem-loop and then stall (Coburn & Mackie, 1996a), can actually interfere with further decay by converting ends that are susceptible to PNPase to ends that are not (Hajnsdorf et al., 1994; Pepe et al., 1994; Coburn & Mackie, 1998; Mohanty & Kushner, 2000). As a result, thermodynamically stable structures appear to require repeated steps of adding single-stranded poly(A) extensions followed by exonuclease digestion before breathing or shortening of the stem permits complete passage of exonucleolytic degradation through a decay intermediate. Although the evidence is now strong that polyadenylation does not have a general role in initiating decay of most mRNAs, there is almost certainly a class of RNAs that require polyadenylation for degradation to begin. These include tightly folded RNAs lacking RNase E cleavage sites [for example, RNA-OUT (Pepe et al., 1994) and phage f1 RNA T (Goodrich & Steege, 1999)] and any structured decay intermediates released from the degradosome before being broken down completely.

DECAY PATHWAYS VARY AS A FUNCTION OF THE POSITION OF THE INITIATING ENDONUCLEOLYTIC CLEAVAGE

To what extent can molecular models be formulated for the degradation of individual mRNAs, using the enzymes currently known to be involved? One of the most difficult problems continues to be assigning PNPase or RNase II to 3′ → 5′ exonucleolytic steps in specific decay pathways. RNase II is the predominant activity available in *E. coli* (Deutscher & Reuven, 1991) to act on RNA 3′ ends not engaged in the degradosome, but it is not yet clear whether free PNPase is also available to compete for 3′ ends as well. Recent in vitro complementation experiments with free wild-type PNPase and degradosomes bearing the Pnp-13 mutant protein suggest that PNPase does not readily cycle between a free and complexed form (Coburn et al., 1999). If the principal source of PNPase proves to be the degradosome, then PNPase will be implicated at upstream ends left from endonucleolytic cleavage by RNase E. As specific mRNAs are studied in more detail, in vitro data become available to indicate whether decay intermediates from an mRNA are susceptible to digestion by both exonucleases or only the less structure-sensitive PNPase. However, in cases where both enzymes can digest a fragment to completion, possibly with stimulation by polyadenylation, in vitro data still leave unanswered the question of which enzyme actually functions in vivo. Likewise, where results from studies in exonuclease-deficient strains implicate one enzyme over the other as being important in certain steps during the degradation of an mRNA in vivo, it is often not clear which enzyme is involved in other steps of the decay pathway. A second but similar difficulty is posed by the existence of at least two mechanisms for clearing the barriers posed by RNA secondary structure, polyadenylation by PAP I and unwinding by the RhlB helicase activity of the degradosome. Given present information, it is hard to know under what conditions in vivo which mechanism comes into play or whether both cooperate to make decay more efficient.

These difficulties aside, in-depth study of several protein-coding mRNAs over the past decade has given a more complete picture of their degradation pathways and hence has improved our understanding of the principal mechanisms by which bacterial mRNAs are degraded. Present evidence clearly shows that multiple pathways exist, and that the mode and overall appearance of a decay pathway is determined to a great extent by the position of the initiating endonucleolytic cleavage. Three examples of protein-coding mRNAs will be described to document this conclusion. The mechanism of decay for the filamentous phage f1 mRNAs (Goodrich & Steege, 1999) probably illustrates the pathway followed by most mRNAs (Fig. 2). After transcription, most of the f1 transcripts move through the RNase E-dependent endonucleolytic cleavage pathway, presumably in the degradosome. In view of evidence that 5′-terminal triphosphate groups inhibit RNase E cleavage (Lin-Chao & Cohen, 1991; Mackie, 1998), the initiating RNase E cleavages probably occur somewhere in the 5′-proximal region of overlapping genes II and X. Although these cleavage sites have not yet been mapped, the finding that RNA A is stabilized more than RNA B following inactivation of RNase E (Kokoska & Steege, 1998; Goodrich & Steege, 1999) suggests that the first cleavages occur in sequences unique to RNA A. Once the degradosome has accessed the large primary transcripts near their 5′ ends, apparent movement in a 5′ → 3′ direction then occurs slowly over a period of ~10 min to generate upstream products of endonucleolytic cleavage that are degraded quickly (Kokoska et al., 1990) and downstream products that serve as the longer-lived processed mRNAs. A characteristic 5′ → 3′ wave of intermediates results. Over the course of the endonucleolytic pathway, as many as 30% of the processed mRNA species are degraded via the poly(A)-dependent 3′ exonucleolytic decay pathway, but the marked heterogeneity of poly(A) addition sites near the

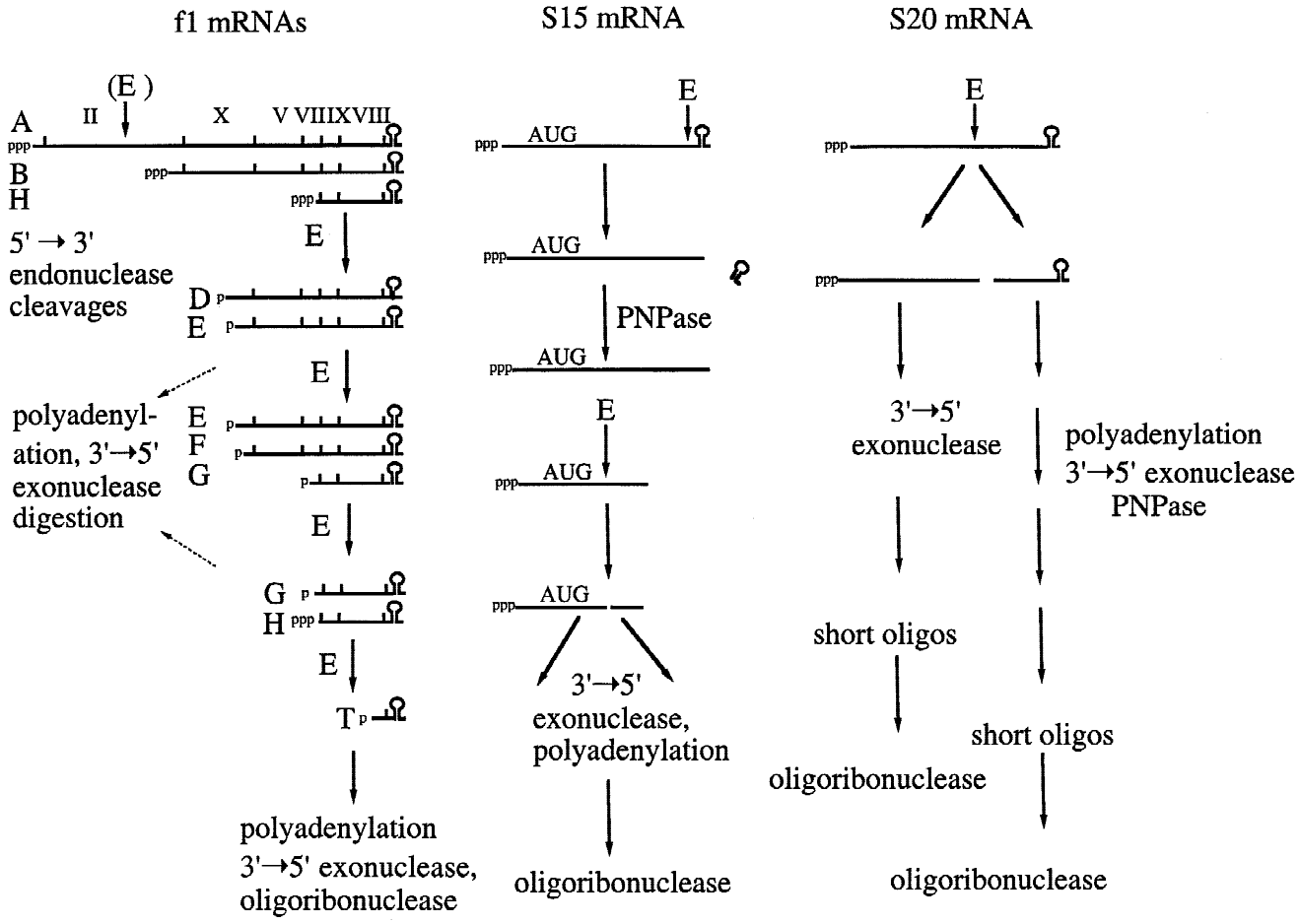

FIGURE 2. Pathways established for processing and decay of the f1 mRNAs (Goodrich & Steege, 1999) and degradation of the mRNAs for ribosomal proteins S15 (Hajnsdorf & Régnier, 1999) and S20 (Coburn & Mackie, 1998; Coburn et al., 1999). The final step of breakdown to mononucleotide units is presumed to be digestion of small RNA fragments by oligoribonuclease (Ghosh & Deutscher, 1999). Major steps in the pathways are denoted by arrows with solid lines, and minor steps are indicated by arrows with dashed lines. The positions of the major RNase E cleavage sites that have been mapped precisely are designated E, and those sites not yet located, (E).

3′ end is a good indication that the stable stem-loop poses a significant barrier to exonuclease digestion. The final RNase E cleavage removes the structured 3′ end from RNAs G and H. RNA T, the downstream product bearing the terminator stem-loop, then requires polyadenylation and 3′ → 5′ exonuclease digestion for its breakdown. With no evidence for degradosome activity near the 3′ end of the f1 mRNAs until the last step in the endonucleolytic pathway, the most likely candidate for exonucleolytic activity over time on the 3′ end beyond the terminator is RNase II. By contrast, PNPase is the probable candidate for the enzyme that digests the upstream products of RNase E cleavage. The 3′ end of the upstream product from the cleavage generating RNA T appears not to be a site of poly(A) addition, suggesting that it is rapidly degraded in the degradosome before becoming accessible to poly(A) polymerase. An interesting possibility is that once the degradosome finally reaches the region near the 3′ terminus of the RNAs, PNPase becomes available and more successfully degrades polyadenylated RNA T into substrates for oligoribonuclease. In any case, present evidence finally makes it clear that the primary basis for the long half-lives of the f1 mRNAs is their resistance to exonuclease digestion. Also, ribosomes on gene VIII prevent access of the degradosome to the 3′-most distal RNase E cleavage site, delaying the removal of the structured 3′ end.

In contrast to the pathway for decay of the f1 mRNAs, the ribosomal protein S15 mRNA degradation pathway illustrates a predominantly 3′ → 5′ exonucleolytic mode of decay. Of pathways examined in *E. coli*, this is the one best characterized in vivo, and recent evidence has uncovered additional intermediate steps (Hajnsdorf & Régnier, 1999). In this case the initiating cleavage by RNase E is near the 3′ end of the principal form of the mRNA studied (Fig. 2), just downstream from the stop codon but upstream of the terminator stem-loop

(Régnier & Hajnsdorf, 1991). This means that functionally, the degradosome accesses this mRNA at the 3′ end, and that the first step of decay is removal of the structural barrier to exonuclease digestion. Whether the degradosome is also tethered to the mRNA elsewhere is not known. In keeping with the location of the degradosome at the 3′ end of the mRNA, PNPase is strongly implicated in the initial wave of 3′ → 5′ exonucleolytic degradation (Braun et al., 1996). A second RNase E cleavage then occurs at codons 7 and 8 at the beginning of the coding sequence, presumably by transfer of the degradosome in some way to this region of the mRNA (Hajnsdorf & Régnier, 1999). Following this cleavage, the internal decay intermediate becomes susceptible to polyadenylation, which activates its decay. The two RNase E cleavage sites are appropriately positioned to assure rapid degradation of S15 mRNA with minimal synthesis of nonfunctional peptides when further synthesis of ribosomal proteins is not needed. Under conditions of S15 excess, S15 protein acts as a translational repressor by binding to a site in the promoter-proximal region of the mRNA. As translation ceases and ribosomes are released from the end of the coding region, RNase E cleavage just beyond the stop codon is stimulated (Braun et al., 1998). Removal of the ribosome binding site by cleavage at the beginning of the coding region then prevents further loading of ribosomes on the truncated mRNA decay intermediate (Hajnsdorf & Régnier, 1999).

The third example, decay of ribosomal protein S20 mRNA, illustrates that decay can also be initiated at internal positions in an mRNA. In a *tour de force* effort, Mackie and colleagues have reconstituted many steps of this pathway in vitro using purified proteins and degradosome preparations (Coburn & Mackie, 1998; Coburn et al., 1999). In this mRNA, the initiating RNase E cleavage is located two-thirds of the way from the 5′ end of the smaller form of the mRNA produced (Fig. 2). Further steps of decay then depend on the secondary structure of the two decay intermediates. The upstream fragment is sensitive to exonuclease digestion and does not show a requirement for polyadenylation in vitro for complete degradation, but polyadenylation does significantly stimulate its decay (Coburn & Mackie, 1996b). As indicated in the discussion of polyadenylation, decay of the highly structured downstream fragment is strongly dependent on polyadenylation by PAP I in vivo and in vitro (Coburn & Mackie, 1998). The fragment requires repeated steps of poly(A)-tail addition and exonuclease digestion for complete decay in vitro. RNase II cannot digest this fragment to completion, even with the stimulation of polyadenylation (Coburn & Mackie, 1998). This finding and earlier results (Mackie, 1989) strongly suggest that PNPase is the exonuclease operating in vivo on this decay intermediate. Under the in vitro conditions used to reconstitute the degradation pathway with purified proteins (Coburn & Mackie, 1998), PAP I, PNPase, ATP, and phosphate alone suffice to degrade the structured fragment to completion, without the involvement of RhlB. In agreement with this, decay of S20 mRNA under the same assay conditions using degradosome preparations is supported by the nonhydrolyzable analog ATPγS and hence independent of ATPase activity. However, when decay of the structured fragment is examined under the conditions of Blum et al. (1999) using the minimal degradosome (Coburn et al., 1999), ATP-activated RhlB is required for complete decay. Activation of RhlB is dependent on the presence of a poly(A) tail, in agreement with the finding by Blum et al. (1999) that poly(A) and certain other single-stranded extensions stimulate the activation of RhlB in degradosome preparations. On the basis of these in vitro findings, it is likely that RhlB activity and polyadenylation cooperate in vivo to minimize the number of rounds of polyadenylation required for complete degradation of the highly structured downstream fragment.

CONCLUDING REMARKS

Exciting discoveries of the 1990s have provided the needed impetus and support for increased effort to understand the mechanisms by which cells recycle the nucleotide units in mRNA to regenerate energy. It had been appreciated for some time that breaking down these polynucleotides to nucleoside mono- and diphosphates suitable for resynthesis into NTPs is an essential and important process. It is now clear that mRNA decay in bacteria is proving to be far more interesting enzymatically than might have been predicted, involving a multiprotein machine to coordinate steps and polyadenylation to maintain the momentum of exonucleolytic degradation through RNA structure. Much work remains for the future. As discussed in each section of this review, major issues remain to be resolved before biochemical models can be formulated in mechanistic terms for most mRNA pathways. The structures and interactions of nearly all the known components need to be better characterized, and additional components may need to be identified. It is not unlikely that such an important process as mRNA decay is under complex regulation, but at present very little is known about such controls. Given the significance of issues outstanding at present, it is anticipated that results from work on the problem of mRNA decay in the next decade could be even more revealing than those obtained in the last.

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