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DIALOG

Processing Endoribonucleases and mRNA Degradation in Bacteria

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Forty years have passed since the dramatic identification of mRNA, the unstable carrier of genetic information from DNA to protein (15, 48, 56). During the last decade, there have been scores of papers and reviews that assume that RNase E is the central enzyme for degradation of mRNA. (There have been hundreds of papers and many reviews on RNase E and mRNA degradation, with at least 60 just in the last 2 years. I regret the unintentional omission of worthy ones but only refer to examples in this short presentation.) It is appropriate to consider evidence for and against this conclusion since it bears on our understanding of overall pathways of metabolism. RNase E was identified in 1978 by Apirion et al. (4, 44, 96) as an endoribonuclease (endo-RNase) that catalyzed the maturation of 5S rRNA by two sequential cleavages at specific sites of the 9S RNA of *Escherichia coli*. About the same time, Kuwano (recently from training with Apirion) et al. isolated an unusual temperature-sensitive mutant called the *ams* (for altered mRNA stability) mutant (73, 107). About a decade later, it was shown that the *ams* mutation maps in the gene for RNase E, *rne* (9, 95, 99, 130). This identification contributed to the now widely held view that RNase E is the principal RNase for initiation of mRNA decay (e.g., see references 33, 34, 35, 49, 57, 87, 122, and 124).

FUNCTIONAL DECAY AND MASS DECAY SHOULD BE CONSIDERED SEPARATELY

It is important to recognize the useful compartmentalization of the degradation of an mRNA population for a specific protein (message) into two distinct components: inactivation or loss of function (the ability to synthesize protein) as opposed to loss of mass. It is also important to recognize that decay rates are measured on populations of millions of molecules and are used to infer how a single molecule is degraded. Loss of mass of a heterogeneous population of mRNAs is defined empirically by loss of trichloroacetic acid-precipitable material; the lost fraction generally includes oligonucleotides of less than 12 to 15 nucleotides (nt). For mRNA for a specific protein (message), it has been defined by loss of oligonucleotides that cannot form stable hybrids with their cDNA. The minimal size depends on the stringency of the hybrid reaction and the base composition but is often in the 15- to 30-nt range. A 500-nt mRNA population would only show loss of mass if cleavages occurred near the ends or if it were degraded by an exonuclease. Other terms have been used, such as "bulk decay" and, most commonly, "chemical decay." Mass decay is more specific, since "chemical" could refer to any number of parameters from chemical modifications to structural alterations, as well as loss of mass.

Functional decay (inactivation) is defined here by loss of the "capacity" of an mRNA molecule to participate in the initiation of synthesis that leads to the normal functional protein product. It is the rate-limiting event for determining the amount of functional protein from the message, as well as for the onset of rapid degradation of the inactive molecule to reusable nucleotides. The rate of functional decay of the total cell mRNA is measured by the decline in the rate of total protein synthesis with time, after blockage of transcription initiations, in either of two protocols (12, 73). The functional decay rate of a specific message, or total mRNA, has been measured by very different assays. During the past 2 decades, most assays have been for the decline of incorporated amino acid into a full-length protein product(s) (or incorporated nucleotide into a full-length message) measured by a band(s) in gel electrophoresis.

Besides not being an assay for active product, the gel assay can give misleading results for a number of technical reasons. For example, instead of simply counting the label in protein or RNA with an instrument with a linear response to all levels of the label, many studies measure half-lives of total mRNA by summation of the intensity of all of the bands in a gel or film after autoradiography. Since the abundance of messages and proteins can range more than 500-fold in *E. coli* (60), many bands have to be overexposed in order to "see" a fair sampling of many proteins. These overexposed bands can remain overexposed for three or four half-lives; i.e., their bands remain as "black as black" on film, and by contributing to the resultant half-life of total mRNA, the latter will appear longer than it actually is.

Soon after the recognition of mRNA, Kepes devised an ingenious and simple procedure by which to measure the inactivation rate of a specific message (65, 66). Transcription initiations are blocked shortly (e.g., 30 s) after induction, and the amount of enzyme is assayed with time until no further enzyme is synthesized (capacity is zero). The difference between enzyme activity at any time and this plateau level gives the remaining capacity of the message population. After the induction lag (capacity = 100%), the capacity declines exponentially. This assay is extremely powerful because function is defined by the activity of a specific enzyme product and is valid even under various abnormal growth conditions. For example, the only effect of a block at an internal codon from a fraction of faulty ribosomes or tRNAs would be to lower the final

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enzyme plateau level but the derived half-life would measure the functional half-life correctly, as seen in a variety of cases, such as amino acid starvation, when protein synthesis is only ~5% of normal (64).

Any attack on a message molecule would likely inactivate it or promptly lead to its incapacitation, so the inactivation must be the first, or an early, event that initiates the decay process, e.g., a cleavage at the 5′ end that blocks further translation initiations or at the 3′ end that leads to 3′-to-5′ exonucleolytic attack.

By this qualification, mass decay could never be faster than functional.

Half-lives for loss of function and of mass are usually the same for reasons discussed later. However, if one reviews the literature published when investigators were still grappling to understand the basic mechanics of mRNA degradation, it can be seen that there are a variety of conditions in which mass decay can be slower than is the rate of functional decay (51, 62, 109, 110, 117, 120, 141).

When functional and mass decay rates are uncoupled, the mass of inactive mRNA fragments should reach a higher level in the cells. (Since mRNA decays exponentially, its rate of decay is defined by the formula $D = kA$, where A is the amount of degradable RNA and k is the decay constant. When k is lower (longer half-life), A is higher, and at any instant in the steady state, $D = S$ (the rate of synthesis) (61). For example, if S were the same in RNase E^+ and RNase E^- cultures (99) but the loss of mass were slower in the RNase E^- cells, then the level of inactive mRNA per cell would be higher in the mutant than in the parent).

RNase E AND FUNCTIONAL DECAY OF mRNA

The original observations by Kuwano, Ono, et al. are probably still the most incisive for the function of RNase E in total mRNA degradation in vivo (73, 107), and the primary results have been verified in subsequent studies with a different *rne* mutant strain (99). However, generally, they have been ignored, although a careful study of the results yields many important insights that will be referred to throughout this report. (i) Decay rates of total pulse-labeled RNA and mass of two *trp* (for tryptophan synthesis) mRNAs are significantly slower (compared to growth at 30°C) during growth of the temperature-sensitive *ams* mutant at 42°C (from three- to at least eightfold, depending on the preincubation time at 42°C). (ii) The functional decay rates of *trp* mRNA and β-galactosidase (β-Gal) mRNA are not affected at all by growth at 42°C in the temperature-sensitive *ams* mutant, and the functional decay rates of total mRNA for total protein synthesis are barely affected (only 1.2-fold slower even at longer times at 42°C). In the Kepes assay for functional inactivation, the final plateau levels of both tryptophan synthetase and β-Gal are reduced by the loss of RNase E; as discussed in the preceding section, this result is consistent with the fact that (iii) protein synthesis slows progressively with time in the temperature-sensitive *ams* mutant at 42°C.

If the preceding results are correct, loss of RNase E has no effect on functional inactivation of the large majority of mRNAs.

DISTINCTIONS BETWEEN PROCESSING AND DEGRADATIVE RNases

The enzymes and pathways for biosynthesis of DNA, protein, polysaccharides, and other macromolecules are completely distinct from those for their degradation. While the separation between degradation and synthesis may not be as distinct for RNA, it is important to recognize differences between them. The enzymes known to participate in maturation of stable RNAs from precursor RNAs (the final steps in their biosynthesis) have been designated processing RNases, as opposed to the known RNases for degradation. In general, processing endo-RNases have stringent specificity in order to recognize only one site, or a limited number of sites in order to process a large RNA molecule on its path of biosynthesis without destroying it. Besides differences in degrees of specificity, the processing and degradative endo-RNases of *E. coli* generate different end groups.

RNase E, like other known processing *E. coli* endo-RNases, generates 5′ P and 3′ OH ends, while the known degradative endo-RNases generate 5′ OH and 3′ P ends (20). An identification of the observable 5′ ends of representative sizes of RNA oligonucleotides in growing *E. coli* showed that about 60% have 5′ OH ends and the remaining ~40% have 5′ ppp-purine ends from transcription starts. 5′ P ends were at much lower levels (20, 22). Except for the 5′ ppp ends of nascent transcripts, almost all of the remaining oligonucleotides must be derived from mRNA degradation. Fifty-five percent of the 5′ OH ends of larger oligonucleotides were 5′ OH-adenosine (Ade) and were 65 to 70% of all of the 5′ OH ends in cells lacking RNase I*, an endo-RNase that lacks bond specificity (19). Also, the sequenced in vivo cleavage sites of lactose operon mRNA (*lac* mRNA) showed a marked preference for pyrimidine (Pyd)-Ade bond cleavages with generation of 5′ OH-Ade (28).

The 3′-end nucleotides from such cleavages would probably represent <5% of the mononucleotides generated, on the basis of the *lac* mRNA results (discussed below). Presumably, they would originate as 2′,3′ cyclic mononucleotides, which is the usual initial product of cleavages by the known endo-RNases with Pyd-N (N is any nucleotide) specificity. RNase II, a major 3′ exonuclease involved in mRNA degradation (39, 69), can initiate degradation of the oligonucleotide with a neutralized 2′,3′ cyclic P almost as effectively as to one with a 3′ OH group and to a 3′ P group at least half as fast (21). We estimated that at least 5% of the mononucleotide pool of *E. coli* spheroplasts is composed of 2′,3′ cyclic mononucleotides (19). As far as we know, these cyclic mononucleotides could only arise from the degradative endo-RNases and this is further (perhaps seemingly obscure) evidence of involvement of the degradative endo-RNases in mRNA degradation. That *E. coli* can handle nucleotides phosphorylated in any way was shown long ago by Seymour Cohen, who found that *E. coli* can grow on 3′,5′, or 2′,3′ cyclic mononucleotides (77).

PROCESSING ENDO-RNases AFFECT mRNA METABOLISM

It may be found that every processing RNase affects the metabolism of some specific mRNA(s), e.g., even the highly

specific RNase P on the histidine operon mRNA (*his* mRNA) (1, 34), but many examples are known for RNase III and RNase E, and it is interesting to compare them.

(i) RNase III. Probably the earliest study, in 1973, of the activity of a processing endo-RNase on mRNA concluded that the early mRNAs of phage T7 are cut from a large polycistronic mRNA by RNase III (40, 115) and was followed by identification of RNase III sites on phage T3 (55) and the left transcript of phage λ (82, 83). These early observations led to a flurry of activity, lasting several years, to determine if RNase III is the primary endo-RNase for the initial attack on *E. coli* mRNAs.

The original RNase III$^-$ strain, AB301-105 (68), showed a slower decay rate of pulse-labeled RNA (120). Many other mRNAs were shown to decay more slowly in the *rnc* mutant, e.g., the three messages of the galactose (*gal*)operon (129), and another paper reported slower mass decay of *trp* mRNA but normal functional decay rates of two *trp* messages (120). As will be discussed below, it was concluded that many of these diverse responses resulted from secondary effects of the mutation rather than from a direct effect of RNase III deficiency. However, it was several years before there was general agreement (now taken for granted) that RNase III is not the primary enzyme for initiation of mRNA degradation.

The most influential observations were the unexpected reports that RNase III activity at a site (*sib*) about 250 nt downstream from the *int* gene of phage λ could result in degradation of the early λ *int* mRNA (47, 50, 116), and the term "retroregulation" was introduced into the scientific vocabulary. It is not clear if this impressive terminology or the observation itself was responsible, but most of the literature on mRNA degradation in the 1980s focused on the importance of 3′-to-5′ degradation of *E. coli* mRNA (104), even suggesting that it is the primary mechanism of mRNA degradation (e.g., see references 102 and 103). Since translation termination almost always precedes transcription termination by a sufficient distance to ensure a stem-loop structure and protect from 3′-to-5′ exonucleolytic degradation, removal of this structure would usually be the initial step in such a mechanism.

Subsequent studies have shown that RNase III cleavage can either increase mRNA stability or decrease it and that target sites can be either at the 5′ or the 3′ end of a message. For example, some messages in the T7 early polycistronic mRNA are more resistant to RNase III cleavage while others are not affected (41). Even λ *int* mRNA made later in infection from a different promoter is stabilized by RNase III activity, so the enzyme has both negative and positive effects on the mRNA from the same gene during the brief growth phase. The many diverse effects of RNase III on the metabolism of many mRNAs are discussed in the very interesting chapter by Court (36), with many citations that cannot be given here.

(ii) RNase E. Most studies of the effect of RNase E emphasize cases in which an mRNA is degraded faster by its activity. However, other studies, especially earlier ones, reported examples of a possible positive effect on the stability and translation of messages, e.g., the *papA* mRNA of the *pap* operon (10, 101), a downstream *his* operon message (2), an upstream *malE* message (102, 103), the *dicF* mRNA (43), and certain phage T4 mRNAs (11, 100). In the last case, gel patterns seem to show several T4 proteins that are synthesized for longer times in RNase E$^+$ than in RNase E$^-$ cells; i.e., RNase E increases the stability of mRNA and/or its translatability.

However, a later study by the same group (98) differed by the addition of rifampin at 6 min and the gel patterns were quite different from those in the earlier study. The authors concluded that RNase E increased the functional and mass decay rates of all of the T4 messages examined. It is a very complex system, with the T4 phage burst size in the RNase E$^-$ cells reduced to half of that which it reaches in the RNase E$^+$ culture (98), and the measured functional decay half-life is three times longer than the total mass decay half-life (theoretically impossible; see "Functional and mass decay rates"), which may or may not be accountable for by the technical considerations given. In the RNase E$^-$ culture, phage development may be blocked for any number of reasons in the 50% that do not mature fully and those messages simply continue making their proteins (as seen even after the maximum burst size is reached). Nonetheless, the studies identified RNase E sites on the T4 polycistronic mRNA whose cleavage led to faster message degradation while other studies reported cleavages that led to slower degradation.

A more recent study with a deletion of almost the entire carboxy half (477 residues) of RNase E reported that both mass and functional decay rates of bulk mRNA were marginally (1.7 times) slower than in the *rne*$^+$ strain (81). Functional decay was monitored by measuring the total label in all of the gel bands as a function of time of decay, as in the earlier T4 mRNA studies, and, as in those studies, also showed slower functional than mass decay rates for bulk mRNA in all four of the strains compared. As noted above, in theory this is not possible. One strain was the original *ams* mutant strain, with a functional half-life at 37°C 1.6 times that of the *rne*$^+$ strain and a mass half-life only1.4 times that of the *rne*$^+$ strain. This ratio of functional half-lives was close to that in the deletion strain and somewhat greater than the original 1.2-fold increase at 42°C in the *ams* mutant (73, 99, 107) and, as noted, could reflect limitations in the functional assay (see "Functional and mass decay rates"). In any case, the evidence continues to show that loss of RNase E activity has no effect on the functional decay rates of most *E. coli* mRNAs.

Several studies of mRNA degradation in the absence of RNase E have noted that rapid degradation continues in the absence of the enzyme. For example, Hajnsdorf et al. (53) reported that the *rpsO* message continues to be degraded rapidly in the *ams pnp rnb* triple mutant, supporting an alternate background mechanism in an mRNA whose decay is slower in the absence of RNase E activity.

As discussed above, the initial event that inactivates a message is rate limiting not only for the yield of functional protein product but for the subsequent decay rate of inactive mass. Ow et al. (108) measured the changes in mass decay rates of several messages in strains with different deletions in the *rne* gene. If RNase E activity were rate limiting for decay, the same proportional increase in half-life might have been observed for all messages with each decline in RNase E activity. However, the half-life changes varied over a wide range (less than 2-fold to >10-fold).

It appears that sites for both of the processing endo-RNases RNase III and RNase E are unusually prevalent on polycistronic mRNAs of bacteriophages. Court (36) noted this obser-

vation for RNase III and referred to a very early speculation that their presence could reflect an evolutionary adaptation by bacteria to "fight off phage infections" (114).

The emphasis of interest in RNase E has focused on cases in which it increases the decay rate rather than increases the protein yield. Whether this emphasis is warranted because RNase E really is the major endo-RNase for mRNA degradation or whether it is simply expected from the original observations of slower mass decay in *rne* mutant cells remains to be seen.

A MODEL OF RNase E AND RNase III ACTIVITIES

From the positive effect of unpaired nucleotides at the 5′ end, Bouvet and Belasco (14) proposed a decade ago that RNase E interacts at the 5′ end before effecting internal cleavage. More recently, Coburn and Mackie (34) proposed a mechanism that they suggest is applicable to most mRNAs and called it the "5′ tethering model." It is based on their conclusion, mainly from in vitro experiments with oligonucleotide constructs, that RNase E only binds to a free 5′ end (85, 87) and preferably to a 5′ monophosphate (rather than a triphosphate) (79, 85, 87). In the latter work, the target mRNA is circularized, resulting in reduced degradation to support the proposed need for a free 5′ end. To account for initial cleavages at the 3′ end of a message or many messages, as in a polycistronic mRNA, this initial binding to the 5′ end is maintained while the mRNA loops back to give simultaneous binding at the 5′ end and at some distal site, which latter binding can lead to cleavage to give the new 3′ end. In some cases, there can be an initial binding via polynucleotide phosphorylase (PNPase) to a 3′ end with subsequent simultaneous binding to the free 5′ end (3′ tethering model).

As a consequence of this constraint, the postulated "degradosome" (a degradation complex) must exist with at least two molecules of RNase E (or a higher oligomer) plus two (perhaps more than two) molecules each of PNPase, RhlB helicase, and enolase. Other proteins and RNA molecules have also been reported to be associated with degradosomes (reviewed in references 34, 80, and 81). For example, in the case of the *malEFG* operon, the *malE* message is actively translated, so this operation would occur simultaneously with translations in progress during the initial binding to the 5′ end. In fact, the authors propose that the ribosomes on *malE* would block the distal binding, which would then occur at a more poorly translated site somewhere in the distal *malF* message. Mackie maintains that the initiating step in the decay of *E. coli* mRNA is usually mediated by RNase E (87), so this mechanism would apply to most messages and mean that many of these large (at least 1,500-kDa) complexes are active at any instant. However, the significance of this postulated complex is unclear, since degradosome assembly is not necessary for normal mRNA decay (108).

A remarkably similar model of mRNA degradation was suggested, but with more reservations, for RNase III when comparable efforts were being made in the waning years of that period to show that that processing endo-RNase could be the primary enzyme for initiation of mRNA decay. On the basis of studies with an in vitro protein synthesis system (118) using a 479-bp template DNA fragment of the 5′ end, Shen et al. (119) concluded that *lac* mRNA is inactivated by RNase III by either of two RNase III cleavages at stems formed by base pairings in the center of the 479-nt mRNA or by a looping back of the distal segment (401 to 421 nt) to the 5′ end. While this is consistent with the observed in vitro cleavages, they did not know if the same mechanism applies in vivo. This extraordinary mechanism was later shown to be untenable, since the 5′ end of the β-Gal message starts to be degraded in vivo at its final exponential rate as soon as it is synthesized (17).

ARGUMENTS FOR MULTIPLE ENDONUCLEOLYTIC CLEAVAGES ALONG THE ENTIRE LENGTH OF mRNAs

Degradation of bacterial mRNA during growth is a very major metabolic activity. As we noted years ago, since mRNA accounts for ~50% of the synthesized RNA but only 2 to 3% of the total RNA mass (60), the rate of mRNA degradation is equivalent, in nucleotides per second, to about half of the rate of total RNA synthesis in the cell. Furthermore, and not as a necessary condition for the preceding relationship, after inactivation of a message at the 5′ end that prevents further ribosome loading, the ensuing mass decay of the naked end is so fast that it almost keeps up with the last translating ribosome (117). The regenerated mononucleotides are reutilized very efficiently to provide about half of the needs for biosynthesis. With this major metabolic burden and with selection pressure for more rapid growth, it seems reasonable that millions (or billions) of generations of evolution would have resulted in a very efficient primary mechanism for degradation of the bulk of the inactive mRNA mass.

There is no evidence of 5′-to-3′ exonuclease activity in *E. coli*. The mRNA fragments released by endo-RNase activities are believed to be degraded by the two major 3′ exo-RNases RNase II and PNPase (39, 69). However, these 3′ exo-RNases are blocked by double-stranded stem-loop structures. The addition of oligoadenylates to some 3′ ends of these fragments facilitates binding by the exonucleases (54, 97, 139) but probably plays a very minor role in the attack on the 3′ end of a finished transcript, since very few *E. coli* mRNAs have added oligoadenylate ends (0.01 to <2%) (30, 97).

RNase II cycles on and off stem-loop structures, which could lead to some probability of their elimination when RNase II is bound simultaneously with an opening at the stem base during the normal "breathing" of any duplex (24). Also, RNase II binding to a secondary structure can facilitate endonuclease attack (24, 26). RNase I* can cleave any RNA bond but, as opposed to RNase I, is extremely specific for single strands (19, 25) and is involved mainly in degradation of small oligonucleotides that would lack secondary structures (19, 20) and complements the RNase II reaction, which slows dramatically when the oligonucleotide is less than 12 to 15 nt and the RNase II reaction becomes essentially nonprocessive (21, 26). Also involved in the final step to degrade very small fragments of 2 to 5 nt is oligo-RNase (37, 45, 106, 142).

Despite these cooperative activities to eliminate released RNA fragments, primary endo-RNase cleavages that release inactive mRNA fragments that might be hundreds of nucleotides long would be less efficient than an initial activity that released smaller fragments of 20 to 40 nt sequentially along the entire mRNA length. The statistical probability of the occur-

rence of stable stem-loops in any random sequences becomes high when the RNA oligonucleotide is longer than about 70 nt (L) and increases approximately as L^2 (61).

EVIDENCE OF ENDONUCLEOLYTIC CLEAVAGES ALONG THE ENTIRE LENGTH OF mRNA

The first evidence of multiple cleavages along the entire length of an mRNA during its translation in vivo came from regions of the *lac* operon (28). From S1 mapping and direct sequencing of the 5′ and 3′ ends, the cleavages were generally, at most, 10 to 50 nt apart (28) (at most because weaker bands may not have been seen, let alone sequenced). However, note that this distance is for the entire population of millions of molecules and any given molecule could have cleavages farther apart, but nonetheless, these closely spaced sites indicate that there are vulnerable bonds along the entire length of the mRNA. The cleavages were at Pyd-N bonds, with most at Pyd-Ade bonds. Since then, multiple fragments have been observed from many mRNAs, including other regions of *lac* mRNA (127). In fact, multiple gel bands from such fragments have been observed in mRNAs with an RNase E site(s) and even with the mRNAs degraded more slowly in the absence of RNase E activity. It is significant that the sizes of fragments from RNase E^+ and RNase E^- cells are generally similar (e.g., see references 7, 8, 31, and 140).

The Pyd-purine (Pur) cleavage specificity was also reported by Kai et al. (58) for middle and late transcripts from phage T4 in *E. coli*-infected cells. In a very recent paper, the observations were extended to mapping of cleavages in the 300-nt *soc* mRNA of phage T4. All cleavage sites were at Pyd-Pur or Pyd-Pyd bonds (59) and were independent of RNase E, III, or G activity (personal communication).

In order to visualize the cleavage fragments, it is necessary to expose films for such long times that the original full-length sequence is a very dark band (28). This relationship results from the odd fact, which we showed mathematically, that when a population of macromolecules is attacked with exponential, i.e., random, kinetics and the degradation is exclusively net unidirectional, the ratio of numbers of any two sizes is invariant during the entire time of decay, so the shape of the size distribution curve does not change (13). As noted, the bulk of the mass remains the original full-length size, as shown clearly for decaying β-Gal message (78) because, again, the breakdown to mononucleotides is so rapid that it is not far behind the last translating ribosome (117).

Although it has only been shown rigorously for β-Gal mRNA (17), it is likely that degradation is usually 5′ to 3′ (discussed below) and ribosome-free ends of other messages are presumably degraded as rapidly. The net unidirectional mechanism has been shown every time an investigator has seen a sharp gel band for a full-length message without visible intermediates; it was also shown mathematically that if degradation were by random endonucleolytic cleavages, the most abundant molecules would be of a decreasing size with time of decay, and the resulting pattern for the total steady-state population would be a fairly broad smear of mRNA (13, 78).

DO THE MANY ENDONUCLEOLYTIC CLEAVAGES OCCUR IN VIVO, OR ARE THEY ARTIFACTS?

The critical question is whether these many low-concentration mRNA fragments are normal endonucleolytic products or artifacts. In our 1986 work, we recognized at least two major sources that could have led to an erroneous conclusion (28). First, the possibility of internal breaks in the RNA during preparation had to be eliminated. Purification procedures were developed that allowed the recovery of large RNA molecules (27, 29), and full-length *lac* mRNA (~5,100 nt) was reacted in the hybridization to the 380-nt DNA probe and, upon elution, showed no detectable cleavages (28).

Second, the purification included RNase A to eliminate spurious RNA that might be "sticking" to the hybridization filters. Even though the RNase A concentration was 100-fold lower than that usually used, the fact that RNase A also prefers Pyd-Ade bonds when at very low activity (92) made this a serious consideration, since it was concluded that most in vivo cleavages were at Pyd-Ade bonds. One control included ^{32}P-poly(A-C-U) (300 kDa) added to the hybridization filter during the elution step, and no degradation of it was seen. Most significantly, when RNase A was completely eliminated from the purification and replaced with RNase T1, which only cuts after guanosine (Guo) residues, we still observed the Pyd-Ade cleavage specificity (28).

WHAT IS THE RNase E RECOGNITION SITE?

On the basis of its multiplicity of cleavages and its sequence specificity, RNase E is not a candidate for these many mRNA cleavages. A sampling of RNA with reported RNase E sites shows only one or a small number of such sites on a given RNA. For example, besides the large 9S RNA with two sites and RNA I with one, the mRNAs for S20 (86), for *rpsO* (52, 53), for *trxA* (8), for the *rpsU-dnaG-rpoD* operon (140), for the *pap* operon (105), and for phage sites (for T4 [42] and polycistronic f1 [71]) generally have only one RNase E site within 200 or more nucleotides. They cannot account for the many cleavages along the entire mRNA described above unless a great many RNase E sites on these messages have not been detected.

On the basis of a limited number of in vivo RNase E sites, a stringent recognition of 10 nt was proposed (131). With identification of more in vivo RNase E sites in different messages, the consensus recognition site continued to be quite complex, with a sequence of five nucleotides proximal to a downstream stem-loop structure; four of the five nucleotides in the sequence included A and U residues (42). Stable stem-loops are adjacent to most in vivo cleavage sites (34).

More recent studies have used synthetic substrates that change the sequence of the RNA I site (91) or the S20 mRNA site (88). From these in vitro experiments, it has been concluded that there is no sequence specificity other than an enrichment for AU residues but, adding to earlier conclusions, secondary structures, either nearby or even at a distance, can affect activity. RNase E is a very large protein (114 kDa [32]), which, in itself, could explain cases of interference by secondary structures (33, 88) and if RNase E is part of the postulated "degradosome" which includes PNPase, an even larger protein

(258 kDa [112]) and other proteins, distant structures could be even more of a problem for the positioning of this very large complex (estimated to be at least 1,500 kDa [34]) and, as noted, explain why RNase E cleaves complex RNA at a limited number of sites (91).

Defining in vivo RNase specificity and activity from in vitro assays can be very treacherous. First, you have to guess the ionic, compartmentalization, etc. conditions in the cell's microenvironment. From early estimates of component volumes, the *E. coli* interior may be more like a gel matrix than a "sea" of protoplasm (63). More easily measured but no less important is the fact that most endo-RNases change bond specificities as a function of enzyme/substrate concentrations and reaction time, e.g., pancreatic RNase A; RNases U2, T2, and C3; etc. A notable exception is RNase T1. RNase I cleaves any phosphodiester bond at all concentrations. It is listed, and sold, as single strand specific, but as the enzyme/RNA ratio is increased, it starts attacking double strands and is capable of degrading all cell RNA (23, 25) so that, in this case, the structural specificity changes with enzyme activity. Thus, one must do a very broad titration of ratios.

The evidence obtained so far suggests that RNase E cleaves complex RNA at a limited number of sites (91) and does not have the broad specificity to recognize the thousands of different sites that can be cleaved during degradation of the hundreds of different messages in growing *E. coli*. One very old insight in enzymology is that, in general, the more stringent the recognition site, the larger the enzyme, while enzymes with broad specificity are usually smaller. This is generally true of the best-known broad-specificity endo-RNases, e.g., RNase A (13 kDa), RNase T1 (11 kDa), and *E. coli* endo-RNases I and I* (27 kDa).

When crude extracts of *E. coli* lacking RNase I or I* were fractionated by size, the only fractions with the ability to eliminate the original sizes of a mixture of heterogeneous *E. coli* mRNA were in the 25- to 33-kDa range (123), even when the reaction occurred in the prescribed RNase E assay mixture and the protein fractions covered all sizes to >300 kDa.

WHY IS THE MASS DECAY RATE OF mRNA SLOWER IN THE ABSENCE OF RNase E ACTIVITY?

The most common assumption in the literature is that RNase E is involved directly in the degradation of mRNA. The reasoning, which has served genetic analyses so admirably, is that if mutant A^- (RNase E^-) gives B^- (slower mRNA decay), then A^+ gives B^+; i.e., RNase E causes mRNA degradation. However, the equation can be more complex, especially in the case of an enzyme that is known to affect many metabolic pathways and to be involved in the biosynthesis of many functional products. It could be that A^- gives X^- gives B^- but A^+ does not give B^+. X could be any number of candidates.

Besides the original discovery that it is necessary for maturation of 5S rRNA, recent work has shown that RNase E is required for maturation of the 5′ end of 16S rRNA (76, 132). RNase E also functions in the maturation of tRNAs (75, 111), M1 RNA of RNase P (67, 67a, 84), and *ssrA* RNA (80), which functions in the elimination of incomplete peptides. That there may be a defect in translation is consistent with (but not proof of) the third important observation of Kuwano et al. obtained with the original *ams* mutant, i.e., that protein synthesis becomes progressively slower in the absence of RNase E activity (46, 73).

It is also important that the slower mass decay rate of mRNA becomes more pronounced with the time of growth at the nonpermissive temperature, going from 3.5 times slower than that at 30°C with no preincubation at 42°C to >8 times slower with preincubation at 42°C for 15 min (73). This pattern suggests that either some component(s) that influences the rate of decay is being inactivated or a population of normal components is being diluted by additions of abnormal members. Of course, RNase E itself could be the component being inactivated with time, but it was reported that when a temperature-sensitive *rne* mutant was transferred to the nonpermissive temperature, "RNase E was almost instantly inactivated" (46). Also, how would inactive RNase E directly slow protein synthesis or, conversely, is there any reason to suggest that active RNase E acts directly to stimulate translation? RNA synthesis continues at a normal rate (99), with a normal fraction being mRNA (107). If anything, slower decay of messages might be expected to result in faster, rather than slower, protein synthesis. Of course, the reason it does not do so is that messages continue to be inactivated at normal rates at the nonpermissive temperature (73, 99, 107).

No one knows why the close linkage between functional and mass decay rates becomes uncoupled in RNase E^- cells, because so many phenotypic functions could be affected by loss of a biosynthetic enzyme for a major growth component or for a specific mRNA. For example, Apirion reported that RNase E^- cells are blocked in cell division for unknown reasons (46). Later work identified an RNase E processing site in the *fts*A-*fts*Z mRNA whose products are important for cell division, but the authors could not conclude that the RNase E cleavage affects cell division (16).

Defective translation might account for the uncoupling of the two decay components. As noted, the occasional translation block by a defective ribosome or tRNA would lower the final enzyme yield but not affect the observed functional message half-life in the Kepes assay. As shown by a number of agents, when translation is slowed, the mass decay rate becomes slower and can be completely arrested with a complete inhibition of protein synthesis (e.g., see reference 117). An uncoupling of functional and mass decay rates was reported when streptomycin-dependent *E. coli* grew on various concentrations of the ribosome antibiotic, leading to much slower mass decay than functional decay of bulk mRNA (51).

However, the slower mass decay in RNase E-defective cells cannot be attributed to a translation defect sufficient to prevent continuous growth. Specific *rne* deletion mutants can process 9S RNA sufficiently to 5S rRNA and do continue to grow, although more slowly, but the mass decay of several mRNAs measured was still slower than normal (81, 108). This condition was seen in other cases, such as in streptomycin-dependent growth (51), and was also shown in the original work of Kuwano et al. (73; Fig. 2). Protein synthesis slowed gradually in the *ams* mutant at 42°C; it was still half as fast as in the parent strain up to the first 60 min, while pulse-labeled RNA decayed eight times slower after only 15 min at 42°C.

RNase E has been implicated in the maturation of several stable RNAs. Among the many possible candidates for affect-

ing mRNA mass decay, there is a site-specific RNase E cleavage near the 3′ end of the precursor of a small stable RNA, *ssrA* RNA (10Sa RNA) (80). *ssrA* RNA is necessary for the elimination of truncated peptides. The authors speculated that these could arise from mRNA fragments derived by endonucleolytic action, with translation of these fragments producing N-terminal peptides lacking a stop codon. They would be trapped with 70S ribosomes to impede degradation of the peptides and mRNA. It may be relevant that *ssrA* mutants grow more slowly, especially at higher temperatures (72). The temperature sensitivity of many *rne* mutants could reflect a secondary defect of some of them for a stable RNA, such as the *ssrA* RNA, rather than of RNase E directly.

DIRECT EVIDENCE THAT RNase E DOES NOT CAUSE FASTER MASS DECAY OF BULK mRNA

Loss of RNase III results in multiple pleiotropic effects. The reason for many of them is not known, but as discussed above, this processing enzyme influences many functions in positive or negative ways.

Even more puzzling is the fact that some of these phenotypes can be dramatically ameliorated by subculturing, as we observed with *lac* operon expression (129) in a constructed isogenic *rnc* strain (126). The mechanism is not known, but apparently the cells adapted by marshalling compensatory activities ("phenotypic suppression"), since the original *rnc* allele was still present and it seemed unlikely that suppressor mutants could have been selected in so few generations (129). Such strain transformations are frustrating, since the original phenotype to study has mysteriously disappeared.

The original RNase III$^-$ strain, AB301-105 (68), was defective for growth on a variety of sugars (6), and Studier had also noted the unique auxotrophic requirements of that strain (but not the parent, AB301) for biotin and succinate (126). However, it was peculiar that the *suc* mutation mapped near 39 min, which is far from the *sucA* and *sucB* loci at 16 min, and *suc*$^+$ "revertants" from AB301-105 occurred at a very high frequency but were still RNase III$^-$. He concluded that the Suc$^-$ phenotype resulted from the *rnc* genotype on some unknown component that somehow caused the succinate requirement. There were other phenotypes resulting from the loss of RNase III that could be suppressed while maintaining the *rnc* genotype (129).

Phenotypic or genetic suppression could explain the unexpected results of Apirion and Gitelman (3). Working with a triple mutant temperature sensitive for three processing RNases—RNase P, RNase III, and RNase E—they reported that functional decay was normal at the nonpermissive temperature, but unexpectedly, the half-life for degradation of pulse-labeled RNA was also normal. The half-lives were identical in the triple mutant at 30 and 43°C, even after its incubation for 40 min at 43°C. In comparison, pulse-labeled RNA in the original *ams* mutant was degraded three times slower after incubation at 42°C for only 8 min (Fig. 3 in reference 73). As noted, the degradation rate declines progressively with time at high temperature, so after 40 min, it would have been considerably more than three times slower. They concluded that none of the enzymes, including RNase E, "participate in decay of mRNA in the cell" (3) (of historical interest, this is an opinion maintained by the discoverer of RNase E, e.g., in one of his last publications [5]).

That mass decay is not affected by loss of RNase E disagrees with the earlier observations by Kuwano et al. (73, 107) and later ones by Mudd et al. (99). Barring some experimental error, these results support the suggestion that the slower mass decay of bulk mRNA results not from loss of RNase E but from some other component that is affected by loss of RNase E but which was compensated for by adaptation of the bacterial stock in that laboratory.

It might be instructive to compare the observed effect of 42°C on the decay rate of pulse-labeled RNA in the original *ams* strain in different laboratories. For example, it was reported in a 1999 paper that, after 30 min at 42°C, its half-life was only 2.5 times longer in the *ams* mutant than in the parent strain (Table 1 in reference 81). This compares with an eightfold difference after only 15 min at 42°C observed 20 years earlier with the same strains using the same assay (Fig. 3 in reference 73). This significant difference could reflect adaptation with subculturings over time.

If the slower decay of bulk mRNA mass does not result from a direct role of RNase E in the degradation of inactive mRNA fragments, then the functions of RNase E become analogous to the functions of other processing endo-RNases, in particular, RNase III. Their primary function is the biosynthesis of stable RNAs, but they also participate in endonucleolytic attacks at various mRNA sites, especially on polycistronic mRNAs and prominently on polycistronic mRNAs of bacteriophages. Their activity can lead to increased or decreased mRNA stability and/or translation of a limited number of specific messages. RNase E, like RNase III, continues to be worth studying for its enzymological mechanism and to identify potential targets.

However, if the available data are correct, the primary and major pathways of mRNA inactivation and mass degradation do not include either of these biosynthetic enzymes.

RNase M

If most cleavages along the entire length of mRNA are at Pyd-Ade bonds, there should be an endo-RNase that accounts for such specificity. An enzyme with such specificity has been found throughout the living world. Pancreatic RNase A, the most widely studied, has been found in scores of eucaryotic species and many tissues (113). The specificity has been reported in a variety of microorganisms, including an *Enterobacter* sp. (89), yeast (74, 125), *Rhizopus oligosporus* (138), and *Bacillus subtilis* (90). As noted, the immediate products have neutral 5′ OH and 2′,3′ cyclic P ends, which would allow passage into the periplasm for dephosphorylation. It is interesting that the *B. subtilis* enzyme appears to be ~15 kDa, close to the size of RNase A, while the enzymes from yeast and *R. oligosporus* are in the 26- to 30-kDa range, about twice as large. The secondary preferences can vary among species; e.g., the yeast enzyme has a more stringent specificity for Pyd-Ade bonds than does RNase A (125) and the *B. subtilis* enzyme degrades poly(C) 10 times faster than it degrades poly(U) (90).

It is important to recognize that secondary preferences can be minor and be a function of a specific context and do not describe the defining specificity. For example, we noted a pos-

sible secondary preference for U (uridine) residues adjacent to the Pyd-Ade nucleotides (28), but it was not statistically significant, and even if it were, it would not necessarily apply in other sequence contexts. The in vivo cleavage specificity in *E. coli* is for Pyd-N bonds, with a preference for Pyd-Ade, not for a UUAU sequence, which has been an incorrect interpretation of the data (7, 8).

We found low levels of such an activity in spheroplasts, but the enzyme appeared to be in the size range of another very abundant broad-specificity endo-RNase with much stronger activity and greater mass that had never been reported. It turned out to be a 27-kDa endo-RNase that showed little activity against polymer cell RNA but degraded homopolymers and small oligonucleotides. We called it RNase I* since it could be a cytoplasmic precursor to periplasmic 27-kDa RNase I (19, 94). As noted, evidence suggests that RNase I* participates in mRNA degradation, especially in the terminal stages against small oligonucleotides (19, 20).

To avoid these enzymes we used free-living strain MRE600, which was reported to have ~1% RNase I activity (133) since at that time there were no constructed strains with zero RNase I (18). An endo-RNase with Pyd-N specificity was found and called RNase M. Peptide maps of RNase M from strain MRE600 and RNase I from the K-12 strain used in the studies showing the cleavage specificity (28) were compared (93). Most of the peptides overlapped, with only a few separating differently. We proposed the possibility that the gene for RNase M had some evolutionary origin in common with the gene for RNase I/I* since they seemed to be so closely related in structure. However, several years later, the sequence of the entire genome of *E. coli* was completed and it could be shown that there was no other gene sequence that was homologous to the *rna* gene, which had by then been sequenced (94). This fact made it unlikely that the gene for RNase M was similar to that of RNase I and that the peptide mapping was incorrect either for technical reasons or because the protein used for peptide mapping was the wrong one.

In a recent paper, Subbarayan and Deutscher (128) reported that the low level of activity in strain MRE600 resulted from a multiply altered form of RNase I with eight nucleotide changes that could account for the peptide map data. However, no assays for specificity were performed on the altered RNase I, so it was not shown to be the same enzyme purified earlier that had the stringent specificity of RNase A based on sequenced cleavage sites of 5S rRNA (18). The assay is only comparable to that of Cannistraro and Kennell (18) in that the same 5S rRNA substrate was used, but they only measured loss of alcohol-precipitable counts per minute and found significant degradation (128).

The assay showing RNase M activity gave cleavages at five specific sites, all Pyd-Ade bonds, on the 5S rRNA molecule that were identified by sequencing of the ends (18). These five sites were the same as cleaved by very low concentrations (10 to 100 pg/ml) of pancreatic RNase A. It is not clear if any degradation to acid- or alcohol-soluble products would have resulted from the five cleavages, but it is really irrelevant without identifying the actual cleavage sites. It is remotely conceivable that the amino acid changes in RNase I could give RNase A specificity. However, in view of the fact that there are no sequences homologous to *rna* in the genome, it seems more likely that the RNase purified later by Meador et al. for peptide mapping (93) was predominantly the much more abundant altered RNase I, which is not RNase M. As noted, it is difficult to separate the two RNases and RNase M is at very low levels in the cell. This explains why there is no sequence region homologous to *rna* in the *E. coli* genome.

Whatever the explanation, it does not detract from the likelihood that there is an endo-RNase in *E. coli* with a specificity for Pyd-N bonds and a preference for Pyd-Ade bonds. However, the endo-RNase is not homologous to RNase I in sequence or necessarily in size. With regard to size, as noted, a mutant of the K-12 strain used to determine cleavage specificities (28) was constructed with zero RNase I/I* activity (123), and the only fractions from crude extracts showing activity were in the ~24- to 33-kDa range (123). With strains now available with zero RNase I/I* activity (123, 143), it should be possible to determine if an RNase M is present (our *rna*::*kan* strain, DK533, is available from the *E. coli* Genetic Stock Center at Yale University).

It is of interest that strain MRE600 was also used by Spahr and Gesteland (121), who reported an activity that they called RNase IV (~31 kDa), which cleaved phage R17 RNA at one site and led to differential control of phage protein synthesis. Subbarayan and Deutscher have suggested that RNase IV might be the altered RNase I, but its limited cleavage specificity also does not seem to be consistent with that of RNase I, which attacks any bond, and it is possible that RNase M and RNase IV are the same.

Five points summarize why it seems likely that RNase M, an RNase A enzyme (cleavage specificity for Pyd-N bonds with limiting activity for Pyd-Ade bonds), is present in *E. coli*. (i) Many mRNAs have been shown to have closely spaced endonucleolytic cleavages throughout their lengths. These cleavages occur whether or not RNase E is active in the cells. (ii) The closely spaced cleavages of *lac* mRNA (28) and of the phage T4 mRNAs (58, 59) that have been examined are predominantly at Pyd-N bonds, with most at Pyd-Ade bonds. (iii) About 60% of the oligonucleotides in growing *E. coli* have 5′ OH ends, and of these, ~55% start with 5′ OH-Ade and ~25% start with 5′ OH-guanosine (Guo), consistent with the specificity preferences of a very low level of RNase A activity. Almost all of the remaining 40% are nascent transcription starts with 5′ pppAde or 5′ pppGuo ends (20). Processing RNases, including RNase III and RNase E, generate 5′ P ends. (iv) Apirion's laboratory identified endo-RNase F, which they concluded is a processing enzyme for a T4 phage tRNA (134) and later indicated that it cleaves a cytosine-Ade (Cyt-Ade) bond (135). RNase F could be RNase M and was identified in a characterized *E. coli* K-12 strain (not MRE600). (v) Finally, RNase A activity has been found in organisms throughout nature, including other bacteria and yeast, and it would be remarkable if it were missing in *E. coli*.

However, the stringent Pyd-Ade specificity of pancreatic RNase A exists in a concentration range 1,000- to 1,000,000-fold lower than the usual ~1 μg/ml at which it is used in laboratories as a reagent to cleave any Pyd-N bonds. Of the 19 cleavage sites sequenced, 15 were Pyd-Ade bonds (28), indicating a very low concentration of the postulated RNase M in *E. coli*.

Pyd-Ade BONDS AND NONENZYMATIC CLEAVAGES

Before searching for an enzyme activity with the expected specificity, we considered the possibility that the preferred Pyd-Ade cleavages could be spontaneous in vivo nonenzymatic breaks, since earlier work had shown that Pyd-N bonds are more labile (70), but assumed that it was enzymatic partly because the RNase A specificity appears to be ubiquitous in nature. Since then, interesting studies of the nonenzymatic cleavages of synthetic oligonucleotides have been done (67). These studies have shown that the frequencies of spontaneous cleavages between adjacent nucleotides are the same frequencies, or preferences, of RNase A that were identified many years ago (Pyd-Ade > Pyd-Guo > Pyd-Pyd) (92), with further secondary preferences influenced by adjacent residues, the number of adjacent residues, etc. (67).

In one study, the possibility of contaminating RNases was eliminated by observing crystallized tRNAs, and among 49 spontaneous breaks, 41 preceded an Ade and 37 followed a Pyd (38). Watson et al. reported spontaneous cleavages at two sites in precursor p2Sp1 RNA of phage T4. They were between Cyt and Ade residues (135). As previously noted (67), 17 of the 19 cleaved phosphodiester bonds of *lac* mRNA were preferred bonds in nonenzymatic cleavage (28).

However, spontaneous cleavage in vitro requires a polyamine present in a narrow concentration range and is very slow (measurements for 24 h) even when accelerated severalfold by the presence of 0.1% polyvinylpyrrolidone (67). The spontaneous reactions are quenched by formamide. Putrescine and spermidine are polyamines present in *E. coli*. While it is remotely conceivable that nonenzymatic cleavages could account for the extremely rapid endonucleolytic reactions in vivo, it is much more likely that some endo-RNase with RNase A specificity accounts for such activity. Both spontaneous and enzymatic reactions generate 2′,3′ cyclic P ends, but the former react further to give an equal mixture of 2′ and 3′ phosphate ends while the enzyme reactions give 3′ phosphates only (136, 137).

Why would evolution have selected probably the most universal endo-RNase with a specificity that recognizes the most labile phosphodiester bonds in RNA? It seems like overkill. Kierzek speculated that if RNA originally cleaved at sites intrinsically susceptible, then it is likely that this reaction would be facilitated by an evolutionarily derived RNase A activity. RNase A enzymes may not require interaction specifically with the adjacent Pyd-Pur residues but act on the phosphodiester bond simply because they are the most labile chemically, just as the polyamines assist in the nonenzymatic reaction (67). In any case, the lability of those bonds and the abundance of an RNase for them guarantee the instability of any RNA segment, unless it is protected (e.g., by double strandedness or the association of a ribosome or proteins), and thus guarantee that the RNA that carries the genetic information will be unstable.

RNA is made to be destroyed unless it is protected. rRNAs and tRNAs have extensive secondary structures but, perhaps more importantly, are bound tightly by proteins. However, when 5S rRNA was extracted from *E. coli* ribosomes with care to avoid degradation, a small fraction (<1%) of the population of molecules showed cleavages at three sites in single-strand regions between nt 14 and 15, 65 and 66, and 103 and 104, which are all 5′ uridine-Ade bonds (Cannistraro and Kennell, unpublished results). These could have resulted from RNase M activity before the 5S molecule was fully protected or from a very small probability of cleavage in its final state.

NO UNIQUE TARGETS FOR ENDONUCLEOLYTIC ATTACK

We proposed that every Pyd-Ade bond (and, to a lesser extent, other Pyd-N bonds) in the entire mRNA molecule has a certain probability of attack and that the initial cleavage is usually nearer to the 5′ end simply from statistical considerations, since that end would be the first to be free of protecting ribosomes. There is no unique target for the initial attack. The initial cleavage could prevent further translation initiations, and transit of the last loading ribosome would expose more Pyd-N bonds to cleavage (28).

We came to that perspective partly by examination of the sequences of many mRNA leaders that showed no common sequences or structures that would be recognized by a specific endo-RNase, and it seemed unlikely that, in general, the recognition sequence could be a part of the translated message (61). Although mRNA degradation occurs with random kinetics, it was shown that there is a wide range of half-lives among *E. coli* messages (12). At that time, the random kinetics argued that all messages could be subject to the same jeopardy of attack and thus all would decay at the same rate. That they do not was crucial for any further analyses.

Before obtaining the results, we expected either "all same or all different" with the same probability. Also, the observations that a long message could decay slower than a much shorter one and that a distal one could decay faster than a proximal one on a polycistronic mRNA established that the random kinetics only apply to the kinetics of each message population but not to relative rates of different messages, i.e., properties of each message and/or other components determine its exact half-life. Half-lives could vary as a function of the frequencies of translation initiations, as is consistent for the first versus last *lac* messages (63), to the extent of double strands on the exposed RNA or other factors.

Whether or not these differences in stability represent a selected control mechanism is not known. The unique target for a processing RNase could reflect a specific selected control and provide a rationale for the presence of such targets on specific RNAs.

ARGUMENTS FOR 5′-TO-3′ DECAY BUT A POSSIBLE ADVANTAGE OF SOME INACTIVATIONS AT THE 3′ END

It is important to differentiate rational speculation from established fact. For example, we have speculated (28, 61), and others have casually concluded, that the vast majority of messages in bacteria are degraded unidirectionally from the 5′ end to the 3′ end. Our speculation was based on direct evidence from studies of *lac* mRNA degradation that showed, from size analyses of the decaying population, that the long message for β-Gal is degraded in a net unidirectional wave (13, 78) and that the direction is physically 5′ to 3′ (17). There are examples of messages that have a cleavage target near the 5′ end, and

eliminating the target, or a presumed enzyme for the target, changes the decay rate. Such cases suggest that degradation starts at the 5′ end but do not prove a net 5′-to-3′ mechanism. As far as I know, only the β-Gal mRNA results provide such proof.

Other reasons to favor the 5′-to-3′ direction simply invoke common sense. We noted that degradation from the 3′ end to the 5′ end, a popular model at the time (61), was inefficient, with perhaps 20% or more of the protein product from an average message being incomplete peptides (13). Note that this does not apply to 3′-to-5′ exonucleolytic degradation of the naked fragments released by the endonucleolytic attacks but only to 3′-to-5′ degradation (inactivation) from the original 3′ end. Also, it presents the mechanical problem of degradation proceeding "uphill" against the 5′-to-3′ passage of ribosomes. Since selection in bacterial populations is for faster growth, it seems unusual that millions (or billions) of generations of evolution would have maintained a mechanism that would waste so many resources on worthless polypeptides (and maintain slower growth). However, no one knows. I think experiments could be designed to find out.

To derive order from chaos, we can search for a plausible advantage for 3′-to-5′ decay. As opposed to 5′-end inactivation with ribosome runoff, inactivation of the 3′ end would halt the synthesis of functional protein immediately. Such a mechanism, although inefficient, might be advantageous in the case of the phage-infected cell, in which the entire development phase occurs in 10 to 15 min, with transitions from early, mid, and late proteins being made in a rapid-fire fashion and sequentially. It could also explain why evolution apparently selected so many processing endo-RNase sites between messages of phage polycistronic mRNAs. Another possible reason, to "fight off infections," has been proposed (114).

CONCLUDING STATEMENT

It is also clear from many studies that some *E. coli* mRNAs are attacked by processing endo-RNase III or E. In some cases, the resultant cleavages may be necessary for translatability, and in other cases, they may lead to slower or faster degradation. Perhaps all of the investigations of the past decade that concluded that RNase E is a/the major RNase for mRNA degradation will be proven correct. Alternatively, these forays into degradative processes by biosynthetic enzymes are exceptions that occur by some evolutionary design or simply by accident.

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DIALOG

The comments below on Sidney Kushner's inclusive review of competing hypotheses regarding the molecular mechanisms of mRNA decay in E. coli (J. Bacteriol. ***184:****4658–4665, 2002) relate to the field of mRNA degradation in general. Kushner states that it appears that RNase E and its homolog RNase G play an important role in the initiation of mRNA decay. Many reviews and papers assume that RNase E is the primary RNase in mRNA degradation. Contrary evidence and alternate mechanisms are ignored (except in some of Kushner's references).*

The greatest disregard of fact concerns the ams mutant results reported by Kuwano et al. (Mol. Gen Genet. ***154:****279-285, 1977) that RNase E is not involved in the initial inactivation of almost all E. coli messages. Inactivation is the crucial physiological event determining protein product yield. It is also the primary biochemical event initiating rapid destruction of the inactive mRNA mass. Few RNase E papers distinguish between them.*

Kushner notes that the ams-1 mutation leads to slower decay of pulse-labeled RNA without noting that functional decay is normal, even though the section is on "Initiation of mRNA decay." Mudd et al. repeated the experiments of Kuwano et al. (possibly to show them wrong) and, to their credit, reported the same results. Coburn and Mackie (Prog. Nucleic Acid Res. Mol. Biol. ***62:****55-108, 1999) interpreted the observations as "paradoxical." The belief that RNase E initiates degradation disagrees with the paradoxical fact that it does not.*

Why the universal denial? Perhaps investigators cannot accept the Kepes assay for message inactivation or direct one for total cell mRNA. Vague allusions to "faulty translation" or the "rate-enhancing effect of higher temperature" have no basis in theory or observation. Kushner states that "If RNase E does initiate mRNA decay, why do the 5′ ends . . . ¦. ¦. ¦. contain hydroxy termini?" It was nice to recognize the hydroxy-terminal data, but RNase E does not initiate decay!

There is probably one primary degradative mechanism for virtually all cell mRNAs directed by a limited number of enzymes, with some messages also affected by specific enzymes with other primary functions. Loss of RNase E and/or III never completely "stabilizes" an mRNA (infinite half-life), but countless papers simply state that "loss of RNase E stabilizes the mRNA." It may decrease the mass decay rate, but the primary mechanism is still operative.

What is the primary mechanism? A good clue is the closely spaced cleavages along the entire length of decaying mRNA. Close spacing suggests a broad-specificity endo-RNase and, as a corollary, no unique target. It could be RNase M or some comparable activity. Evidence of Pyd-Ade cleavage has generally been ignored—partly for technical reasons. The half-lives of these intermediates are very short to give very low concentrations. From 6 liters of cells, we had to sequence by the "wandering-spot" method (Cannistraro et al., J. Mol. Biol. ***192:****257-274, 1986).*

Kushner dismisses RNase M as an altered RNase I. There is no basis for that conclusion, as explained in the review above. RNase activity lost by an rna gene deletion depends on the assay. Acid solubilization would not detect stringent-specificity RNases, e.g., RNases E, III, P, and H, and probably not those with intermediate specificities, such as RNases M and I. Also, Pyd-Ade specificity (four papers cited in the review above) was found in K-12 strains having normal RNase I. It became clear after the entire E. coli genome was finally sequenced that the peptides mapped (Meador et al., Eur. J. Biochem.* ***187:****549-553, 1990) were not derived from RNase M. It had not been purified clear of the much more abundant altered RNase I of strain MRE600.*

This mistake resulted from deviation from the previous RNase M purification procedures (Cannistraro and Kennell, Eur. J. Biochem. ***181:****363-370, 1989). Vin Cannistraro, a 20-year collaborator and an excellent enzymologist, did all of the earlier enzymology research but did not participate in any of the later work, and I obviously exercised poor oversight. Unfortunately, we did not continue the work. We felt that the primary mechanism had been identified. With limited funds, the only other person in the laboratory was Cannistraro, whose primary interest is enzyme mechanisms. In the short time remaining, we studied the mechanism of movement in a processive reaction using RNase II (reviewed in Methods Enzymol.* ***342:****309-330, 2001).*

Kushner also discounts RNase I because a zero-RNase I/I* mutant seems to have a normal phenotype, but he noted compensating enzymes for PNPase and RNases II and III masking normal function. As with RNase M, the biochemical data are overlooked. 5′ ends of oligonucleotides from mRNA differ when RNase I* is missing (Cannistraro and Kennell, Eur. J. Biochem.* ***213:****285-293, 1993).*