Bacterial Toxin RelE Mediates Frequent Codon-independent mRNA Cleavage from the 5’ End of Coding Regions in Vivo*

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The enzymatic activity of the RelE bacterial toxin component of the Escherichia coli RelBE toxin-antitoxin system has been extensively studied in vitro and to a lesser extent in vivo. These earlier reports revealed that 1) RelE alone does not exhibit mRNA cleavage activity, 2) RelE mediates mRNA cleavage through its association with the ribosome, 3) RelE-mediated mRNA cleavage occurs at the ribosomal A site and, 4) Cleavage of mRNA by RelE exhibits high codon specificity. More specifically, RelE exhibits a preference for the stop codons UAG and UGA and sense codons CAG and UCG in vitro. In this study, we used a comprehensive primer extension approach to map the frequency and codon specificity of RelE cleavage activity in vivo. We found extensive cleavage at the beginning of the coding region of five transcripts, ompA, lpp, ompF, rpsA, and tufA. We then mapped RelE cleavage sites across one short transcript (lpp) and two long transcripts (ompF and ompA). RelE cut all of these transcripts frequently and efficiently within the first ~100 codons, only occasionally cut beyond this point, and rarely cut at sites in proximity to the 3’ end. Among 196 RelE sites in these five transcripts, there was no preference for CAG or UCG sense codons. In fact, bioinformatic analysis of the RelE cleavage sites failed to identify any sequence preferences. These results suggest a model of RelE function distinct from those proposed previously, because RelE directed frequent codon-independent mRNA cleavage coincident with the commencement of translation elongation.

The RelE family of bacterial toxins consists of the HigB, RelE, YafQ, and YoeB toxins (1), each inhibiting translation through related, but distinct, mechanisms. The Escherichia coli YafQ toxin is a ribosome-associated endoribonuclease that cleaves in-frame AAA codons that are followed by either an A or G in the subsequent codon (2). Likewise, HigB from the Rts1 plasmid (from Proteus spp.) is a ribosome-associated endoribonuclease that cleaves mRNA at A-rich regions, regardless of frame (3). In contrast to the other family members, YoeB expression leads to marginal mRNA cleavage. The cleavage activity of ribosome-associated YoeB does not appear to underlie toxicity because a YoeB mutant lacking endoribonuclease activity retains toxicity (4). Instead, YoeB apparently inhibits translation by destabilization of the initiation complex (4). The RelE toxin also interacts with the ribosome and induces mRNA cleavage. In vitro studies have demonstrated that RelE exhibits a preference for cleavage at the UAG codon among the three stop codons tested (5, 6). Enzyme kinetic studies also identified sense codons CAG and UCG as the most efficiently cleaved codons in vitro (6). Structures of enzymatically active versus inactive E. coli RelE associated with the Thermus thermophilus 70 S ribosome complex have shed light on RelE properties in vitro (7). However, it is unclear whether these in vitro activities accurately depict the mechanism of cleavage that occurs in vivo.

In this work, we investigated the frequency and sequence specificity of RelE-mediated cleavage in vivo. In contrast to its reported rapid cleavage at UAG stop codons (and thus at the 3’ end of the mRNA) in vitro, our data revealed that RelE expression resulted in frequent cleavage early in mRNA coding regions (within the first 100 codons) in vivo. Furthermore, we did not observe any codon specificity. In fact, the use of bioinformatics software to search for common features among the major RelE cleavage sites did not reveal any statistically significant sequence preferences for this toxin. The activity we documented is more consistent with the two hallmarks of RelE expression in living cells (i.e., rapid, comprehensive mRNA degradation and concomitant growth arrest) than the existing model where preferential cleavage occurs at only two sense codons in the coding region (one of which is very rare) plus UAG and UGA stop codons at the 3’ end of mRNAs.

EXPERIMENTAL PROCEDURES

Strains, Plasmids, and Reagents—The E. coli strain BW25113 was used for all protein expression and toxicity studies. Mach1 T1 E. coli cells (Invitrogen) were used for all cloning experiments. The relE ORF was PCR-amplified from E. coli cells with 5’-Ndel/Xhol-3’ ends and cloned into the corresponding sites of pBAD24 (8) to create pBAD24-relE. The wild type and mutant ompA genes were PCR-amplified from E. coli with 5’-Ndel/Xhol-3’ ends and ligated to the corresponding sites of pBAD33-MCS5 containing a ribosome-binding site and a modified polylinker (M. Inouye laboratory). These plasmids were then both transformed into an E. coli K12 BW25113 ΔompA strain obtained from the KEIO collection (9). All bacterial liquid cultures were grown in M9 minimal media supplemented with either 0.2% glucose or 0.21% glycerol at 37 °C, unless otherwise noted. The working concentration of ampicillin was 100

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The accuracy of the DNA sequences of PCR products used for cloning was confirmed by automated DNA sequence analysis.

**Primer Extension**—Total RNA was extracted and primer extension analysis was carried out as described previously (2). The sequences of the primers used were as follows: lpp, 5’-TTA-CTTGCAGTTATTAAGTACCC-3’; ompA1, 5’-GGTGATACCCAGTTAAGGCTGTTGATGAAACC-3’; ompA2, 5’-GGTACCCAGTTAAGGCTGTTGATGAAACC-3’; ompA3, 5’-GGTACCCAGTTAAGGCTGTTGATGAAACC-3’; ompA4, 5’-GGTACCCAGTTAAGGCTGTTGATGAAACC-3’; ompA5, 5’-GGTACCCAGTTAAGGCTGTTGATGAAACC-3’; ompF, NWO1172, 5’-AAACCAAGACGGGCATAGGTC-3’; NWO1173, 5’-TGTAACCCAGTGATTACAAGCACCACC-3’; NWO1174, 5’-TTAAGGCTGTTGATGAAACC-3’; rpsA, 5’-CTTATGCTTGAGACTTGACC-3’; tufA, 5’-CTTATGCTTGAGACTTGACC-3’; and primer extension analysis of the OmpA mutant, NWO1577, 5’-CTTATGCTTGAGACTTGACC-3’.

**RESULTS**

**Comprehensive in Vivo Approach to Study RelE Codon Specificity**—Expression of RelE facilitates rapid and complete degradation of all transcripts analyzed by Northern analysis (3, 5). We also observed that RelE induction leads to rapid (within 5 min) and nearly complete (to 5% of wild type levels) translation arrest as assessed by [35S]Met incorporation (3). In contrast, translation inhibition is more gradual with RelE family member HigB, with the maximal impairment occurring 20 min post-induction (20% of wild type levels) (3).

Previous in vivo studies of RelE-mediated mRNA cleavage were limited to primer extension analysis of very short regions of lpp and transfer-messenger RNA transcripts (5). For lpp, only the first 21 codons of the wild type transcript and three lpp mutants each containing a unique premature stop codon at position 21 were analyzed. For transfer-messenger RNA, only the last eight codons at the 3’ end were analyzed. The rationale behind these limited in vivo experiments was to substantiate earlier in vitro studies using purified ribosomal complexes containing RelE that revealed rapid cleavage at the stop codon UAG (6). RelE exhibited a preference for cleavage at CAG (Gln) and UCG (Ser) codons among sense codons (6).

Because we determined that HigB acts by specifically cleaving mRNA at A-rich sequences along the entire length of mRNA, the rapid kinetics of RelE cleavage in vivo seemed incongruent with the proposed target sequence at the 3’ end of the mRNA (i.e., at stop codons) and at two sense codons CAG (Gln) and UCG (Ser). Also, of the two sense codons identified, UCG is a rare codon (10). Therefore, it seemed plausible that the rapid inhibition of translation by RelE in vivo might involve a less restrictive cleavage mechanism. We sought to understand the mechanism of RelE toxicity by analyzing the frequency of

**FIGURE 1.** RelE expression leads to frequent cleavage at the 5’ end of mRNAs. Primer extension analysis of tufA (A), ompF (B), and rpsA (C and D) transcripts using a primer that annealed 150 nts from the translation start site. Exact cleavage positions of mRNAs identified near the top of the gel were determined with more adjacent primers (data not shown). Numbers indicate time (min) after RelE induction; *60wt* lanes are minutes after growth without RelE induction; FL, full-length products. Labeled cut sites correspond to those in Tables 1–3. DNA sequencing ladders were prepared using the same primers used for primer extension reactions; start codons are indicated on panels containing reactions closest to the 5’ end of transcripts.
cleavage and codon specificity of this toxin using a comprehensive in vivo approach. Primer extension analysis was performed on the five mRNAs (*lpp*, *ompA*, *ompF*, *rpsA*, and *tufA*) that we previously demonstrated were rapidly degraded upon RelE expression (3). We initially used a primer that annealed ~150 nts downstream of the translation start site for each mRNA analyzed (Figs. 1 and 2). Additional primer extension experiments were subsequently performed with oligonucleotides spanning the entire length of three of the five mRNAs (*lpp*, *ompA*, and *ompF*; Figs. 2 and 3).

In total, 196 RelE-specific cleavage products were detected among all five mRNAs (Tables 1–5). Under the steady state conditions of our study and based on band intensities on the same film exposure, of these 196 sites, 78 were relatively abundant (designated as “major” cleavage sites) and 41 were products whose intensities were estimated as ≤25% that of the major sites (“minor” sites). In addition, we also detected another 77 cleavage products that were only clearly discernable after long exposures of the films (designated as “rare”), indicating that these products constituted a very small percentage of the total RelE-mediated cleavage events under steady state conditions. Nevertheless, it was useful to identify these rare sites because they contributed to the body of information on mRNA sequences that are targeted across the entire length of a transcript. Analysis of the location and codon sequences of all cleavage sites revealed several features of mRNA cleavage that are unique to RelE activity in the intact bacterial cell are addressed below.

**Extensive RelE Cleavage Is Detected within 100 Codons from the Translation Start of All Five Transcripts**—We observed a consistent trend among all five transcripts after primer extension analysis: each mRNA was cut preferentially at the beginning of the coding region (RelE only cleaves within mRNA-coding regions (5)). The two transcripts in which only the first ~150 nts of the coding region were assessed, *tufA* (Fig. 1A and Table 1) and *rpsA* (Fig. 1, C and D, and Table 3), showed evenly dispersed and extensive RelE cleavage. Comprehensive analysis of the three full-length transcripts, *lpp*, *ompA*, and *ompF*, was even more instrumental in demonstrating RelE cleavage trends. First, the relatively short 237-nt coding region of the *lpp* mRNA was efficiently cut at 35 sites throughout its

3 The abbreviation used is: nt, nucleotide.
entire length (Fig. 2A and Table 4). Second, based on the results from ompA and ompF, RelE predominantly targeted mRNA for cleavage within the first 70–100 codons from the translational start site. Past this point we were only able to detect products using 10-fold longer exposure times than those needed to detect major and minor sites. Thus, ompA or ompF transcripts that were cut at these rare sites represented a very low percentage of the total pool of the respective mRNAs cut by RelE.

The illustration in Fig. 3 summarizes the RelE cleavage patterns of the full-length lpp, ompA, and ompF coding sequences, as well as the first ~150 nts of tufA and rpsA mRNA coding regions. In each case, we documented extensive RelE-mediated cleavage early in the coding region. Also notable, the mapped cleavage sites within the long ompA and ompF coding regions revealed that only rare sites were detected past the first ~100 codons for ompA and ~70 codons for ompF.

RelE Does Not Exhibit Codon Specificity in Vivo—Measurement of $k_{cat}/K_m$ values (representing enzymatic efficiency) for 21 codons cleaved by RelE at the ribosomal A site in vitro revealed the following: 1) of the three stop codons, UAG was cleaved at the fastest rate; 2) among the sense codons, UCG and CAG were cleaved most rapidly (6). Therefore, RelE exhibited codon specificity under these conditions. This specificity influenced the construction of the synthetic RNA template, engineered with a UAG at the A site, used to determine the structure of ribosome-bound RelE (7).

Although we identified RelE cleavage at 14 of the 17 CAG codons covered by our primer extension analysis, overall we failed to document a pattern supporting a codon-specific cleavage model. Among the 196 RelE cut sites we examined, CAG codons represented only 7% of total. UCG was also previously identified as another sense codon with a high $k_{cat}/K_m$ value (6). However, this is a rare codon (10) and was not represented in any of the coding regions of the five mRNAs we analyzed by primer extension. Examination of residues flanking RelE cleavage sites exposed only one discernable feature, 40% of the cut sites were before or after a G (Table 6). Interestingly, RelE exhibits structural relatedness to the microbial endoribonu-
clease RNase Sa (11) and fungal RNase T1 (7), both of which cleave single-stranded RNA on the 3' side of G residues.

RelE exhibited preferential cleavage at codons containing a G or C base in the third position \textit{in vitro} as well as in limited \textit{in vivo} studies (5, 6). In agreement with this, we found that 66% of all codons cut by RelE in \textit{lpp} mRNA possessed a Go rC at position 3, 66% for \textit{ompA} (Table 5), 61% for \textit{tufA} mRNA codons (Table 1), 43% for \textit{ompF} (Table 2), and 67% for \textit{rpsA} (Table 3). Overall, 64% of the 196 codons cleaved by RelE \textit{in vivo} ended with a G or C.

Neubauer \textit{et al.} (7) also analyzed the perceived preference of RelE for a 3-nt consensus of pyrimidine-purine-G in the A site (6). In the precleavage structure of the RNA-ribosome complex, it was noted that a smaller pyrimidine appears to be a better conformational fit at position 1, although the stacking visualized in positions 2 and 3 was thought to be stabilized by larger purine residues. However, our data did not support this model;
of the 196 RelE cleavage sites, only 9% of the A sites contained this consensus.

Bioinformatic Analysis of Major RelE Cut Sites Does Not Uncover Clear Cleavage Sequence Preferences—To assess the statistical significance of the trends we identified in vivo, we tested whether perceived sequence preferences would hold up upon more rigorous computational analysis using the HMMER software tool (12). We first used the 51 major sites from full-length lpp and ompA transcripts. We built hidden Markov models from 2-base (one on each side of the cut site), 4-base (two on each side), 6-base (three on each side), and 8-base (four on each side) major ompA cuts. These models were then used to predict the major and minor cuts of lpp; however, none were identified. Conversely, hidden Markov models were built from the major cuts of lpp, and they were used to predict the major and minor cuts of ompA. Again, no hits were identified. As a final test of statistical modeling, the models built from ompA major cuts were used to predict the cuts in ompA itself, and again none were identified; the same result was obtained for lpp. Thus, it was concluded that the motifs around the cut sites are so nonspecific that the best, and widely used, HMMER statistical tool was unable to produce models of enough statistical power. The development of new algorithms may enable identification of RelE sequence preferences in the future.

Parameters Influencing RelE Cleavage in Vivo May Be Relatively Complex—We performed experiments to assess how deletion or addition of a major cleavage site to the beginning of the ompA transcript affected RelE cleavage (Fig. 4, A and B). We began with a bacterial strain in which the chromosomal copy of the nonessential ompA gene was deleted, and we transformed it with arabinose-inducible plasmids for OmpA (wild type control or mutant) and RelE expression. Unexpectedly, the cleavage pattern was altered slightly in the ompA wild type mRNA control transcribed from the pBAD plasmid compared with that transcribed from within the native chromosomal context; the plasmid mRNA was cleaved at two positions in the 4th codon (Fig. 4C, top line), whereas the chromosomally derived transcript was not cleaved at these positions (1st line, left side of Table 5). Mutation of codon 4 from ACA to AAG, which was predicted to add a RelE cut site, behaved as expected. This mutated 4th codon was cut at the same position (AA↓G) as the preceding codon that contained a major cut site (Fig. 4C, middle line). However, clear interpretation of this result was not possible since we also observed cleavage of the control plasmid at this codon. Finally, mutations engineered into codons 2 and 3 were predicted to remove two contiguous
RelE cut sites by changing AAG-AAG to ACA-ACA. However, instead of preventing RelE cleavage at both mutated codons, mutagenesis precluded cleavage of only one (codon 3) of the two (Fig. 4C, bottom line). This result suggests that RelE cleavage is influenced by more than the RNA sequence of the codon. In fact, the mutated third codon was not cut because the sequence was changed from AAG to ACA. However, the mutated second codon was cut at the same position as the AAA codon it replaced (i.e. the original AAA was changed to ACA but cleavage still occurred as AC2A). Therefore, the ACA was cut at codon 2 but not at codon 3. In fact, we noticed that the second codon was cleaved between the second and third base regardless of the sequence in all five wild type transcripts we studied (first sequences are listed in Tables 1–5).

These limited mutant studies revealed the following: 1) the second codon seems to be favored for RelE cleavage; 2) the sequence determinants of RelE cleavage are not predictable (consistent with the conclusions of our bioinformatics analysis); and 3) neither codon sequence nor position alone dictates RelE cleavage, although both appear to contribute to the process.

RelE Cleave Codons Most Frequently after the Second or Third Base in Vivo—In vitro studies have reported that RelE typically targets codons for cleavage between the second and third base (6). In vivo, we observed that 45% of the 196 RelE sites cut after the third base of the codon (XXX). However, another 40% were cut between the second and third base (XX2X). Only 15% were cut after the first base (X2XX). Therefore, in our 196-site sample set, RelE cleaved codons after the second or third base with highest frequency and infrequently cut after the first base of the codon occupying the ribosomal A site. These results are consistent with models stemming from structural data of RelE bound to programmed ribosomes. Because RelE appears to contain a single active site, the mRNA likely shifts inside the ribosome, allowing cleavage at other locations in addition to that between positions 2 and 3 in the A site (7).

### TABLE 5

| mRNA 5′ → 3′ | Cut site | Major (M) minor (m) rare | mRNA 5′ → 3′ continued | Cut site | Major (M) minor (m) rare |
|--------------|----------|--------------------------|-------------------------|----------|--------------------------|
| ompA         | AAG AAG  | A1 M GAG UAC | GCG AUC A48 nes       | A1 M GAG UAC | A48 nes |
| AAA AAA     | ACA ACA  | A2 M UAC GCG | AUC AUC A49 nes       | A2 M UAC GCG | A49 nes |
| ACA GCG     | AUC CCG  | A3 M ACU CCG | GUA AUC A50 nes       | A3 M ACU CCG | A50 nes |
| GUC AUC     | GCG AUC  | A4 m ACU GCC | AUC AUC A51 nes       | A4 m ACU GCC | A51 nes |
| AUC UAG     | AUU UCA  | A5 M GGU UCA | GUA UAC A52 nes       | A5 M GGU UCA | A52 nes |
| AEU GCG     | AGG GCA  | A6 M GUC AUC | GUA AGC A53 nes       | A6 M GUC AUC | A53 nes |
| AUC GCA     | GUC GCA  | A7 M AGC CUG | GUU GGU A54 nes       | A7 M AGC CUG | A54 nes |
| GCA CUG     | GCA CUG  | A8 M UCC GUC | CAG GGC A55 nes       | A8 M UCC GUC | A55 nes |
| CCC GGC     | GUC GGG  | A9 M GGU CAG | GGA GAA A56 nes       | A9 M GGU CAG | A56 nes |
| UCC GGC     | ACC GUA  | A10 M GCU CCA | GUA GUU A57 nes       | A10 M GCU CCA | A57 nes |
| GCU AGG     | GCA CCG  | A11 M GC CCG | GUC AAG A58 nes       | A11 M GC CCG | A58 nes |
| GCA CGG     | GGC GCU  | A12 M GCC GCG | AUC GCA A59 nes       | A12 M GCC GCG | A59 nes |
| GCA CCG     | GCC GCU  | A13 M GCU CCG | GUA GCA A60 nes       | A13 M GCU CCG | A60 nes |
| GCU CUG     | GCG CCA  | A14 M GCA CCG | GAU GAA A61 nes       | A14 M GCA CCG | A61 nes |
| CCC GAG     | AAA GAA  | A15 m GUA CAG | ACC AAG A62 nes       | A15 m GUA CAG | A62 nes |
| AAA AAA     | AAC ACC  | A16 m ACC AAC | GUC UAA A63 nes       | A16 m ACC AAC | A63 nes |
| AAC ACG     | UCG UAC  | A17 m ACU CUG | GAC UGG A64 nes       | A17 m ACU CUG | A64 nes |
| AAC CCG     | GAC AUC  | A18 m UAC GCU | AAC UAC A65 nes       | A18 m UAC GCU | A65 nes |
| ACC CCG     | AAC GAC  | A19 m CUG GUC | UCG UGC A66 nes       | A19 m CUG GUC | A66 nes |
| GCC UGG     | AGU UGC  | A20 m CUG GUC | AUC AAC A67 nes       | A20 m CUG GUC | A67 nes |
| UAC AUC     | GGU GCU  | A21 m GUC CAG | UGU AAC A68 nes       | A21 m GUC CAG | A68 nes |
| GGU GCU     | AAA CUG  | A22 m UCC AUC | AAC AAG A69 nes       | A22 m UCC AUC | A69 nes |
| UAC UAC     | GCC AAC  | A23 m ACC UAA | GAC AAA A70 nes       | A23 m ACC UAA | A70 nes |
| ACC CCA     | AAA CAG  | A24 m AAC AGG | GGA GCG A71 nes       | A24 m AAC AGG | A71 nes |
| ACC GAC     | GAA AAG  | A25 m GGA CAG | GCC GGU A72 nes       | A25 m GGA CAG | A72 nes |
| ACC CAG     | CUG GCG  | A26 m GCU CAG | GUA AAG A73 nes       | A26 m GCU CAG | A73 nes |
| ACC CAU     | GGC GCA  | A27 m GCC CAG | UAC AGC A74 nes       | A27 m GCC CAG | A74 nes |
| ACC GGA     | GGC GCA  | A28 m GCC CAG | AGC AAC A75 nes       | A28 m GCC CAG | A75 nes |
| GCU UGU     | GUU GGU  | A29 m AGC AAC | GUA AAG A76 nes       | A29 m AGC AAC | A76 nes |
| UAC CUG     | GGU AAC  | A30 m AAA GAC | GGG UCC A77 nes       | A30 m AAA GAC | A77 nes |
| GCU GCC     | GCC CUG  | A31 m GGU GCC | CUG GUU A78 nes       | A31 m GGU GCC | A78 nes |
| GAA AAA     | GGU UAC  | A32 m GAC GGU | CUC UCC A79 nes       | A32 m GAC GGU | A79 nes |
| GUC UCU     | UCG UGG  | A33 m UCC GAG | CGC CGU A80 nes       | A33 m UCC GAG | A80 nes |
| GAC CUG     | UGU GGG  | A34 m CGC UGG | GUC GGG A81 nes       | A34 m CGC UGG | A81 nes |
| UAC CUG     | GGU UGC  | A35 m CUG GCC | AUC UGC A82 nes       | A35 m CUG GCC | A82 nes |
| CUG GGU     | CUC UAC  | A36 m UAC GUC | AUC UAC A83 nes       | A36 m UAC GUC | A83 nes |
| AUG UAC     | UAC AAA  | A37 m AUC GGU | CAG GAA A84 nes       | A37 m AUC GGU | A84 nes |
| GCU GGU     | GCC GUC  | A38 m GCC GUG | AUC AUC A85 nes       | A38 m GCC GUG | A85 nes |
| AAC GUC     | GAC ACG  | A39 nke UCC GCA | CGU GGU A86 nes       | A39 nke UCC GCA | A86 nes |
| AGU UGU     | GGU GCC  | A40 nke UCC GCA | GGC AAG A87 nes       | A40 nke UCC GCA | A87 nes |
| GUC AUG     | GUU UGC  | A41 nke UCC GCA | GAC AGC A88 nes       | A41 nke UCC GCA | A88 nes |
| AAC AUG     | GUG UGC  | A42 nke UCC GCA | AAG UUG A89 nes       | A42 nke UCC GCA | A89 nes |
| GUG UAU     | UUG AAA  | A43 nke UCC GCA | AGG GGU A90 nes       | A43 nke UCC GCA | A90 nes |
| AAC CAC     | GAC ACC  | A44 nke UCC GCA | AAA CAG A91 nes       | A44 nke UCC GCA | A91 nes |
| GAC CAC     | GCC GUC  | A45 nke UCC GCA | GUA AAG A92 nes       | A45 nke UCC GCA | A92 nes |
| GUC CUG     | GCC GUG  | A46 nke UCC GCA | CAG AAG A93 nes       | A46 nke UCC GCA | A93 nes |
| GUU CUG     | CUG GGU  | A47 nke UCC GCA | CAG AAG A94 nes       | A47 nke UCC GCA | A94 nes |
Interestingly, we noted several examples in all five transcripts of a G/U in the first position of the codon following the cleavage site, e.g. XXX XXX ↓ (G/U)XX. This suggests that RelE somehow recognizes bases in the next codon. This observation can be reconciled by features derived from structural data of the pre- and post-cleavage states of the RelE 70 S ribosome complex (7). First, the electron density of two nucleotides downstream of the A site was visible. Second, in the structure of the RelE-bound ribosome, the mRNA path was longer than with ribosomes alone, so it was projected to require threading of one or more additional nucleotides in the 30 S ribosomal subunit entry channel. Finally, the basic side chains of RelE were proposed to pull mRNA into the active site (7). Although further experimentation will be required to address this possibility, recognition of a downstream G/U is consistent with the known structure of the RelE-ribosome complex.

**DISCUSSION**

Our study complements, extends, and clarifies earlier, mostly in vitro, approaches that reported RelE properties and cleavage preferences. In contrast to the existing models for RelE function, we did not observe codon-dependent cleavage by RelE in vivo. As a consequence, all five transcripts we studied were cleaved at numerous codons with high frequency shortly after elongation commenced. This observation precludes a model favoring RelE cleavage at stop codons in vivo. If the majority of the transcripts were already cut at their 5′ ends, the percentage of RelE toxin associated with the translating ribosome to the end of the transcript is predicted to be negligible.

The rapid translational shutdown mediated by RelE appears to result from its ability to frequently and efficiently cleave mRNAs from their 5′ ends. In fact, upon analysis of RelE cut sites across the >1000-nt *ompA* and *ompF* transcripts, we observed a transition from major to only rare sites after ~70–100 codons. The exact position of the transition from major to rare cleavage sites appears to be transcript-dependent and does not occur at all in the short *lpp* transcript. It remains unclear how RelE preferentially exerts its effects from the 5′ end of the coding region. Because we did not observe robust cleavage by RelE across the length of the mRNA as seen for HigB (3), RelE appears to specifically recognize a conformation or component of the translation complex that is unique to initiation or early elongation. Further structural studies of more complex versions of the translation machinery should shed more light on

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**FIGURE 4. Mutations in the *ompA* 5′ end alter RelE cleavage.** A, primer extension analysis of wild type (WT) *ompA*, an *ompA* transcript in which a strong cut site was added (+ site), and an *ompA* transcript in which strong cut sites were removed (− sites) using a primer that annealed ~90 bases from the start site. Mutated bases are highlighted in black. RNA was extracted after induction for the time (min) indicated. Note that there are slight changes in the cleavage pattern in the primer extensions of the chromosomally versus plasmid-expressed *ompA*. B, to clearly highlight changes in cleavage that were observed at the mutated sites in *ompA*, only the 15 min post-induction time points were aligned next to the WT control. Crossed out scissors indicate positions where sites were no longer visible. C, in summary, cleavage sites observed by primer extension are shown in the context of the sequence of each transcript. Arrows indicate cleavage positions; as in A, mutated bases are highlighted in black.
the mechanistically intriguing properties of RelE-mediated translation arrest.

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