Stem-loops direct precise processing of 3′ UTR-derived small RNA MicL

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ABSTRACT

Increasing numbers of 3′UTR-derived small, regulatory RNAs (sRNAs) are being discovered in bacteria, most generated by cleavage from longer transcripts. The enzyme required for these cleavages has been reported to be RNase E, the major endoribonuclease in enteric bacteria. Previous studies investigating RNase E have come to a range of different conclusions regarding the determinants for RNase E processing. To better understand the sequence and structure determinants for the precise processing of a 3′ UTR-derived sRNA, we examined the cleavage of multiple mutant and chimeric derivatives of the 3′ UTR-derived MicL sRNA in vivo and in vitro. Our results revealed that tandem stem–loops 3′ to the cleavage site define optimal, correctly-positioned cleavage of MicL and probably other sRNAs. Moreover, our assays of MicL, ArcZ and CpxQ showed that sRNAs exhibit differential sensitivity to RNase E, likely a consequence of a hierarchy of sRNA features recognized by the endonuclease.

INTRODUCTION

While bacterial small RNAs (sRNAs) that act by limited base pairing to increase or repress synthesis from target mRNAs initially were found to be encoded as independent genes in intergenic regions, more and more base pairing sRNAs derived from the 3′ UTRs (untranslated regions) of mRNAs are being discovered (1–5). Some of these sRNAs, such as Salmonella enterica CpxQ, arise from the cleavage of the longer mRNA transcript, while others, such as Escherichia coli MicL, are transcribed from promoters internal to the protein coding sequence. Even the sRNAs transcribed from the internal promoters can be cleaved to give rise to a shorter product, which, in the example of MicL, contains the region for target base pairing (4).

The cleavage of the 3′ UTR-derived sRNAs characterized thus far has been observed to be well defined, often at or very near the stop codon of the corresponding upstream gene, raising the question of how this specific cleavage occurs. For the S. enterica CpxQ sRNA, which is cleaved from the cpxP mRNA, the cleavage was found to be dependent on the conserved endonuclelease RNase E (1). In addition, recent RNA-Seq analysis comparing the 5′ ends of transcripts with and without inactivation of a temperature-sensitive RNase E mutant in S. enterica indicated that the majority of 3′ UTR-derived sRNAs are generated by RNase E (6).

Tetrameric RNase E (encoded by rne), the major endoribonuclease in enteric bacteria, forms the core of the degradosome (reviewed in (7,8)). Given the central role of RNase E and the degradosome in the processing of mRNA, rRNA, tRNA and sRNA (reviewed in (9)), the determinants of RNase E-dependent cleavage have been studied extensively. Multiple studies have shown that the site of cleavage generally is single stranded and AU-rich (6,10). Initial characterization of the RNase E cleavage site in the bacteriophage T4 mRNA led to a proposed recognition sequence of (G,A)AUU(U,A) (11). In vitro assays examining the cleavage of poly(A) or poly(U) oligonucleotides with substitutions at specific positions further showed that while A- or U-rich sequences are uniformly cleaved, specific nucleotides near the cleavage site impact the position of cleavage (12,13). The recent genome-wide analysis of RNase E cleavage sites in S. enterica further led to the proposal of a RN/WUU core motif (R = A or G, N = any nucleotide and W = A or U), in which the location of a uridine residue two nucleotides downstream of the cleavage site is most critical (6).

Considering the degenerate nature of the proposed cleavage motifs, it is not surprising that additional factors have been proposed to impact RNase E-dependent cleavage including the status of the 5′ nucleotide, secondary structure and proteins bound to the RNA. A number of studies have shown that a 5′ monophosphate stimulates RNase
E-dependent cleavage at sites near this end (14). However, transcriptome-wide comparisons of total RNA in the presence and absence of the RppH RNA pyrophosphohydrolase, which generates the 5′ monophosphate ends showed that a large percentage of cleavage sites are not impacted by the 5′ end but rather are ‘direct entry’ sites for RNase E (15). This study also suggested that the presence of multiple single stranded regions enhanced direct entry by RNase E. Another genome-wide characterization of the secondary structure of the \textit{E. coli} transcriptome via parallel analysis of RNA structure (PARS) coupled to deep sequencing revealed sequences 5′ to 1800 known RNase E cleavage sites were significantly structured (10). For at least one RNA, the structure was shown to be critical for RNase E cleavage (11). Finally, the binding of proteins can impact the position and extent of cleavage both positively and negatively. This is illustrated by RNase E cleavage of the GlmZ sRNA, which is blocked by the binding of the Hfq RNA chaperone protein but is stimulated by the binding of the RapZ protein (16). In this example, it was found that RNase E cleaves at a site 6 nucleotides downstream of the stem–loop binding site, and conversion of the single stranded region from AU rich to GC rich did not prevent RNase E cleavage, while removal of the upstream stem–loop binding site did.

In contrast to the \textit{S. enterica} CpxQ sRNA, the levels of the truncated \textit{E. coli} MicL sRNA were not strongly reduced in a temperature-sensitive \textit{rne} mutant strain, though levels of the full length transcript increased (4). Given the prevalence of 3′ UTR-derived sRNAs, the precise cleavage observed, and our previous, ambiguous results regarding the ribonucleases acting on the 3′ UTR-derived MicL sRNA, we set out to define the determinants for MicL cleavage.

**MATERIALS AND METHODS**

**Bacterial strains and plasmids**

The bacterial strains and plasmids used in the study are listed in Supplementary Tables S1 and S2, respectively. For plasmid construction, the desired gene fragments were either cloned into the vectors using the Gibson Assembly Cloning Kit (New England Biolabs) or were generated by PCR amplification using MG1655 genomic DNA as a template and, after digestion with restriction enzymes, were cloned into the corresponding sites of the indicated vectors. All oligonucleotides used are listed in Supplementary Tables S1 and S2, respectively. For northern analysis, total RNA was extracted with TRIzol Reagent as described (20). Briefly, 1–5 ml cells grown to OD$_{600}$ ∼1.0 (or indicated otherwise) were collected and resuspended in 1 ml of TRIzol Reagent (Thermo Fisher Scientific) with repetitive pipetting to lyse the cells. The mixture was incubated at room temperature for 5 min, 0.2 ml of chloroform was added, and the sample was vortexed vigorously. The sample was then centrifuged for 15 min at 4°C at 11 000 rpm. The top ∼0.6 ml of the aqueous phase was transferred to a new Eppendorf tube and 0.5 ml of isopropyl alcohol added. After 10 min incubation at room temperature the sample was centrifuged at 11 000 rpm for 5 min at 4°C. The precipitated RNA pellet was washed with 1 ml of 75% ethanol and finally air-dried and dissolved in DEPC treated dH$_2$O. Total RNA concentration was determined based on OD$_{260}$.

Northern blots were performed as described (20). Briefly, 10 μg of total RNA was separated on an 8% polyacrylamide–7M urea gel (USB Corporation) in 1× TBE and transferred to Zeta-Probe membrane (Bio-Rad) overnight at 20 V in 0.5× TBE. Oligonucleotides were end-labeled with γ$^32$P-ATP by T4 polynucleotide kinase (New England Biolabs). Membranes were UV cross-linked and hybridized overnight with the labeled probe at 45°C in UltraHyb (Ambion) hybridization buffer. Following hybridization, membranes were washed once with 2× SSC + 0.1% SDS followed by a 10 min incubation at 45°C with 2× SSC + 0.1% SDS. Membranes were subsequently washed 5× with 0.2× SSC + 0.1% SDS, allowed to air dry for 5 min, and exposed to KODAK Biomax X-ray film at −80°C. Where indicated, band intensities were quantitated using ImageJ 1.50i software.

**Growth conditions**

Unless indicated otherwise, strains were grown aerobically at 37°C in LB (10 g tryptone, 5 g yeast extract, 5 g NaCl/l) to an OD$_{600}$ ∼1.0. The following compounds were added at the following final concentrations where indicated; IPTG at 1 mM, ampicillin at 100 μg/ml, kanamycin at 30 μg/ml, chloramphenicol at 25 μg/ml and tetracycline at 12.5 μg/ml.

To deplete RNase E, \textit{E. coli} strain KSL2000, with the pBAD-RNE plasmid (19), was grown overnight at 37°C in LB supplemented with ampicillin, chloramphenicol, tetracycline and 0.1% arabinose. Strains without arabinose failed to grow. The overnight culture (100 μl) was diluted into 30 ml of LB supplemented with ampicillin, chloramphenicol, tetracycline and 0.1% arabinose and grown at 37°C to OD$_{600}$ ∼0.5. The culture was then washed 3× with 40 ml of LB media and resuspended in LB with ampicillin, chloramphenicol, tetracycline to an OD$_{600}$ ∼0.2. The culture was then split into two 15 ml-cultures with one culture having 0.1% arabinose and the other 0.1% glucose. The two cultures were then grown at 37°C to OD$_{600}$ ∼1 and ∼1.2, and 1 ml of each culture was harvested and subject to total RNA extraction and northern analysis as described below. As expected, the culture with glucose grew slower than the culture with arabinose.

**Northern analysis**

For northern analysis, total RNA was extracted with TRIzol as described (20). Briefly, 1–5 ml cells grown to OD$_{600}$ ∼1.0 (or indicated otherwise) were collected and resuspended in 1 ml of TRIzol Reagent (Thermo Fisher Scientific) with repetitive pipetting to lyse the cells. The mixture was incubated at room temperature for 5 min, 0.2 ml of chloroform was added, and the sample was vortexed vigorously. The sample was then centrifuged for 15 min at 4°C at 11 000 rpm. The top ∼0.6 ml of the aqueous phase was transferred to a new Eppendorf tube and 0.5 ml of isopropyl alcohol added. After 10 min incubation at room temperature the sample was centrifuged at 11 000 rpm for 5 min at 4°C. The precipitated RNA pellet was washed with 1 ml of 75% ethanol and finally air-dried and dissolved in DEPC treated dH$_2$O. Total RNA concentration was determined based on OD$_{260}$.

Membranes were UV cross-linked and hybridized overnight with the labeled probe at 45°C in UltraHyb (Ambion) hybridization buffer. Following hybridization, membranes were washed once with 2× SSC + 0.1% SDS followed by a 10 min incubation at 45°C with 2× SSC + 0.1% SDS. Membranes were subsequently washed 5× with 0.2× SSC + 0.1% SDS, allowed to air dry for 5 min, and exposed to KODAK Biomax X-ray film at −80°C. Where indicated, band intensities were quantitated using ImageJ 1.50i software.

**In vitro RNA synthesis**

The MicL (308 nt), Δ222 (86 nt), Δ222-stemAΔ2 (72 nt), Δ222+10 (96 nt), Δ222+15 (101 nt), S8 (243 nt) and ArcZ (121 nt) RNAs were synthesized using a MEGAscript T7 Transcription kit (Ambion) using manufacturer’s guide-
In vitro assay of RNase E activity

The in vitro cleavage assays with the purified RNase E derivatives and in vitro transcribed RNAs were carried out with slight modifications of a previously reported protocol (1). Briefly, the RNA was diluted in Buffer A (25 mM Tris pH 7.5, 50 mM NaCl, 50 mM KCl, 10 mM MgCl₂, 1 mM DTT and 0.5 U μl⁻¹ RNase Out (Thermo Fisher Scientific)), and heat denatured at 95°C for 1 min and cooled on ice for 5 min. In a 10 μl reaction, 300 nM RNA was incubated in buffer or with 300 nM Hfq₆ for 10 min at 30°C. Buffer or purified RNase E (1-529) or D26N,D28N,D338N mutant RNase E (1-529) or 8× mutant RNase E (1-529) (300 nM) was added, and reactions incubated for an additional 30 min at 30°C. Ammonium acetate stop solution (Ambion) (7.5 μl) was then added along with 82.5 μl dH₂O and 100 μl of phenol:chloroform:IAA. The aqueous phase was separated from the organic phase by PLG-tubes (5PRIME) and the RNA was precipitated from the aqueous phase by the addition of 10 μl of glycogen and incubation at −80°C for 30 min. The RNA was then centrifuged at 15 000 rpm for 30 min at 4°C, and the pellet washed with 200 μl of 70% ethanol and resuspended in 20 μl of Gel Loading Buffer II (Ambion). An aliquot (10 μl) was analyzed via northern analysis as described above.

RESULTS

ArcZ, CpxQ and MicL are differentially sensitive to the rne-3071 allele

RNase E is capable of cleaving hundreds of RNAs and has been shown to play the most prominent role in sRNA cleavage in E. coli (reviewed in (9)). The enzyme is essential though overexpression of RNase G, which can cleave many of the same targets, can rescue an rne- strain (19). To determine the impact of RNase E and RNase G on cleavage of MicL, total RNA was isolated from strains carrying combinations of wild-type and temperature-sensitive rne-3071 (21) and Δrng alleles. Strains initially grown at 30°C to OD₆₀₀ ~1.0 were split and cultured for an additional h at either 30°C or 43.5°C, after which total RNA was extracted.

We first assayed the levels of ArcZ and CpxQ, previously reported to be cleaved by RNase E (1,6). As expected, while the levels of the cleavage products were similar for all of the strains grown at 30°C (Figure 1A, lanes 1-4), the levels of the processed transcripts were significantly reduced in the rne-3071 single mutant while the levels of the longer, uncleaved transcripts increased at the non-permissive temperature of 43.5°C (Figure 1A, lane 6). The products detected for the Δrng single mutant were similar to the products seen for the wild type strain at 43.5°C (Figure 1A, lanes 5 and 7). In contrast, the cleavage products were almost entirely ab-
Δrng double mutant strains. The levels of MicL increased, and the pattern of MicL-S was changed somewhat with the detection of one slightly shorter transcript in both mutants as well as somewhat shorter RNase G-dependent products in the rne-3071 mutant (Figure 1A, lanes 6 and 8).

ArcZ, CpxQ and MicL are differentially sensitive to RNase E depletion

We also examined the consequences of depleting wild type RNase E in an rne- strain with RNase E expressed from a P_BAD promoter on a plasmid (19). A culture of these cells was grown to mid-exponential phase in LB with arabinose, washed, split and the two halves grown in either LB with arabinose, which induces expression from the P_BAD promoter, or LB with glucose, which represses expression. Total RNA isolated from the two cultures further grown to either OD_{600} ∼ 1.0 or ∼1.2 was again probed for the ArcZ, CpxQ and MicL sRNAs (Figure 1B). Full-length and processed ArcZ as well as CpxQ were not present at approximately wild type levels for the cells grown with arabinose with a greater increase for full-length MicL (Figure 1B, lanes 1 and 3). In contrast, the levels of MicL-S were reduced. The shorter form of the sRNA may be further digested by elevated RNase E likely expressed from the plasmid. As expected, depletion of RNase E by growth in glucose led to increased levels of full-length and decreased levels of the processed ArcZ though cleavage was not completely abolished (Figure 1B, lanes 2 and 4). The levels of CpxQ, as well as intermediate cleavage products observed with the rne-3071 strain, all increased for the glucose-grown cultures (Figure 1B, lanes 2 and 4). MicL and MicL-S levels also were both increased with glucose with a greater increase for full-length MicL (Figure 1B, lanes 2 and 4). Thus, as observed for the rne-3071 strain, the three sRNAs showed differential sensitivity to altered RNase E levels.

MicL is cleaved by RNase E in vitro

To further test whether RNase E is capable of cleaving MicL at the position observed in vivo, we carried out in vitro cleavage assays with the purified catalytic N-terminal domain (NTD; residues 1–529) of RNase E in the presence and absence of the RNA chaperone Hfq. As shown in Figure 2, when we incubated full length MicL with RNase E (1–529) and Hfq, we observed faint cleavage of MicL at the position where cleavage is seen in vivo, possibly due to secondary structure occluding the cleavage site (see below). However, the levels of this product increased when the RNA was incubated with Hfq and a D26N,D28N,D338N mutant RNase E (1–529) that is more catalytically active than wild-type RNase E (1–529) (22).

A truncated version of MicL lacking the first 222 nt, which is processed as efficiently as wild type MicL in vivo (see below), is cleaved robustly by both wild type RNase E (1–529) and the hyperactive D26N,D28N,D338N mutant RNase E (1–529) in the presence of Hfq (Figure 2). Together the results of the in vivo and in vitro assays indicate that MicL is cleaved by RNase E. We suggest that MicL may be a particularly sensitive substrate since it is still processed by low levels of the endonuclease in vivo.

Cleavage of MicL is not affected by sequences at the 5’ end

We next wanted to examine what sequences directed the very specific cleavage of MicL to give MicL-S. To determine if sequences 5’ of the MicL cleavage site are important, we examined the consequences of one insertion as well as sequential deletions of this region (Figure 3A and Supplementary Figure S1A). The insertion and series of 5’ truncations were cloned into the pBR* expression plasmid and introduced into a ΔcutC strain, which lacks the native micL promoter and the region of micL overlapping the cutC coding sequence (4). Total RNA isolated from these strains was examined by northern analysis using an oligonucleotide probe complementary to the 5’-end of MicL. We suggest the in vitro transcribed full-length RNA may fold into some non-native configurations that could contribute to the low levels of cleavage observed for the full-length RNA with wild type RNase E (1–529).

Figure 2. Purified RNase E (1–529) cleaves MicL in vitro. In vitro transcribed full-length MicL (308 nt) and Δ222 (86 nt) with 5’PPP was incubated with purified Hfq and purified wild type RNase E (1–529) or D26N,D28N,D338N mutant RNase E (1–529) at 30° C for 30 min. The RNA was then subject to northern analysis using an oligonucleotide probe complementary to the 5’-end of MicL. We suggest the in vitro transcribed full-length RNA with wild type RNase E (1–529).

Figure 3. Cleavage of MicL is not affected by sequences at the 5’ end. We next wanted to examine what sequences directed the very specific cleavage of MicL to give MicL-S. To determine if sequences 5’ of the MicL cleavage site are important, we examined the consequences of one insertion as well as sequential deletions of this region (Figure 3A and Supplementary Figure S1A). The insertion and series of 5’ truncations were cloned into the pBR* expression plasmid and introduced into a ΔcutC strain, which lacks the native micL promoter and the region of micL overlapping the cutC coding sequence (4). Total RNA isolated from these strains was examined by northern analysis using an oligonucleotide probe complementary to the 5’-end of MicL (Figure 3B and Supplementary Figure S1B).

All of the constructs still gave rise to the MicL-S cleavage product, though the levels were reduced for +20, Δ70, Δ72, Δ82, Δ130, Δ132 and particularly Δ162. Structure predictions suggested that for the constructs with reduced MicL-S levels, the cleavage site might be occluded by the formation of an alternative stem–loop (Supplementary Figure S1A and C). To test this possibility, we mutated residues 177–180 and 204–208 predicted to be involved in this pairing and observed that cleavage is increased (Supplementary Figure S1D). Overall, given that the same MicL-S cleavage product was observed for all 20 constructs assayed, we concluded that the sequences 5’ of the cleavage site are not important for specific cleavage, but that, as has also been
Sequences at the 5′ end of cleavage site are not required for MicL cleavage. (A) MicL RNA sequence. Lower case sequence and arrow indicate cutC stop codon and RNase E cleavage site, respectively, and brackets denotes the portion of MicL 5′ end deleted (as indicated above the sequence) in constructs assayed in Figure 3B. (B) Wild-type (wt) and the indicated MicL mutants with 5′ end deletions were cloned into the pBR* plasmid. Total RNA isolated from a ΔcutC strain carrying these constructs was subject to northern analysis using an oligonucleotide probe complementary to the 3′ end of MicL. The bottom band:top band ratio is given below each lane.

RNA isolated from strains expressing these derivatives of Δ222 was subject to northern analysis. Interestingly, none of the mutations eliminated cleavage and the substitution of the A residues 5′ of the cut site, which did not match the consensus, led to the most reduced cleavage (Figure 4B). Similar results were seen for full-length MicL, where a plasmid overexpressing MicL carried mutations disrupting the cleavage site sequence or the flanking single-stranded sequences (Supplementary Figure S2). In this case, the UG|A cut site sequence was mutated to GG|A, UC|G, UC|A or GC|C and the flanking 5′ AAA and 3′ UUU sequences (relative to the UG|A cut site) were mutated to UUU and AAA respectively. None of the aforementioned mutations eliminated cleavage. These data indicate MicL processing is not greatly influenced by the sequence directly adjacent to or overlapping the cleavage site.

Sequences surrounding the cleavage site can be promiscuous

Studies in multiple organisms have indicated that RNase E preferentially cleaves at sequences rich in adenine and uridine (6,10,11). Additionally, a genome-wide analysis led to a proposed RNase E consensus sequence of RN|WUU, with the U at the +2 position being the most critical (6). The MicL cleavage site of AAAUG|AUUU is a good match to the consensus sequence. To examine the contribution of this sequence to cleavage, three mutants were constructed in which three adjacent residues, AAA preceding the site, UGA overlapping the site and UUU after the site, were mutated to CCC (Figure 4A) given that C residues were previously shown to inhibit RNase E cleavage, particularly two nucleotides downstream of the cleavage site (6,11). Total RNA isolated from strains expressing these derivatives of Δ222 was subject to northern analysis. Interestingly, none of the mutations eliminated cleavage and the substitution of the A residues 5′ of the cut site, which did not match the consensus, led to the most reduced cleavage (Figure 4B).

Similar results were seen for full-length MicL, where a plasmid overexpressing MicL carried mutations disrupting the cleavage site sequence or the flanking single-stranded sequences (Supplementary Figure S2). In this case, the UG|A cut site sequence was mutated to GG|A, UC|G, UC|A or GC|C and the flanking 5′ AAA and 3′ UUU sequences (relative to the UG|A cut site) were mutated to UUU and AAA respectively. None of the aforementioned mutations eliminated cleavage. These data indicate MicL processing is not greatly influenced by the sequence directly adjacent to or overlapping the cleavage site.

Stable 3′ stem–loops are required for cleavage

Having ruled out the sequences 5′ to and overlapping the cleavage site as necessary determinants of MicL processing, we next examined the requirement for the sequences 3′ of the cleavage site. This region contains two secondary structural elements: stem–loop A (ΔG = −11.7 kcal/mol) and the terminator, stem–loop B (ΔG = −17.2 kcal/mol) (Figure 5A and Supplementary Figure S3). Given the presence of stem–loops adjacent to other RNase E cleavage sites in E. coli (10), we wondered whether the stem–loops found in the Δ222 derivative might affect its cleavage. We first tested whether the sequences of the two stem–loops were important by replacing them with stem–loops from Spot 42.
sRNA, which have a similar size and stability despite significantly different sequences (Figure 5A). Analysis of total RNA isolated from cells expressing these constructs revealed that the heterologous stem–loops did not affect the location or extent of cleavage; the pattern was very similar for Δ222, stemA = Spot 42 (ΔG = −10.8 kcal/mol) and stemB = Spot 42 (ΔG = −21.2 kcal/mol) (Figure 5B) indicating the sequences of the 3′ stem–loops do not strongly impact processing.

To further examine whether stem–loop A was important, we shortened this stem in two mutants. In stemAΔ1 (ΔG = −8.7 kcal/mol), the stem was shortened by the removal of three base pairs towards the top of the stem–loop and in stemAΔ2 (ΔG = −13.0 kcal/mol), the bulges in the lower portion of the stem were removed thereby increasing stability (Figure 5C). Again, synthesis from these constructs was examined by northern analysis. While the stemAΔ1 construct showed cleavage similar to the control Δ222 construct, the stemAΔ2 derivative, which is predicted to be more stable than the wild type stemA, gave higher levels of MicL-S (Figure 5D). These data suggest that stem–loop A influences cleavage efficiency, possibly along with contributing to transcript stability.

To further explore the effect of stem A stability on processing, we additionally introduced mutations to destabilize the stem in the context of Δ222 with stemAΔ2; C245A246Δ1-G245C246 ΔG = −4.1 kcal/mol) and C245→G245 (ΔG = −5.8 kcal/mol) (Figure 5E). The transcripts made from these constructs were then compared to the Δ222-stemAΔ2 (ΔG = −13.0 kcal/mol) RNA. Both destabilized constructs showed a decrease in the cleavage product when compared to Δ222-stemAΔ2, further suggesting that the stability of stem–loop A affects cleavage efficiency (Figure 5F).

**Stem-loop A determines position of cleavage**

To investigate whether stem–loop A also governs the position of cleavage, we inserted heterologous sequences of ACACAC, UCUCUC or UGUUGUG in the single-stranded region between the cleavage site and stem–loop A and examined the influence of the insertions on the cleavage of the Δ222 construct (Figure 6A). Northern (Figure 6B) and primer extension (Supplementary Figure S4) analysis of all three mutants expressed from a plasmid clearly shows cleavage to generate a product similar to the size of MicL-S, again suggesting cleavage can occur at a very precise distance from stem–loop A regardless of sequence. We also detect an extra band, particularly for the UCUC, which might be directing cleavage at the AAAUG sequence. However, an extended stem–loop A that can be predicted for the UCUCUC and UGUUGUG constructs also might be directing cleavage at the second position.

**Distances between two 3′ stem–loops of MicL affect cleavage**

We noted that the two stem–loops are directly adjacent to each other and next examined the consequences of altering the distance between the two structures by inserting repeating U and C ‘spacers’ of 10 or 15 nucleotides between...
stem–loop A and B in the Δ222 context (Figure 6C). Secondary structure predictions indicate the spacer constructs create a single stranded region between the stem–loops and do not alter the stem–loop structures and their stabilities. Again, total RNA isolated from strains carrying the resulting constructs was subjected to northern analysis (Figure 6D). Compared to the Δ222 control, both spacer constructs showed decreased levels of cleavage with less product for the +15 construct than for the +10 construct. These data indicate that two stable stem–loops in close proximity to the RNase E cleavage site are required for efficient MicL cleavage, superseding recognition of a specific sequence.

Strongest cleavage is observed with two adjacent 3′ stem–loops

To further test whether two stem–loops are needed for efficient cleavage and to examine the influence of these two stem–loops with respect to the distance from and position 5′ or 3′ of the cleavage site, we synthesized a number of synthetic constructs (Figure 7A) starting with the Δ222–stemAΔ2 MicL derivative. For all of the synthetic constructs, the same linear sequence of four repeats of the MicL cleavage site (UGAUU) separated by a CC or UC spacer was flanked by different stem–loops. The repeat sequence was predicted to be single-stranded and not affect the structures of added or altered stem–loops in each of the constructs. Total RNA extracted from the strains harboring the resulting constructs was subjected to northern analysis (Figure 7B). For the construct with the repeats followed by stemAΔ2 and the wild type stem–loop B terminator (repeats+stemAΔ2+term, Figure 7B, lane 2), the 95 nt transcript gave the same predominant MicL-S product as the Δ222–stemAΔ2 construct (Figure 7B, lane 1), though one additional minor cleavage product was observed. These data suggest that although additional putative cleavage sites are present in the 5′-single stranded region, cleavage was directed towards the site nearest the two 3′ stem–loops.

The requirement for having two 3′ stem–loops was again tested by deleting stemA (repeats+term). Analysis of the total RNA from the strain carrying this construct showed there was very little cleavage of the 69 nt transcript with just stem–loop B (Figure 7B, lane 3). We suggest that the four minor products observed correspond to cleavage at the four consensus sequences in the linear repeat. The addition of a Spot 42 stem–loop in place of stem A (repeats+Spot 42 stem+term) restored strong cleavage at the position closest to the stem–loops (Figure 7B, lane 4), further indicating that efficient cleavage requires two stable stem–loops, independent of the sequences of the stem–loops.

To test whether stem–loops at the 5′ end of the repeat sequence could also influence cleavage, we examined one additional construct carrying stemAΔ2 upstream and the stem–loop B terminator downstream of the repeat sequence (stemAΔ2+repeats+term). Only very faint cleavage products were detected for this construct (Figure 7B, lane 5). Thus, two adjacent stem–loops are required for robust cleavage.

Mutations in MicL and RNase E decrease MicL cleavage in vitro

Finally, we tested whether the effects of the MicL mutations and insertions similarly impacted cleavage by purified RNase E. In vitro transcribed Δ222, stemAΔ2, +10 and +15 were all incubated with purified RNase E (1–529) and examined by northern analysis. The results of the in vitro experiments are consistent with the in vivo findings with stemAΔ2 showing somewhat increased cleavage and +10 and +15 showing decreased cleavage (Figure 8A).

Two recent crystal structures of a D303R,D346R catalytically-inactive RNase E (1–529) with sRNA fragments (22) have shown that helices of both RprA and SdsR are recognized by RNase E. The structures led to the identification of eight amino acid residues that when mutated (8× RNase E 1-529) impaired RNase E binding to and cleavage of RNA substrates with helical elements adjacent to the cleavage site. Among the substrates tested was the 9S rRNA, previously reported to contain structural elements important for RNase E recognition (23). We similarly did not observe cleavage of the 9S rRNA with the 8× RNase E mutant unless the in vitro transcript was treated with calf intestinal phosphatase (CIP) to convert the 5′ triphosphate
Figure 7. Strongest cleavage is observed with two 3′ stem–loops. (A) Predicted secondary structures of constructs with repeat sequences between different stem–loop structures. Secondary structures were predicted using the Mfold software package. The arrow indicates the site of RNase E cleavage. UGAUU repeats are underlined in red. (B) Cleavage is directed to the site nearest to two stable stem–loops. The constructs from Figure 7A were expressed from pBR* in ΔcutC background. Total RNA was extracted and probed for MicL as in Figure 3B. Full length products are denoted by asterisks, and prominent cleavage products are denoted by arrows.

to monophosphate to allow 5′ end recognition (Supplementary Figure S5). Consistent with RNase E recognition of stem–loop structures in MicL, the Δ222 derivative was not cleaved by the 8× RNase E mutant (Figure 8B). In contrast, in vitro synthesized ArcZ was partially cleaved by this mutant (Supplementary Figure S6).

As also observed in Figure 2, in the absence of Hfq, we no longer detected Δ222 cleavage with wild type RNase E (1–529), but the RNA was completely degraded by the hyperactive D26N,D28N,D338N mutant (Figure 8B). The ArcZ RNA was almost completely degraded by both the wild type and the D26N,D28N,D338N mutant proteins and partially degraded by the 8× mutant in the absence of Hfq (Supplementary Figure S6). Together these results indicate that Hfq may have two roles in promoting MicL cleavage, positioning RNase E to help direct cleavage at a specific position as well as protecting the cleaved product from excess cleavage.

DISCUSSION

The 308 nt 5′-dependent MicL sRNA, transcribed from within the cutC gene, was previously reported to be cleaved to give a 80 nt derivative capable of binding Hfq and repressing the synthesis of the abundant lpp mRNA (4). The results presented here show that MicL is cleaved by RNase E, and that precise cleavage is dependent on the presence of two adjacent hairpin structures 3′ to the cleavage site. Cleavage was found to be affected by both the stability of the two hairpins and the distance between them, with less impact of the sequence of the two hairpins or the sequence in and around the cleavage site. We also observed that the first stem–loop dictated the position of cleavage, and that Hfq promoted the specific cleavage. Our results suggest that RNase E in conjunction with Hfq is recognizing helical structures leading to cleavage at a specified distance from this recognition element.

sRNAs are differentially affected by rne-3071

The sequence of the MicL cleavage site is similar to the RNase E core motif identified in a recent study mapping RNase E cleavage sites transcriptome-wide by transient inactivation of RNase E followed by high-throughput RNA sequencing in S. enterica (6). However, even in this high-
throughput study, the RNA levels of MicL and MicL-S were not severely affected by the transient inactivation of the RNase E, at least compared to other known RNase E sRNA substrates, analogous to what we observed (Figure 1).

The RNase E temperature-sensitive allele (rne-3071) in E. coli has a C742T transition resulting in the F68L substitution near the nucleotide binding motif (24,25). This temperature-sensitive mutation was found to increase the chemical half-life of total pulse-labeled RNA (26) and affect the steady state levels of individual RNAs cleaved by RNase E (27). Our results show different degrees of sRNA cleavage for the rne-3071 strain at the non-permissive temperature; cleavage is almost completely abolished for ArcZ, significantly decreased for CpxQ and only slightly decreased for MicL (Figure 1A). Nevertheless, we think MicL is in fact a substrate for RNase E since the extent of MicL processing is decreased upon RNase E depletion in vivo (Figure 1B) and cleavage is observed at the same position with the purified NTD of RNase E in vitro (1-529) (Figure 2).

Our observations are consistent with early studies of the known RNase E substrates RNAI and T4 mRNA, for which it was found that not all RNase E decay intermediates are reduced in the rne-3071 strain at the non-permissive temperature (11,13). In fact, when comparing RNAI cleavage by wild-type and rne-3071 at the non-permissive temperature, some decay products were decreased but several products were similar between the two strains and others were enhanced. Furthermore, the rne-3071 strain seemed to generate new RNAI decay bands not seen in the rne+ strain at the non-permissive temperature, leading the authors to warn about assigning RNase E cleavage sites based entirely on changes in RNA abundance in the rne-3071 strain (13).

Recognition of MicL stem–loops by RNase E

From our mutational analyses, we propose that the structural elements at the 3′ end of the MicL RNA are being recognized by RNase E in what is a ‘direct entry’ pathway for cleavage. The 5′ end of in vivo transcribed MicL RNA is triphosphorylated (28) and thus is less likely to be recognized by RNase E. It is interesting to note that several known RNase E substrates have two stem–loop structures either preceding or following the cleavage site. This is the case for 9S RNA where two stem–loop structures either precede or follow the cleavage site. This is the case for 95 RNA where two stem–loop structures are predicted to disrupt the first stem–loop structure of RNAI, while 5′ sequences are dispensable (13). Although the predominant cleavage site occurs four nucleotides from the first 3′ stem–loop structure of RNAI, a less intense cleavage site also occurs 4 nucleotides upstream of the second hairpin. Thus, it would seem, like with MicL, two closely spaced hairpins dictate cleavage of RNAI at a specified distance from an adjacent hairpin.

We suggest that the regions of RNase E, the RNase H like domain and small domain located distal to the catalytic active site of RNase E, observed to interact with helical structures of RprA and SdsR in the crystal structures (22) comprise the domain responsible for structural recognition of MicL. Consistent with this view, the RNase E (1−529) 8× mutant (with mutations to R3, Q22, H268, Q270 and Y269 in the RNase H domain and K433, R488 and R490 in the small domain) did not cleave Δ222 in vitro (Figure 8D). Due to the tetrameric organization of RNase E, it is conceivable that the two MicL 3′ stem–loops can contact two adjacent subunits within the principle dimer and that these close contacts anchor the single stranded cleavage site near the active site of the protein, explaining the requirement for a specific distance between the two 3′ stem–loops as well as the specific site of cleavage relative to the first stem–loop. Consistent with this possibility, a model of RNase E (1−529) with RprA shows that the distance between the helical recognition domain and the active site of the enzyme is approximately 5 nt (B. Luisi, personal communication). Given that MicL is a known Hfq binding sRNA (4) and Hfq likely interacts with the rho-independent terminator struc-
We have found for MicL help direct cleavage of other RNAs, (1,2,3,31). Thus, we predict that structural features like what specific cleavage such that an sRNA might be recognized by ArcZ, CpxQ as well as other 3′ spaced stem–loops at their 3′ ends, the second one corresponding to the Rho-independent transcription terminator (1,2,31). Thus, we predict that structural features like what we have found for MicL help direct cleavage of other RNAs, especially 3′ UTR-derived sRNAs. We note that features in addition to the stem–loops could contribute to the very specific cleavage such that an sRNA might be recognized by a combination of 5′ sensing and direct entry. The individual recognition elements—5′ end, single stranded AU-rich sequence, 3′ stem loops and Hfq binding—each could contribute differently to the efficiency of cleavage. This possibility is underscored by the different sensitivities of MicL, ArcZ and CpxQ to the ability is underscored by the different sensitivities of MicL, ArcZ and CpxQ to the 

E (1–529) when Hfq is omitted these second stem–loop. The lack of 

complex between MicL, RNase E and Hfq. In this model, 


to a cleavage hierarchy by this hypothesis.

E regulation and function. 

The social fabric of the RNA degradosome. 

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9. Supplementary Data are available at NAR Online.

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