Distinct Requirements for 5′-Monophosphate-assisted RNA Cleavage by Escherichia coli RNase E and RNase G*

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RNase E and RNase G are homologous endonucleases that play important roles in RNA processing and decay in Escherichia coli and related bacterial species. Rapid mRNA degradation is facilitated by the preference of both enzymes for decay intermediates whose 5′ end is monophosphorylated. In this report we identify key characteristics of RNA that influence the rate of 5′-monophosphate-assisted cleavage by these two ribonucleases. In vitro, both require at least two and prefer three or more unpaired 5′-terminal nucleotides for such cleavage; however, RNase G is impeded more than RNase E when fewer than four unpaired nucleotides are present at the 5′ end. Each can tolerate any unpaired nucleotide (A, G, C, or U) at either of the first two positions, with only modest biases. The optimal spacing between the 5′ end and the scissile phosphate appears to be eight nucleotides for RNase E but only six for RNase G. 5′-Monophosphate-assisted cleavage also occurs, albeit more slowly, when that spacing is greater or at most one nucleotide shorter than the optimum, but there is no simple inverse relationship between increased spacing and the rate of cleavage. These properties are also manifested during 5′-end-dependent mRNA degradation in E. coli.

Messenger RNA degradation is among the most important mechanisms for controlling gene expression in all organisms. In Escherichia coli, where mRNA half-lives range from seconds to almost an hour, rates of decay are generally dependent on a pair of homologous endonucleases: RNase E and RNase G (1). These enzymes degrade E. coli transcripts by either of two pathways. Decay via the direct-access pathway begins with internal cleavage by either RNase E or RNase G. The resulting RNA fragments are then rapidly degraded by a combination of further endonucleolytic cleavage and 3′-exonucleolytic digestion. Decay via the 5′-end-dependent pathway, which governs the lifetime of hundreds of E. coli mRNAs, begins instead with removal of two of the three 5′-terminal phosphates by a mechanism involving the RNA pyrophosphohydrolase RppH (2, 3). The monophosphorylated intermediate thereby generated undergoes rapid degradation because of the preference of RNase E and RNase G for substrates that bear a single phosphoat the 5′ end (4–6). This property also helps to explain the lability of the 3′ products of endonucleolytic cleavage, as they too are monophosphorylated (7).

X-ray crystallographic examination of the catalytic amion-terminal domain of RNase E bearing an oligonucleotide ligand has revealed that the preference of this enzyme for monophosphorylated substrates is a consequence of a discrete pocket that can bind a monophosphorylated 5′ end but apparently cannot accommodate a 5′ end that is triphosphorylated (8). This pocket, which is close to but distinct from the catalytic site where the scissile phosphate binds, is lined by arginine and threonine side chains that form hydrogen bonds with the 5′ phosphate of the ligand and are crucial for 5′-monophosphate-assisted cleavage (8–10). Despite the absence of a crystal structure, the conservation and functional significance of these and nearby amino acid residues in RNase G (9, 11), which shares 32% overall sequence identity with the catalytic domain of RNase E, suggests a similar mechanism of 5′ end engagement.

RNase E and RNase G share other properties in addition to their sequence homology and preference for monophosphorylated RNA substrates. For example, both cut with low sequence specificity in single-stranded regions that are AU-rich and disfavor regions that are C-rich (5, 6, 12, 13). However, the exact sites where they cleave RNA are often not identical, in part because of the propensity of RNase E to cut 1–2 residues downstream of G nucleotides (6, 13). Other notable differences between the two enzymes include their location and multimeric state in E. coli, where RNase E tetramers utilize a carboxyl-terminal domain that is absent from RNase G to attach the complex to the inner membrane and to assemble the protein into a heteromeric complex with a 3′ exoribonuclease, an RNA helicase, and a glycolytic enzyme (14–17). By contrast, E. coli RNase G is primarily homodimeric (18) and is not thought to interact with the cell membrane or with other proteins.

It is not uncommon for both RNase E and RNase G to participate in the degradation of a particular mRNA in E. coli; however, RNase E frequently has a greater influence, perhaps because of its higher cellular concentration, which may also help to explain why only RNase E is essential for cell growth (19–22). Nevertheless, RNase G makes a substantial contribution to the degradation of certain messages, whose distinguishing characteristics have not been identified (20–24). In general, the features of triphosphorylated primary transcripts that govern their vulnerability to ribonuclease attack in E. coli are not well understood. Such features include the presence or absence of unpaired nucleotides at the 5′ terminus (25, 26), which helps...
to control susceptibility to 5’-end-dependent degradation by determining the accessibility of the 5’ terminus to RppH and RNase E (3, 4) and presumably to RNase G. For example, pyrophosphate removal by RppH requires two unpaired nucleotides at the 5’ end and prefers three or more (27). However, the minimum number of unpaired 5’-terminal nucleotides needed for 5’-monophosphate-assisted cleavage by RNase E or RNase G or for 5’-end-dependent mRNA degradation in E. coli has not been investigated. Furthermore, although E. coli RppH has been shown to exhibit modest sequence preferences at the 5’ end of RNA (27, 28), it is not known whether the identity of the nucleotides there affects rates of 5’-monophosphate-assisted cleavage by RNase E or RNase G.

To better understand the RNA characteristics that govern rates of mRNA decay in E. coli, we have now conducted a systematic analysis of the effect of 5’-terminal sequence and structure on 5’-monophosphate-assisted cleavage by RNase E and RNase G in vitro. This investigation has shown that, despite their homology, these two endonucleases differ as to the optimal spacing between the 5’ monophosphate and the site of cleavage. Nevertheless, both enzymes can tolerate any sequence of unpaired nucleotides at the 5’ end.

**Experimental Procedures**

Purified N-RNase E and RNase G—The N-RNase E used in these experiments comprised amino acid residues 1–529 of E. coli RNase E joined to a carboxyl-terminal extension containing hexahistidine and Myc epitope tags (GGAAHHHH-HHVAEEQKLISEEDLNGAARSA). The RNase G that was used comprised the entirety of E. coli RNase G bearing the same carboxy-terminal extension. Both proteins were overexpressed from plasmids in E. coli; purified from cell extracts by elution from TALON beads (Clontech) with a buffer containing 20 mM Tris (pH 7.5), 0.25 mM imidazole, and 0.5 mM NaCl; concentrated/exchanged into a buffer containing 20 mM Tris·Cl (pH 7.5), 0.5 mM NaCl, 10 mM dithiothreitol, and 20% (v/v) glycerol; and stored at −20°C.

In Vitro Cleavage Assays—All oligonucleotides used to prepare duplexes for in vitro cleavage (see Table I) were synthesized and HPLC-purified by Integrated DNA Technologies. A fluorescein-labeled 3’-terminal oligonucleotide (F1 or F3, 5 pmol) and a partially complementary 5’-terminal oligonucleotide (10 pmol) were heated to 70°C for 10 min in 25 μl of buffer A (25 mM Bis-tris propane, pH 8.0, 100 mM NaCl) and then allowed to anneal for 60 min at 50°C. A test duplex (4 pmol) and an internal standard (4 pmol) prepared in this manner were combined in 90 μl of buffer C (final composition: 25 mM Bis-tris propane, pH 8.0, 100 mM NaCl, 15 mM MgCl2, 5 μg/ml total E. coli RNA, 44 mM each duplex). The solution was preheated to 30°C, and a 9-μl sample (0-min time point) was withdrawn and combined with 5 μl of loading buffer (75 mM EDTA, pH 7.8, 15% (v/v) glycerol, 0.15 mg/ml bromophenol blue). N-RNase E (9 μl of 20–80 nM enzyme subunits in buffer C) or RNase G (9 μl of 120–480 nM enzyme subunits in buffer C) was then added, and 10-μl reaction samples were quenched at 1-min time intervals with 5 μl of loading buffer. The reaction samples were subjected to electrophoresis on a nondenaturing 12% polyacrylamide gel, and fluorescent bands were detected with a Typhoon Trio imager (GE Healthcare) and quantified with ImageQuant software. The percentage of intact duplex remaining at each time point was calculated from the ratio of cleaved to uncleaved duplex, the resulting values were plotted semilogarithmically, and rates of cleavage were determined by linear regression. Finally, relative cleavage rates were calculated by dividing the cleavage rate of the test duplex by that of the internal standard in the same reaction mixture.

**Measurement of mRNA Decay Rates in E. coli**—Plasmid pYeIp-mini104 was constructed from plasmid pYeIp1mini (29) by inserting DNA (5’-ACACCGCCGCCGGCCGGCGGC-3’) at the transcription initiation site of the mini-yeiP gene. Plasmids pYeIp-mini103, pYeIp-mini102, and pYeIp-mini101 were identical to pYeIp-mini104 but for the absence of 1–3 base pairs near the 5’ end of that insert (ACAC → ACA, AC, or A, respectively). E. coli strains BW25113 (∆[araD-araB]567 ∆lacZ4787]:rrnB-3) ∆[yhaD-rhaB]568 hsdR514 rph-1) and BW25113 ∆rppH transformed with each of these plasmids were grown to log phase at 37°C in MOPS-glucose medium. Rifampicin (200 μg/ml) was added to arrest transcription, and total cellular RNA was isolated at time intervals by hot phenol extraction (30). Equal amounts (10 μg) of each RNA sample were subjected to electrophoresis on a 6% polyacrylamide-urea gel, blotted onto Immobilon-NY+ (Millipore), and probed with a radiolabeled oligonucleotide complementary to mini-yeiP mRNA. Radioactive bands were detected with a Typhoon Trio phosphorimaging device (GE Healthcare) and quantified with ImageQuant software. The half-life of full-length yeiP mRNA in the presence or absence of RNase G was compared by growing triplicate cultures of E. coli strains BW25113 and BW25113 Δrng to log phase at 37°C in MOPS-glucose medium, extracting RNA at time intervals after rifampicin addition, and analyzing the samples for their yeiP mRNA and 165 rRNA (internal standard) content by quantitative RT-PCR, as previously described (22).

**Sites of Cleavage within the yeiP 5’-Untranslated Region**—A monophosphorylated transcript comprising the first 621 nucleotides of yeiP mRNA but bearing a U → G substitution at the second nucleotide was synthesized by in vitro transcription with T7 RNA polymerase in the presence of a 50-fold molar excess of AMP over ATP and gel-purified. The in vitro transcript (37 nmol) was then partially digested with purified N-RNase E (20 nM enzyme subunits) or RNase G (80 nM enzyme subunits) at 30°C in buffer D (25 mM Bis-tris propane, pH 8.0, 100 mM NaCl, 15 mM MgCl2). Reaction samples (0.33 pmol) were quenched with excess EDTA before or after ribonuclease addition, mixed with total RNA (7.5 μg) from E. coli strain BW25113 ∆yeiP, and ethanol-precipitated. These RNA samples and total RNA extracted from E. coli strains BW25113 and BW25113 ∆rpg containing plasmid pYeIp1 (29) were then cleaved within yeiP codon 12 by a site-specific 10–23 DNAzyme (DZyeip69, 5’-GTAATTCAAGCTGCTAGCTACAGCATACCTTTT-3’) as previously described (29) and analyzed by Northern blotting as described above.
5′-Terminal Requirements of RNase E and RNase G

### Results

Substrates for Comparing 5′-Monophosphate-dependent Cleavage Rates—As a prototype for systematically examining the influence of 5′-terminal sequence and structure on monophosphate-assisted RNA cleavage by RNase E and RNase G, we designed a synthetic substrate consisting of two partially complementary oligonucleotides, one of which (pCUCC) was monophosphorylated and the other of which (F1) contained a fluorescein label and a hexanucleotide (GUAUUU) previously shown to be cut by both RNase E and RNase G (Table 1) (5, 6). The structurally unambiguous duplex formed by these two oligonucleotides comprised a 5′-monophosphate followed by four unpaired nucleotides (CUCC), a 23-bp stem, a single-stranded segment containing the fluorescent tag and cleavage site, and a 3′-terminal stem loop (Fig. 1). For use as an internal standard, we also designed a variant of pCUCC, pCUCC+hp5/F1, in which a 15-nucleotide stem loop (hp5) was added to the 3′ end of pCUCC so as to reduce the electrophoretic mobility of the duplex.

A pilot experiment was performed in which the reaction of a mixture of pCUCC/F1 and pCUCC+hp5/F1 with the catalytic domain of RNase E (N-RNase E, amino acid residues 1–529) was monitored as a function of time. Samples of the reaction were quenched periodically with EDTA and examined by electrophoresis under non denaturing conditions (Fig. 2A). Each substrate yielded a single fluorescent product with a distinct electrophoretic mobility, which made it possible to quantify turnover very precisely by calculating the extent of the reaction at each time point from the ratio of their band intensities. The two substrates were cut with indistinguishable kinetics, showing that their overall size did not significantly affect the rate of cleavage. By contrast, little or no turnover was detected for a substrate identical to pCUCC/F1 but for a 5′ hydroxyl (HO-CUCC/F1) (Fig. 2B), demonstrating that rapid cleavage was 5′-monophosphate-dependent. Similar results were obtained when these substrates were treated with RNase G, except that a majority of the cleavage shifted to a site one nucleotide downstream (Figs. 1 and 2). As expected, no substrate turnover was observed when a mixture of pCUCC/F1 and pCUCC+hp5/F1 was treated with a catalytically inactive N-RNase E mutant purified in the same manner (data not shown).

Importance of Unpaired Nucleotides at the 5′ End—To determine the minimum number of unpaired 5′-terminal nucleotides that are required for 5′-monophosphate-assisted cleavage, we tested four additional duplexes—pCUCCA/F1, pCUCC/F1, pCU/F1, and pC/F1 (Fig. 3A)—in which more or fewer unpaired nucleotides were present at the 5′ end but the distance from that end to the site of cleavage was unchanged. Each of these substrates was treated with N-RNase E or RNase G in the presence of the internal standard pCUCC+hp5/F1, and cleavage rates were measured. Normalization to the cleavage rate of the same internal standard in every reaction was crucial because it made very accurate comparisons of reactivity possible. N-RNase E cut pCUCC/F1 1.5 ± 0.1 times faster than it cut pCUCC/F1, which in turn was cut 2.8 ± 0.2 times faster than pCU/F1 and 1.8 ± 0.2 times faster than pCUCCA/F1 (Fig. 3B).

### Table 1

| Name | Sequence* |
|------|-----------|
| F1   | CCAAGCACCAGCACACrCrUrUrCrUrCrArC/pFluorT/rCrGrUrUrUrUrCrUrUrUrUrGAGAAGGTGCCTGCGGCGTGGG |
| F3   | CCAAGCACCAGCACACCTCTTCCUCC/pFluorT/CCCAAGCACCAGACCTCCUCCAGACGACGCTGTCrCrG |
| HO-CUCC | rCrUrCrGGAAGAAGGTGCCTGCGGCGTGGG |
| pCUCC | /5Phos/rCrUrCrGGAAGAAGGTGCCTGCGGCGTGGG |
| pCUC | /5Phos/rCrUrGGAAGAAGGTGCCTGCGGCGTGGG |
| pCU | /5Phos/rCrUrGGAAGAAGGTGCCTGCGGCGTGGG |
| pC | /5Phos/rCrUrGGAAGAAGGTGCCTGCGGCGTGGG |
| pAUCC | /5Phos/rCrUrCrGGAAGAAGGTGCCTGCGGCGTGGG |
| pGUCC | /5Phos/rCrUrCrGGAAGAAGGTGCCTGCGGCGTGGG |
| pCUCC | /5Phos/rCrUrCrGGAAGAAGGTGCCTGCGGCGTGGG |
| pAACC | /5Phos/rCrArCrGGAAGAAGGTGCCTGCGGCGTGGG |
| pAGCC | /5Phos/rCrArCrGGAAGAAGGTGCCTGCGGCGTGGG |
| pCC | /5Phos/rCrArCrGGAAGAAGGTGCCTGCGGCGTGGG |
| pCUC | /5Phos/rCrArCrGGAAGAAGGTGCCTGCGGCGTGGG |
| pUCC | /5Phos/rCrArCrGGAAGAAGGTGCCTGCGGCGTGGG |
| pUC | /5Phos/rCrArCrGGAAGAAGGTGCCTGCGGCGTGGG |
| M6 oligonucleotide | /5Phos/TTGAGAAGGTGCCTCGGCGGTGGG |
| M6 oligonucleotide | /5Phos/TATTTGAGAAGGTGCCTCGGCGGTGGG |
| M6 oligonucleotide | /5Phos/GTTAGAGAAGGTGCCTGCGGCGTGGG |
| M2 oligonucleotide | /5Phos/GTTAGAGAAGGTGCCTGCGGCGTGGG |

* All sequences are written 5′ to 3′. A, G, C, or T indicates 2′-deoxyribonucleotide. rA, rG, rC, or rU indicates ribonucleotide. /FluorT/ indicates internal fluorescein-dT. /5Phos/ indicates 5′-terminal monophosphate.

b pcUCC(U(C)9)n is identical to pCUCC.

c The M6, M5, M4, M3, and M2 oligonucleotides were annealed with F3 to create the M6, M5, M4, M3, and M2 size markers, respectively.
Effect of the 5'-Terminal Requirements of RNase E and RNase G

By contrast, cleavage of pC/F1 was not detected. Unlike N-RNase E, RNase G preferred four unpaired nucleotides at the 5’ end, cutting pCUCC/F1 1.7 ± 0.1 times faster than it cut pCUCC+hp5/F1, 6.2 ± 1.1 times faster than it cut pCU/F1, and 1.5 ± 0.1 times faster than it cut pCUCCA/F1, while failing to cleave pC/F1 detectably. We conclude that 5’-monophosphate-assisted cleavage by either RNase E or RNase G requires a minimum of two unpaired nucleotides at the 5’ end and prefers three or more. Furthermore, RNase G is impeded more than RNase E when fewer than four unpaired nucleotides are present there.

Effect of the 5’-Terminal Sequence—The requirement of RNase E and RNase G for at least two unpaired nucleotides at the 5’ end of RNA substrates raised the possibility that the sequence of those nucleotides might also be important. We therefore examined the effect of systematically varying the identity of the first two nucleotides of pAUCC/F1, a derivative of pCUCC/F1 in which the 5’-terminal C had been therefore removed as an internal standard. 

For details, see Table 1. The depicted base-paired conformations were calculated to be substantially more stable than any other conformation. HO-CUCC/F1 was identical to pCUCC/F1 except for the absence of the 5’-terminal phosphate.
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FIGURE 3. Effect of the number of unpaired nucleotides at the 5'-end. A, sequence and secondary structure of pCUCCA/F1, pCUCC/F1, pCUC/F1, pCU/F1, and pC/F1. Only a portion of the region of intermolecular base pairing is shown, the remainder being the same in all five duplexes (see Fig. 1). Although the number of unpaired 5'-terminal nucleotides differs in these five duplexes, the number of unpaired nucleotides between the 5'-monophosphate and the sites of cleavage is identical. For more details, see Table 1. B, relative susceptibility of pCUCCA/F1, pCUCC/F1, pCUC/F1, pCU/F1, and pC/F1 to cleavage by N-RNase E and RNase G. The reactivity of each substrate relative to the internal standard pCUCC/hp5/F1 was calculated from the ratio of their rates of cleavage in the same reaction mixture, as described in Fig. 2. The substrates described as unreactive are those for which no cleavage product was detected. Abscissa values represent the number of unpaired nucleotides at the 5'-end of each substrate: 5 for pCUCCA/F1, 4 for pCUCC/F1, 3 for pCUC/F1, 2 for pCU/F1, and 1 for pC/F1. Error bars represent standard deviations.

Replaced with A, pAUCC+hp4/F1, which was identical to pAUCC/F1 but for the addition of a 19-nucleotide stem loop (hp4: GTCTCAGCGAAGCTGAGAC) to the 3'-end of the pAUCC oligonucleotide, served as the internal standard for these measurements. As expected, pAUCC/F1 and pAUCC+hp4/F1 were cleaved at the same rate. Changing the identity of either the first or the second nucleotide of pAUCC/F1 to each of the three other possibilities (pNUCC/F1 or pANCC/F1, respectively) had at most a modest effect (≤2.5-fold) on the rate of cleavage by either endonuclease (Fig. 4). N-RNase E exhibited a small preference for U or C at position 1 and for U or G at position 2, whereas RNase G slightly preferred U or A at position 1 and U at position 2. These findings indicate that RNase E and RNase G tolerate any sequence of unpaired
FIGURE 4. **Effect of the sequence at the 5’ end.** A, sequence and secondary structure of pNUCC/F1 and pANCC/F1, where N = A, G, C, or U. Only a portion of the region of intermolecular base pairing is shown, the remainder being the same as in Fig. 1. The internal standard pNUCC = hp4/F1 contained stem loop hp4 (GTCTCAGCGAAGCTGAGAC) instead of hp5 (Fig. 1) to improve the electrophoretic separation of the substrates and products. For more details, see Table 1. B, relative susceptibility of pNUCC/F1 and pANCC/F1 to cleavage by N-RNase E or RNase G. The reactivity of each substrate relative to the internal standard was determined as described in Figs. 2 and 3. Error bars represent standard deviations.

FIGURE 5. **Influence of RNase G on the decay rate of yeIP mRNA in E. coli.** At time intervals after inhibiting transcription with rifampicin, total RNA was extracted from cultures of three isogenic E. coli strains containing or lacking RNase G, and the ratio of yeIP mRNA to 16S rRNA in each sample was determined by quantitative RT-PCR and plotted semilogarithmically. The measured half-life of yeIP mRNA increased from 1.0 ± 0.1 min in WT cells to 2.7 ± 0.1 min in Δrng cells.

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Nucleotides at the 5’ end of their monophosphorylated substrates.

**Destabilization by Multiple Unpaired 5’-Terminal Nucleotides in E. coli—** That purified RppH (27), N-RNase E, and RNase G each require 2 and prefer at least 3–4 unpaired nucleotides at the RNA 5’ end prompted us to examine how many unpaired 5’-terminal nucleotides are needed for 5’-end-dependent mRNA degradation in E. coli, a process in which all three of these enzymes participate. For this purpose, we examined the decay of derivatives of yeIP mRNA, which encodes a paralog of the translation elongation factor EF-P and is degraded by an RppH-dependent mechanism (3, 29). Both RNase E and RNase G contribute to the degradation of yeIP mRNA in vivo, as evidenced by its increased half-life when either of these enzymes is inactive (3) or absent (Fig. 5). Mini yeIP, an in-frame deletion mutant that lacks codons 23–164, decays at the same rate as its full-length counterpart and likewise requires RppH for its rapid degradation (half-life of 1.0 ± 0.1 min in wild-type cells and 14 ± 2 min in ΔrppH cells) (29).

The decay of four derivatives of mini yeIP mRNA was compared in E. coli. Each bore a 5’-terminal extension consisting of a stem loop preceded by 1–4 nucleotides (A, AC, ACA, or ACAC) that were chosen so as to minimize their potential to engage in base pairing (Fig. 6A). Isogenic E. coli strains containing or lacking RppH were transformed with plasmids that encoded these transcripts, grown to log phase, and treated with rifampicin to arrest transcription initiation and unmask degradation. Total cellular RNA was then isolated at time intervals, and equal amounts were analyzed by Northern blotting (Fig. 6B). Like its progenitor, the mini yeIP derivative bearing four unpaired nucleotides at the 5’ end (mini104) decayed much more rapidly in wild-type cells than in ΔrppH cells (Fig. 6C). Reducing the number of unpaired 5’-terminal nucleotides to three (mini103) or two (mini102) significantly retarded the decay of this transcript in wild-type cells, prolonging its half-life from 2.1 ± 0.3 min (mini104) to 6.2 ± 0.4 min or 8.9 ± 0.8 min, respectively, whereas the equivalent transcript bearing only one unpaired nucleotide at the 5’ end (mini101) decayed as slowly

A, G, C, or U. Only a portion, sequence and secondary
in wild-type cells (half-life of 12 ± 2 min) as in ΔrppH cells (half-life of 14 ± 2 min). These results indicate that at least two unpaired 5’-terminal nucleotides and preferably four or more are required for 5’-end-dependent degradation of mini yeiP mRNA in E. coli, a finding consistent with the collective action of RppH, RNase E, and RNase G.

Effect of the Proximity of the 5’ Monophosphate to the Site of Cleavage—The rate of 5’-monophosphate-assisted cleavage by RNase E or RNase G may also be influenced by the distance between the 5’ end and the site of cleavage. To determine the minimum spacing, we compared the rate of cleavage of a set of substrates (p(C)_n GUAUUU/F3, where n = 0–7) that were cut upstream of the base-paired stem at various distances from the 5’ monophosphate (Fig. 7, A and B). In addition, the sites of cleavage were mapped by comparing the electrophoretic migration of the p(C)_n GUAUUU/F3 cleavage products with a set of cognate standards (Fig. 7C). The presence of oligo(C) at the 5’ end of these duplexes minimized opportunities for adventitious base pairing and ensured that neither enzyme would cut upstream of the intended site. N-RNase E cut the substrate rapidly when the cleavage site was 8 or 9 nucleotides from the 5’ end (p(C)_n GUAUUU/F3 or p(C)_n GUAUUU/F3) but only 16–21% as fast when the cleavage site was 7 nucleotides from the 5’ end (p(C)_n GUAUUU/F3) and undetectably when the cleavage site was closer (p(C)_n GUAUUU/F3, where n = 2–4) (Fig. 7, B and C). By contrast, RNase G, whose preferred site of cleavage was one nucleotide downstream of that of N-RNase E but additionally cut at the same position as N-RNase E, cleaved the substrate rapidly when the principal cleavage site was as few as 6 nucleotides from the 5’ end (p(C)_n GUAUUU/F3), 15% as fast when it was 5 nucleotides from the 5’ end (p(C)_n GUAUUU/F3), and undetectably at shorter distances (p(C)_n GUAUUU/F3, where n = 0 or 1) (Fig. 7, B and C). RNase G also cut at greater distances from the 5’ end (p(C)_n GUAUUU/F3, where n = 4–7). Within the tested range, the optimal spacing between the 5’ monophosphate and the cleavage site was 8 nucleotides for N-RNase E (p(C)_n GUAUUU/F3) and 6 nucleotides for RNase G (p(C)_n GUAUUU/F3). For both enzymes, cleavage in close proximity to the 5’ terminus required the presence of the 5’ phosphate (data not shown).

These results indicate that monophosphorylated RNA can be cut closer to the 5’ end by RNase G than by RNase E. To corroborate this conclusion, we examined the role of RNase E and RNase G in cleaving the 5’-untranslated region of yeiP mRNA. In E. coli, this yeiP segment undergoes RppH-dependent cleavage at multiple sites (W, X, Y, and Z), including one (W) just five nucleotides from the 5’ terminus (Fig. 8A) (29). To identify the enzyme(s) responsible for cleavage at these sites, we synthesized monophosphorylated yeiP RNA by in vitro tran-

yeiP-mini104 except that the four unpaired nucleotides at the 5’ end of the latter transcript (ACAC) were replaced by ACA, AC, or A, respectively. B, decay of yeiP-mini104, yeiP-mini103, yeiP-mini102, and yeiP-mini101 mRNA in E. coli. The degradation rate of each plasmid-encoded transcript was compared in isogenic E. coli strains containing or lacking RppH by extracting RNA at time intervals after transcription inhibition with rifampicin and examining equal amounts of total cellular RNA by gel electrophoresis and Northern blot analysis with a yeiP-specific probe. C, semilogarithmic plots of the decay data in WT and ΔrppH cells.

FIGURE 6. Effect of the number of unpaired 5’-terminal nucleotides on mRNA decay in E. coli. A, 5’-untranslated region of yeiP-mini104 mRNA. The Shine-Dalgarno element and initiation codon are underlined, and the region corresponding to the natural 5’-untranslated region of yeiP mRNA is bracketed. yeiP-mini103, yeiP-mini102, and yeiP-mini101 mRNA were identical to
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FIGURE 7. Minimum spacing between the 5′ monophosphate and the site of cleavage. A, sequence and secondary structure of the p(C)_nGUAUUU/F3 duplexes, where n = 0–7. Only a portion of the region of intermolecular base pairing is shown, the remainder being the same as in Fig. 1. Sites of N-Rnase E and RNase G cleavage are marked by arrows. To render it invulnerable to ribonuclease cleavage, oligonucleotide F3 (green) was composed entirely of deoxyribonucleotides. For more details, see Table 1, B, relative susceptibility of p(C)_nGUAUUU/F3 duplexes to cleavage by N-Rnase E or RNase G. The cleavage of each substrate and an internal standard (pCU/F1 or pCUCC/F1, respectively) was monitored as a function of time after adding N-Rnase E or RNase G, and their relative reactivity was calculated as in Figs. 2 and 3. Substrates described as unreactive are those for which no cleavage product was detected. Sites of N-Rnase E or RNase G cleavage are marked by arrows. Abscissa values (n) represent the number of 5′-terminal cytidylate nucleotides in each substrate. Error bars represent standard deviations. C, products of p(C)_nGUAUUU/F3 cleavage by N-Rnase E and RNase G. N-Rnase E or RNase G was added to a mixture of the substrate to be tested (p(C)_nGUAUUU/F3, where n = 0–7) and an internal standard (pCU/F1 or pCUCC/F1, respectively), and reaction samples quenched after 9 min were subjected to electrophoresis on nondenaturing polyacrylamide gels beside unreacted starting materials and a set of cognate size markers (M2–M6). Using the same nomenclature as for the p(C)_nGUAUUU/F3 duplexes, M2 was pTT/F3, M3 was pTTT/F3, M4 was pATTT/F3, M5 was pTATTT/F3, and M6 was pGTATTT/F3 (Table 1). As expected, intact p(C)_nGUAUUU/F3 co-migrated with M6. The N-Rnase E cleavage products of p(C)_5–7GUAUUU/F3 co-migrated primarily with M3 but also with M4. The RNase G cleavage products of p(C)_5–7GUAUUU/F3 co-migrated primarily with M3 but also with M4.

scription, treated it with purified N-Rnase E or RNase G, and examined the cleavage products by Northern blotting. RNase G cut the yeiP 5′-untranslated region at all four sites, whereas N-Rnase E cut it at all but the 5′-proximal site W (Fig. 8B). Consistent with this observation, cleavage at W required RNase G in E. coli, as determined by parallel analysis of the cellular yeiP transcript. We conclude that 5′-monophosphate-assisted cleavage as close as five nucleotides from the 5′ end is a property of RNase G both in vitro and in vivo.

We also investigated the effect of markedly increasing the distance between the 5′ end and the cleavage site by examining two additional synthetic substrates (pCUCCU(C)_9/F1 and pCUCCU(C)_9U(C)_9/F1, equivalent to pCUCCU(C)_9/F1, where n = 1 or 2, respectively) that were cut downstream of the base-paired stem at significantly greater distances from the 5′ monophosphate because of the insertion of uncleavable C-rich extensions with little or no base-pairing potential (Fig. 9A). Increasing the distance from the 5′ monophosphate to the cleavage site from 10 unpaired nucleotides (pCUCCU(F1, equivalent to pCUCCU(C)_9/F1, where n = 0) to 20 unpaired nucleotides (pCUCCU(C)_9/F1 slowed the rate of N-Rnase E cleavage by a factor of eight (Fig. 9B). Interestingly, this rate
\textbf{5'-Terminal Requirements of RNase E and RNase G}

![Diagram](image)

**FIGURE 8.** RNase E and RNase G cleavage sites in the \textit{yeiP} 5'-untranslated region. A, sequence of the \textit{yeiP} 5'-untranslated region. The sites of endonucleolytic cleavage observed in \textit{E. coli} (W, X, Y, and Z) are marked by arrows, and the Shine-Dalgarno element and initiation codon are underlined. B, cleavage of the \textit{yeiP} 5'-untranslated region in vitro and in vivo. Monophosphorylated \textit{yeiP} RNA synthesized by in vitro transcription was partially digested with purified N-RNase E or RNase G, and a 5'-terminal segment of the resulting 3' cleavage products was then released by site-specific 10–23 DNAzyme cleavage within codon 12. In addition, total RNA isolated from isogenic \textit{E. coli} strains containing or lacking RNase G (WT or \textit{Δrng}, respectively) was subjected to site-specific cleavage with the same DNAzyme. The \textit{yeiP} cleavage products in these RNA samples were subsequently examined by Northern blotting. RNase G cut at site W, whereas RNase E did not. Differences between some of the RNase E cleavage products observed in vitro and in vivo (\textit{Δrng} cells) may reflect the greater complexity of the cellular environment.

Reduction was partially reversed by further increasing that distance from 20 unpaired nucleotides to 30 unpaired nucleotides (pCUCCU(C)\textsubscript{9}U(C)\textsubscript{9}/F1). Similar behavior was observed for RNase G, except that the impact of altering the distance was more pronounced. For both enzymes, cleavage as far as 30–31 nucleotides from the 5' end still required the 5' phosphate (data not shown).

**Discussion**

RNase E and RNase G are homologous endonucleases with important roles in RNA processing and degradation in \textit{E. coli}. Previous studies have shown that the activity of both enzymes can be greatly enhanced when the substrate is monophosphorylated and that their cleavage site selectivity is similar but distinct (4–6), findings confirmed here. The present investigation has examined the features of RNA substrates critical for 5'-monophosphate-assisted cleavage by these two endonucleases. The results of these experiments have defined the minimum number of unpaired 5'-terminal nucleotides and the minimum spacing between the 5' end and the site of cleavage required for rapid 5'-monophosphate-dependent RNA cleavage by RNase E and RNase G, as well as the effect of distances greater than the minimum. They have also demonstrated the tolerance of these enzymes for a variety of 5'-terminal RNA sequences.

5'-Monophosphate-assisted RNA cleavage by either RNase E or RNase G requires at least two unpaired nucleotides at the 5' end and is even faster when 3–5 unpaired nucleotides are present there. RNase E is more tolerant of the bare minimum, cutting RNA only three times more quickly when the number of

**FIGURE 9.** Effect of greater distances between the 5' monophosphate and the site of cleavage. A, sequence and secondary structure of the pCUCC/F1, pCUCCU(C)\textsubscript{9}/F1, and pCUCCU(C)U(C)\textsubscript{9}/F1 duplexes. Only a portion of the region of intermolecular base pairing is shown, the remainder being the same as in Fig. 1. Sites of N-RNase E and RNase G cleavage are marked by arrows. For more details, see Table 1. B, relative susceptibility of pCUCC/F1, pCUCCU(C)\textsubscript{9}/F1, and pCUCCU(C)U(C)\textsubscript{9}/F1 to cleavage by N-RNase E or RNase G. N-RNase E or RNase G was added to a mixture of the substrate to be tested (pCUCC/F1, pCUCCU(C)\textsubscript{9}/F1, or pCUCCU(C)U(C)\textsubscript{9}/F1) and an internal standard (pCUCC+hp5/F1, pCUCC+hp4/F1, or pCUCC/F1, respectively; each was chosen to allow the electrophoretic separation of substrates and products), and their relative reactivity was calculated as in Figs. 2 and 3. Abscissa values (n) represent the number of U(C)\textsubscript{n} repeats in each substrate: zero for pCUCC/F1, one for pCUCCU(C)\textsubscript{n}/F1, and two for pCUCCU(C)U(C)\textsubscript{n}/F1. Error bars represent standard deviations.
unpaired 5′-terminal nucleotides is doubled from two to four, in contrast to the 6-fold rate enhancement observed for RNase G. The minimum requirement of two unpaired nucleotides at the 5′ end is consistent with the published structure of the catalytic domain of RNase E bearing a monophosphorylated RNA ligand (8). In that structure, the 5′-terminal nucleotide is completely buried inside the 5′-monophosphate-binding pocket, and the second nucleotide has barely emerged from the pocket and remains in contact with the protein surface, restricting its conformational flexibility. Consequently, neither is available for base pairing. By contrast, the fully emerged third nucleotide is completely exposed and therefore has the potential to engage in base pairing. The reason why RNase E reacts somewhat faster with a substrate bearing three unpaired nucleotides at the 5′ end rather than four or five is not evident from the structure of the liganded enzyme.

5′-end-dependent mRNA decay in E. coli involves not only cleavage by RNase E and RNase G but also prior conversion of the 5′ terminus to a monophosphate by RppH (3); therefore, all three enzymes have the potential to influence the overall rate of degradation (22). Like those two endonucleases, RppH requires two and prefers three or more unpaired nucleotides at the 5′ end of its RNA substrates (27). Consistent with the properties of the purified enzymes, the degradation of a derivative of yeiP mRNA in E. coli is slow and 5′ end-independent when only one unpaired nucleotide is present at the 5′ terminus. Its half-life declines steadily and becomes RppH-dependent as more unpaired nucleotides are added there, falling from 12 min to just 2.1 min when four unpaired 5′-terminal nucleotides are present. This behavior conforms to the consensus requirements of the three purified enzymes, all of which appear to participate in the degradation of yeiP mRNA in vivo.

The requirement of RNase E and RNase G for at least two unpaired nucleotides at the 5′ end raised the possibility that the identity of those two nucleotides, which are known to contact RNase E (8), might also be important. However, both enzymes proved to be relatively permissive when confronted with sequence variation there, slightly favoring U and often an additional nucleotide at the first two positions but tolerating all the others as well. Consistent with this promiscuity, structures of N-RNase E bound to RNA ligands reveal little if any interaction of the enzyme with the edges of the bases of these two nucleotides (8). E. coli RppH is also rather tolerant of sequence variation at the 5′ terminus of its substrates, and the identity of the nucleotides there appears to have at most a modest effect on rates of 5′-end-dependent mRNA degradation in E. coli (27, 28).

Previous reports have differed as to how close to a monophosphorylated 5′ end RNase E can cut (6, 13, 31, 32). Our data indicate that the minimum spacing necessary for efficient 5′-monophosphate-assisted cleavage by this enzyme is seven nucleotides and that eight or more are preferred, results consonant with the findings of Walsh et al. (31) and Kaberdin (13). By contrast, RNase G requires a minimum spacing of just 5 nucleotides and prefers 6 or more. Cleavage closer to the 5′ terminus was not detected at the enzyme concentrations used in the present experiments. Consistent with our findings, cleavage of monophosphorylated yeiP mRNA at a site 5 nucleotides from the 5′ end requires RNase G in vitro and in E. coli. A clue as to why RNase E has difficulty cutting as close to a 5′ monophosphate as RNase G is provided by the structure of N-RNase E bound to a monophosphorylated 13-mer (2′-O-methylated pUUUAACAGUAUUUG) (8). The oligonucleotide ligand follows a circuitous route between the 5′-end-binding pocket and the catalytic site in the same subunit, utilizing eight nucleotides to span that distance. A more direct path is blocked by Arg-373, whose positively charged side chain contacts the phosphates between nucleotides 1 and 2 and nucleotides 5 and 6, forcing the RNA to loop around it. In RNase G, this amino acid residue is replaced by Lys-374, whose smaller, unbranched side chain may be less capable of rerouting an RNA ligand and preventing it from following a direct path from the 5′-end-binding pocket to the active site.

The effect of increased spacing between the 5′ monophosphate and the scissile phosphate was also investigated. In general, cleavage proved to be fastest at the minimum spacing conducive to efficient cleavage: eight nucleotides for RNase E and six nucleotides for RNase G. Increasing that spacing by 1–4 nucleotides caused a modest (20–60%) reduction in the rate of cleavage, whereas substantially larger increases, to a total of 20–31 unpaired nucleotides, resulted in a much greater (severalfold) impediment, especially in the case of RNase G. These findings show that even a cleavage site with a favorable sequence may be cut rather slowly depending on its distance from the 5′ end. Interestingly, the reaction rate did not decline steadily as the intervening distance was increased stepwise from 10–11 to 30–31 unpaired nucleotides, as might have been expected were the relative rate governed simply by entropy loss resulting from RNA loop formation. Instead, a spacing of 20–21 nucleotides was found to be a greater impediment to 5′-monophosphate-assisted cleavage than a spacing of 30–31 nucleotides for both RNase E and RNase G. Because both enzymes are homomultimers, this observation raises the possibility that the 5′ monophosphate of the RNA substrate may bind to the 5′-end-binding pocket of one subunit and the scissile phosphate to the active site of another subunit when the distance between these two RNA elements is sufficiently long, potentially helping to mitigate the entropic cost of constraining the conformation of a lengthier RNA segment by enabling it to interact more extensively with the enzyme.

In view of their sequence homology, it is not surprising that RNase E and RNase G share a number of attributes and can target many of the same transcripts. RNase E, the more abundant of the two, is thought to govern the lifetime of most mRNAs in E. coli, yet the decay rate of a subset of E. coli messages is significantly influenced by RNase G despite its lower cellular concentration. The unique properties of RNase G identified here may provide some clues as to the distinguishing characteristics of this RNase G-dependent subset.
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