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MazF Cleaves Cellular mRNAs Specifically at ACA to Block Protein Synthesis in *Escherichia coli*

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Summary

***Escherichia coli* contains operons called "addiction modules," encoding toxin and antitoxin, which are responsible for growth arrest and cell death. Here, we demonstrate that MazF toxin encoded by "*mazEF* addiction module" is a sequence-specific (ACA) endoribonuclease functional only for single-stranded RNA. MazF works as a ribonuclease independent of ribosomes, and is, therefore, functionally distinct from RelE, another *E. coli* toxin, which assists mRNA cleavage at the A site on ribosomes. Upon induction, MazF cleaves whole cellular mRNAs to efficiently block protein synthesis. Purified MazF inhibited protein synthesis in both prokaryotic and eukaryotic cell-free systems. This inhibition was released by MazE, the labile antitoxin against MazF. Thus, MazF functions as a toxic endoribonuclease to interfere with the function of cellular mRNAs by cleaving them at specific sequences leading to rapid cell growth arrest and cell death. The role of such endoribonucleases may have broad implication in cell physiology under various growth conditions.**

Introduction

In *Escherichia coli*, programmed cell death is mediated through "addiction modules" consisting of two genes, one for a stable toxic protein and the other for a short-lived antitoxin (Engelberg-Kulka and Glaser, 1999). Such genetic systems for bacterial programmed cell death have been reported in a number of *E. coli* extrachromosomal elements for the so-called postsegregational killing effect (Bravo et al., 1987; Jaffe et al., 1985). When bacteria lose the plasmids, these cells are selectively killed because unstable antitoxins are degraded faster than their cognate stable toxins. Thus, the cells are addicted to the short-lived antitoxins since their de novo synthesis is essential for cell survival. The *mazEF* addiction module, the first known prokaryotic chromosomal addiction module (Aizenman et al., 1996), consists of two overlapping genes *mazE* and *mazF*, located downstream of the *relA* gene. MazF is a stable toxin while MazE is a labile antitoxin, which is readily degraded in vivo by an ATP-dependent ClpPA serine protease (Aizenman et al., 1996). The *mazEF* expression is negatively regulated by guanosine 3′, 5′-bispyrophosphate (ppGpp) synthesized by *RelA* under severe amino acid starvation (Aizenman et al., 1996). Moreover, *mazEF*-mediated cell death can be triggered by several antibiotics (rifampicin, chloramphenicol, and spectinomycin) (Sat et al., 2001). From in vivo experiments with *E. coli* cells, it has been suggested that MazF inhibits both protein synthesis and DNA replication (Pedersen et al., 2002). Recently, thymineless death has been reported to be mediated by the *mazEF* module (Sat et al., 2003). Among known *E. coli* addiction modules (Engelberg-Kulka and Glaser, 1999), CcdB, the toxin in the *ccdAB* system, inhibits gyrase A to block DNA synthesis (Bahassi et al., 1999; Bernard et al., 1993; Maki et al., 1992), while RelE in the *relBE* system functions as a global translation inhibitor (Christensen et al., 2001). RelE cleaves mRNA with high codon specificity that is positioned in the ribosome A site, although RelE by itself is not an endoribonuclease and unable to cleave free mRNA (Christensen and Gerdes, 2003; Pedersen et al., 2003). In contrast, little is known about the cellular target of MazF and its underlying toxic mechanisms. Here we used *E. coli* cells permeabilized by toluene treatment to demonstrate that MazF inhibits translation, but not RNA synthesis or DNA replication. We found that MazF is an endoribonuclease, which preferentically cleaves mRNA between A and C residues at the ACA sequence in a manner independent of ribosomes. Recently an X-ray structure of the MazE-MazF complex has been determined (Kamada et al., 2003; de la Cueva-Mendez, 2003), which suggests a possible mechanism by which MazE inhibits MazF function as a sequence-specific endoribonuclease.

Results

MazF is a Protein Synthesis Inhibitor

The *mazF* gene was cloned into an arabinose inducible pBAD plasmid (Guzman et al., 1995). BW25113 (Datsenko and Wanner, 2000) carrying pBAD-MazF could not grow on a glycerol-M9 plate in the presence of arabinose (0.2%) (data not shown). In liquid medium, colony forming units were reduced by 10^4 after the addition of arabinose for a period of 5 min.

To identify the cellular function inhibited by MazF, a cell-free system prepared from *E. coli* BW25113 cells carrying pBAD-MazF permeabilized by toluene treatment was used (Halegoua et al., 1976a, 1976b). As permeabilized cells are unable to synthesize ATP, the addition of ATP is absolutely required for biosynthesis of DNA, RNA, and proteins in the toluene-treated cells. ATP-dependent ^{35}S-methionine incorporation was completely inhibited when cells were preincubated for 10 min

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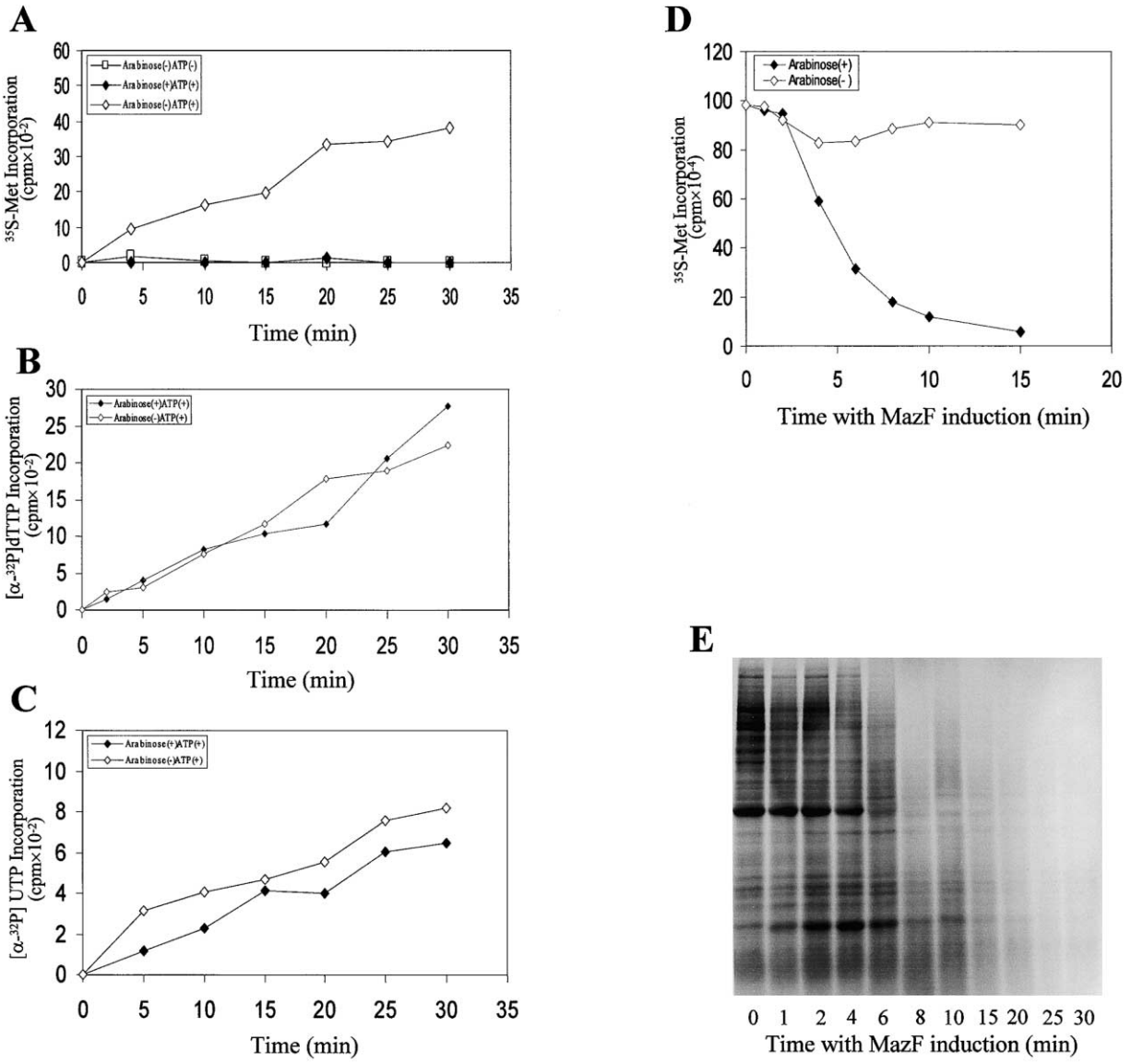

Figure 1. Effect of MazF on Protein, DNA, and RNA Synthesis

(A) Effect of MazF on ^{35}S-methionine incorporation in toluene-treated cells. *E. coli* BW25113 cells containing pBAD-MazF were grown at 37°C in glycerol-M9 medium. When the OD_{600} of the culture reached 0.6, arabinose was added to a final concentration of 0.2% for induction. After incubation at 37°C for 10 min, the cells were treated with toluene (Halegoua et al., 1976a, 1976b). Using toluene-treated cells, protein synthesis was carried out with ^{35}S-methionine as described previously (Halegoua et al., 1976b).

(B) Effect of MazF on [α-^{32}P]dTTP incorporation in toluene-treated cells (Moses and Richardson, 1970).

(C) Effect of MazF on [α-^{32}P]UTP incorporation in toluene-treated cells (Peterson et al., 1971).

(D) Effect of MazF on ^{35}S-methionine incorporation in vivo. ^{35}S-methionine incorporation into *E. coli* BW25113 cells containing pBAD-MazF was measured at various time points as indicated after MazF induction. (E) SDS-PAGE analysis of in vivo protein synthesis after the induction of MazF. The same cultures in (D) were used.

in the presence of arabinose before toluene treatment (Figure 1A). However, the incorporation of [α-^{32}P]dTTP (Figure 1B) and [α-^{32}P]UTP (Figure 1C) was not significantly affected under similar conditions (Moses and Richardson, 1970; Peterson et al., 1971). These results demonstrate that MazF inhibits protein synthesis, but not DNA replication or RNA synthesis. The in vivo incorporation of ^{35}S-methionine was dramatically inhibited after the addition of arabinose using cells not treated with toluene (Figure 1D). SDS-PAGE analysis of total cellular protein synthesis at different time points after arabinose addition (Figure 1E) showed that MazF is a general inhibitor for the synthesis of all cellular proteins. Interestingly, the synthesis of larger proteins was more susceptible to MazF toxicity than that of smaller proteins.

When the polysome pattern of *E. coli* BW25113 cells carrying pBAD-MazF plasmid was analyzed by sucrose density gradient at 10 min after arabinose induction, polysomes completely disappeared with a concomitant

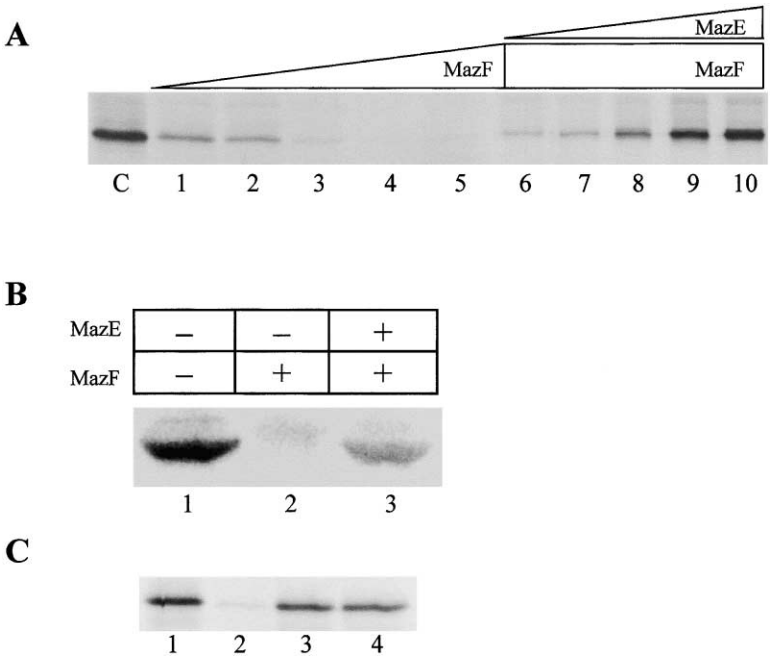

Figure 2. Effect of Purified MazF(His)$_6$ on Cell-Free Protein Synthesis

(A)Effect of MazF(His)$_6$ on protein synthesis in a prokaryotic cell-free system. MazG protein synthesis was performed in the *E. coli* T7 S30 extract system (Promega) with pET-11a-MazG as template. Lane C, without MazF(His)$_6$; lanes 1 to 5: 77, 154, 231, 308, and 384 nM MazF(His)$_6$ were added, respectively; lanes 6 to 10: 384 nM MazF(His)$_6$ and the ratios of (His)$_6$MazE to MazF(His)$_6$ were 0.1, 0.2, 0.4, 0.8, and 1.2, respectively.

(B) Effect of MazF(His)$_6$ on protein synthesis in a eukaryotic cell-free system. A human protein (WP) synthesis was performed in the rabbit reticulocyte lysates system TNT T7 Quick for PCR DNA (Promega). The DNA fragment encoding the human protein, termed WP of unknown function, under a T7 promoter, was used as template. Lane 1, without (His)$_6$MazE and MazF(His)$_6$; lane 2, with 0.66 μM MazF(His)$_6$ and lane 3, with 0.9 μM (His)$_6$MazE and 0.66 μM MazF(His)$_6$, the ratio of (His)$_6$-MazE to MazF(His)$_6$ was 1.4:1.

(C) Effect of preincubation of the *E. coli* cell-free system with MazF on protein synthesis. The cell-free system was preincubated with or without MazF for 15 min at 37°C before the addition of pET-11a-MazG. Lane 1, preincubated without MazF(His)$_6$; lane 2, preincubated with 231 nM MazF(His)$_6$; lane 3, preincubated with 231 nM MazF(His)$_6$ followed by adding 231 nM (His)$_6$MazE together with pET-11a-MazG; and lane 4, preincubated with 231 nM MazF(His)$_6$ and 231 nM (His)$_6$MazE. Then protein synthesis was carried out in the presence of ^{35}S-methionine for 1 hr at 37°C. Reaction products were analyzed by SDS-PAGE followed by autoradiography.

increase of the 70S ribosomal fraction, while no significant changes in 30S and 50S ribosomal fractions were observed (data not shown). These findings suggest that MazF disrupts polysomes either by inhibiting translation initiation or by degrading mRNAs.

Inhibitory Effect of Purified MazF on Cell-Free Protein Synthesis

Subsequently, we examined the effect of purified MazF(His)$_6$ on the synthesis of a candidate protein, MazG, in an *E. coli* cell-free RNA/protein synthesis system. MazF(His)$_6$ was purified from cells coexpressing both MazE and MazF(His)$_6$ as described in Experimental Procedures. The synthesis of MazG (30 kDa) (Zhang and Inouye, 2002) from plasmid pET-11a-MazG was carried out at 37°C for 1 hr using *E. coli* T7 S30 extract system (Promega) in the absence and the presence of increasing concentrations of MazF(His)$_6$ (Figure 2A). MazG synthesis was almost completely blocked at MazF(His)$_6$ concentrations above 231 nM. We next tested the effect of MazE antitoxin on the MazF-mediated inhibition of MazG synthesis. Interestingly, the addition of the antitoxin (His)$_6$MazE rescued MazG synthesis in a dose-dependent manner (Figure 2A). MazF(His)$_6$ was also able to inhibit eukaryotic cell-free protein synthesis (Figure 2B, lane 2), which was also recovered upon coaddition of (His)$_6$MazE (lane 3).

Next, we examined whether the inhibition of MazG synthesis by MazF(His)$_6$ was caused at the translation initiation step using the toeprinting (TP) analysis with 70S ribosomes and the *mazG* mRNA (Moll and Blasi, 2002). Toeprinting of the *mazG* mRNA alone yielded the full-length band (FL) and band TP(s) presumably due to a secondary structure close to the 5′ end of the *mazG* mRNA (Figure 3A, lane 1). In the presence of 70S ribosomes, the toeprinting band [TP(r)] downstream of the initiation codon was detected (lane 2). When increasing amount of MazF(His)$_6$ were added together with 70S ribosomes, a new band TP(F) appeared with concomitant reduction of the TP(r) band (lanes 3–7). This new band, TP(F), corresponds to the region between the Shine-Dalgarno (SD) sequence and the initiation codon as judged from the sequence ladder at the right-hand side. The TP(r) band almost completely disappeared at 260 nM MazF(His)$_6$ (lane 7).

To our surprise, the TP(F) band was detected even in the absence of 70S ribosomes (Figure 3B, lane 2), indicating that MazF is able to bind to the mRNA independent of 70S ribosomes or alternatively that MazF is an endoribonuclease specifically cleaving between A and C residues, which locate between the SD sequence and the initiation codon (Figure 3A). We first examined whether MazF(His)$_6$ and 70S ribosomes compete with each other for the binding to the *mazG* mRNA. When only 70S ribosomes were added, only the TP(r) band was detected as expected (lane 3). When 70S ribosomes were preincubated with the *mazG* mRNA before the addition of MazF(His)$_6$ (lane 4) or when 70S ribosomes were added together with MazF(His)$_6$ (lane 5) before the addition of *mazG* mRNA, both the TP(r) and TP(F) bands appeared. However, when the *mazG* mRNA was preincubated with MazF(His)$_6$ in the absence of 70S ribosomes, the competition between MazF(His)$_6$ and 70S ribosomes was no longer observed (lane 6). This result supports the notion that MazF is an endoribonuclease cleaving the *mazG* mRNA rather than simply binding to the site between the SD sequence and the initiation

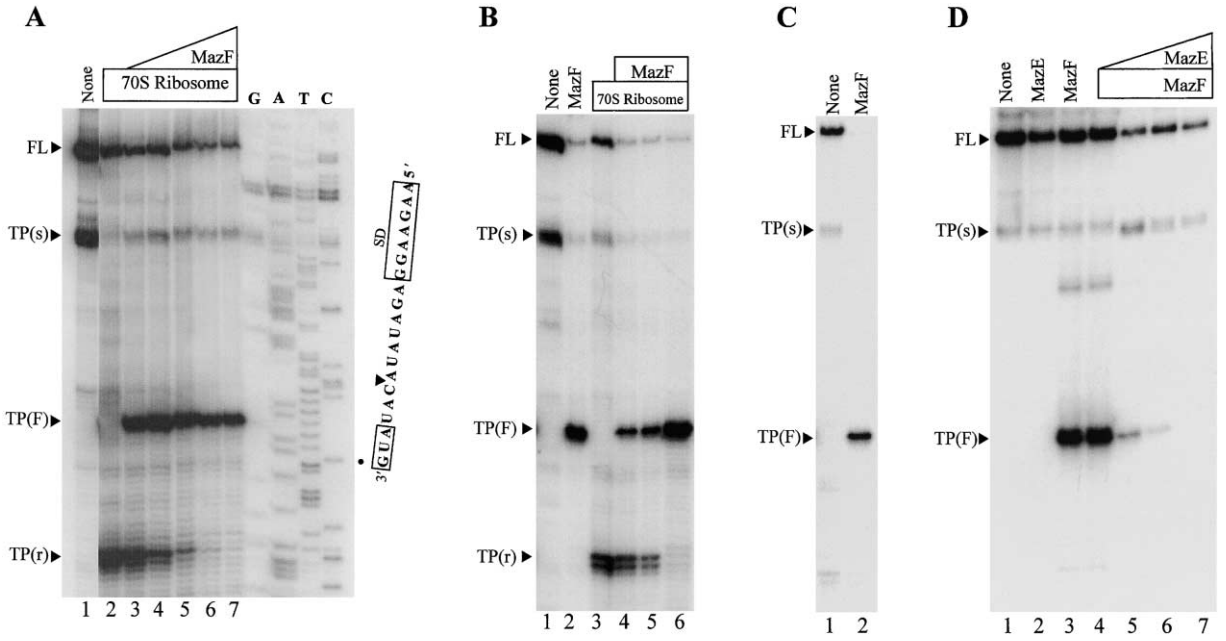

Figure 3. Toeprinting Experiment with the *mazG* mRNA and MazF(His)$_6$

(A) Toeprinting of the *mazG* mRNA was performed in the presence of MazF. mRNA was synthesized in vitro from a 175 bp DNA fragment containing a T7 promoter using T7 RNA polymerase as described in Experimental Procedures. Note that this fragment contains only one ACA sequence. Toeprinting was carried out as described in Experimental Procedures. Lane 1, without MazF(His)$_6$ and 70S ribosomes; lane 2, with 0.5 μM 70S ribosomes and no MazF(His)$_6$ and lanes 3 to 7, with 0.5 μM 70S ribosomes and 35 nM, 70 nM, 140 nM, 210 nM, and 260 nM MazF(His)$_6$, respectively. The sequence ladder shown at the right-hand side was obtained using the same primer used for toeprinting with pET-11a-MazG as template. The mRNA sequence shown is complementary to the sequence ladder starting from the G residue (C in the sequence ladder) of the initiation codon of the *mazG* gene. The initiation codon, AUG, and the SD sequence are boxed. An arrow indicates the position [TP(F)] where toeprinting was stopped in the presence of MazF(His)$_6$. FL, the full-length of the *mazG* mRNA; TP(s), a paused site due to a presumed secondary structure; TP(F), the toeprint site due to MazF cleavage; and TP(r), the toeprint site due to ribosome binding to the *mazG* mRNA.

(B) Effect of 70S ribosomes on MazF cleavage of the *mazG* mRNA. The reaction was carried out as described in Experimental Procedures. Lane 1, without MazF(His)$_6$ and 70S ribosomes; lane 2, with 0.26 μM MazF(His)$_6$ but no 70S ribosomes were added; lane 3, with 0.5 μM 70S ribosomes but no MazF(His)$_6$ was added; lane 4, the *mazG* mRNA and 70S ribosomes were incubated at 37°C for 10 min and 0.22 μM MazF(His)$_6$ was then added to the mixture for another 10 min at 37°C prior to primer extension; lane 5, 70S ribosomes and MazF(His)$_6$ were first mixed and incubated at 37°C for 10 min before the addition of the *mazG* mRNA and further 10 min incubation at 37°C following by primer extension; lane 6, after the *mazG* mRNA and MazF(His)$_6$ were mixed and incubated at 37°C for 10 min, 70S ribosomes were added to the mixture, and the final mixture was then incubated at 37°C for another 10 min before primer extension.

(C) Toeprinting of the *mazG* mRNA after phenol extraction. The experiment was carried out in the same way as described in lane 1 and lane 2 of (B) except that reaction products were phenol extracted to remove proteins before primer extension. Lane 1, without MazF(His)$_6$ and lane 2, with 0.26 μM MazF(His)$_6$.

(D) Effect of MazE on MazF cleavage of the *mazG* mRNA. Lane 1, without MazF(His)$_6$ and (His)$_6$MazE; lane 2, with 0.88 μM (His)$_6$MazE; lane 3, 0.22 μM MazF(His)$_6$ and lanes 4 to 7, with 0.22 μM MazF(His)$_6$ and the ratios of (His)$_6$MazE to MazF(His)$_6$ were 0.25, 0.4, 0.8, and 1.0, respectively.

codon. The observed apparent competition between 70S ribosomes and MazF(His)$_6$ for toeprinting (lanes 4 and 5) may be because the SD sequence and the MazF(His)$_6$ recognition site in the *mazG* mRNA are closely located.

Therefore, in order to further determine whether MazF(His)$_6$ indeed cleaves the *mazG* mRNA, the *mazG* mRNA was incubated with MazF(His)$_6$, then phenol-extracted to remove protein, and then used for primer extension as shown in Figure 3C. The TP(F) band was observed even after phenol extraction (lane 2), indicating that MazF(His)$_6$ indeed directly cleaved the *mazG* mRNA. The cleavage of the *mazG* mRNA was again blocked when (His)$_6$MazE was added together with MazF(His)$_6$ (Figure 3D, lanes 4–7). Note that (His)$_6$MazE by itself had no detectable effect on the mRNA (lane 2). This result indicates that the MazE exerts its antitoxic effect by blocking the endoribonuclease activity of MazF. It is important to note that the present result that MazF is able to cleave mRNA in the absence of ribosomes is distinctly different from the function of RelE whose toxic function requires ribosomes (Christensen and Gerdes, 2003; Pedersen et al., 2003).

Next, we examined if MazF affects ribosome and tRNA functions in the *E. coli* cell-free system. The cell-free system was preincubated without and with MazF for 15 min at 37°C before the addition of pET-11a-MazG (Figure 2C, lane 1 and lane 2, respectively). Comparing the control experiment (lane 1), almost no MazG was produced (lane 2) if MazE was not added together with pET-11a-MazG. However, when MazE was added together with pET-11a-MazG after the preincubation with MazF (lane 3), the level of MazG production was almost identical to that of the control (lane 1) and the experiment in which MazE was added together with MazF during 15 min preincubation (lane 4). These results indicate that ribosomes and tRNA are not susceptible to the MazF endori-

bonuclease activity, and thus we conclude that the primary target of MazF is mRNA.

Sequence Specificity of MazF Endoribonuclease

In order to determine the specificity of the MazF cleavage reaction, we first mutated the *mazG* mRNA SD sequence GGAG to AAUG, and also the initiation codon AUG to AGG or GUG. None of these mutations affected *mazG* mRNA cleavage by MazF(His)$_6$ (data not shown). These results indicate that MazF endoribonuclease requires neither the SD sequence nor the initiation codon for its function. Note that when the SD sequence was mutated, the ribosomes were bound only poorly to the mutated mRNA in contrast to the wild-type mRNA. Interestingly, the AUG to AGG mutation severely affected the TP(r) band formation while the AUG to GUG mutation had little effect on the TP(r) band formation (data not shown). This is consistent with the fact that GUG is known to function as an initiation codon (Reddy et al., 1985).

In order to determine the recognition sequence for MazF, all possible single-base mutations in the UACAU sequence around the MazF cleavage site in Figure 3A were constructed. It was found that both U residues at the 5′ and 3′ ends could be replaced with any other residues (G, A and C), while any point mutations in the ACA sequence (*G*CA, *C*CA, *U*CA, A*G*A, A*U*A, A*A*A, AC*G*, AC*C* and AC*U*) resulted in no cleavage of the mRNA (data not shown), demonstrating that the ACA sequence is absolutely required for MazF function.

To further characterize the specificity of the MazF endoribonuclease activity, two other mRNAs were also examined. Figure 4 shows the MazF cleavage sites in the *lacZ* mRNA (Figure 4A) and the *yeeW* mRNA (Figure 4B). We also analyzed the cleavage of the *mazG* mRNA at two other sites (Figures 4C and 4D) in addition to the site shown in Figure 3A. The 17 base sequences around the cleavage sites in the *mazG* mRNA (Figures 3A, 4C, and 4D) the *lacZ* mRNA (Figure 4A) and the *yeeW* mRNA (Figure 4B) are listed in Table 1. By comparing these sequences, it is evident that an ACA sequence was identified at all of the cleavage sites, and that mRNA is cleaved between the first A and the second C residues in the ACA sequence except for the *lacZ* mRNA (Figure 4A), suggesting that MazF is an endoribonuclease that specifically recognizes the ACA sequence and preferentially cleaves at the 3′ end of the first A residue in ACA. In the case of the *lac*Z mRNA shown in Figure 4A, the cleavage occurred at the 5′ end of the first A residue in an ACA sequence. The reason for this alternative cleavage site is not well understood at present. Further experiments are needed to determine the exact factors that may alter the cleavage site.

Northern Blot Analysis of Cellular mRNAs

On the basis of the above observations, we carried out Northern blot analysis of total cellular RNA extracted at different time intervals after arabinose induction of MazF. Among three genes tested, the *ompA* mRNA (Movva et al., 1980) was observed only at 0 time point, and the *secE* mRNA was detected only up to 2.5 min (Figure 5A). On the other hand, the *lpp* mRNA (Nakamura and Inouye, 1979) was found to be relatively stable. Its band intensity was significantly reduced at 10 min but still detectable at 20 min after MazF induction (Figure 5A). It seems that the observed differences in the stabilities of these mRNAs correlated at least in part with the total number of the ACA sequences present in the mRNA and to the mRNA length. The earlier observation that the synthesis of larger proteins was more sensitive to MazF toxicity (Figure 1E) is probably due to length-dependent susceptibility of their mRNAs to MazF. The *lpp* mRNA is among the shortest mRNAs in *E. coli* consisting of 322 bases with 4 ACA (Nakamura and Inouye, 1979), while the *ompA* mRNA consists of 1229 bases with 24 ACA (Movva et al., 1980). In contrast to the *lpp* and *ompA* genes, the *secE* gene is a member of the essential *secE-nusG* operon and cotranscribed with the *nusG* gene (Downing et al., 1990). The *secE* plus *nusG* ORFs (927 bp) contain a total of 7 ACA sequences. It is important to note that 16S and 23S rRNA were very stable in vivo during the 20 min period of MazF induction, as no significant changes in their band intensities in 1% agarose gel electrophoresis were observed (data not shown), indicating that rRNAs were protected from MazF cleavage in vivo. This is probably due to the protection of rRNA by ribosomal proteins preventing rRNA exposure to MazF cleavage.

In Vivo Cleavage of the *mazG* mRNA by MazF

Next, in order to examine if in vivo degradation of cellular mRNA after MazF induction was indeed caused by cleavage at ACA sequences, a primer extension analysis of the *mazG* mRNA was carried out with the total RNA extracted at different time points after MazF induction as described in Experimental Procedures. The same ACA sequence of the *mazG* mRNA as shown in Figure 4D was tested for the in vivo cleavage. As shown in Figure 5B, a distinct band appeared at 5 min after MazF induction (lane 3), whose intensity further increased at 10 and 20 min (lanes 4 and 5, respectively). This band was hardly detected at 0 and 2.5 min (lanes 1 and 2, respectively). Importantly, the cleavage occurred between the first A and second C residues in the ACA sequence as in the in vitro experiment (Figure 4D), indicating that MazF cleaves the same site both in vivo and in vitro. It should be noted that this ACA sequence is formed between two adjacent codons, AAC for Asn107 and AGU for Ser108, indicating that MazF is able to cleave mRNA at the ACA sequences in vivo regardless of the reading frame. In addition, the region upstream of the ACA site above was examined using a different primer, and two new ACA cleavage sites were found with hardly any other cleavages (data not shown); one was in the coding frame, while the other was out of frame.

Substrate Specificity of MazF Endorinuclease

We synthesized a 30 base RNA identical to the region around the MazF cleavage site in the *mazG* mRNA in Figure 3A with the ACA sequence in the center. The RNA was labeled at the 5′-end with [γ-^{32}P] ATP using T4 polynucleotide kinase and used as a substrate for MazF(His)$_6$. As shown in lane 2, Figures 6A and 6B, the RNA was completely cleaved producing a shorter distinct fragment. This product was confirmed to be 15

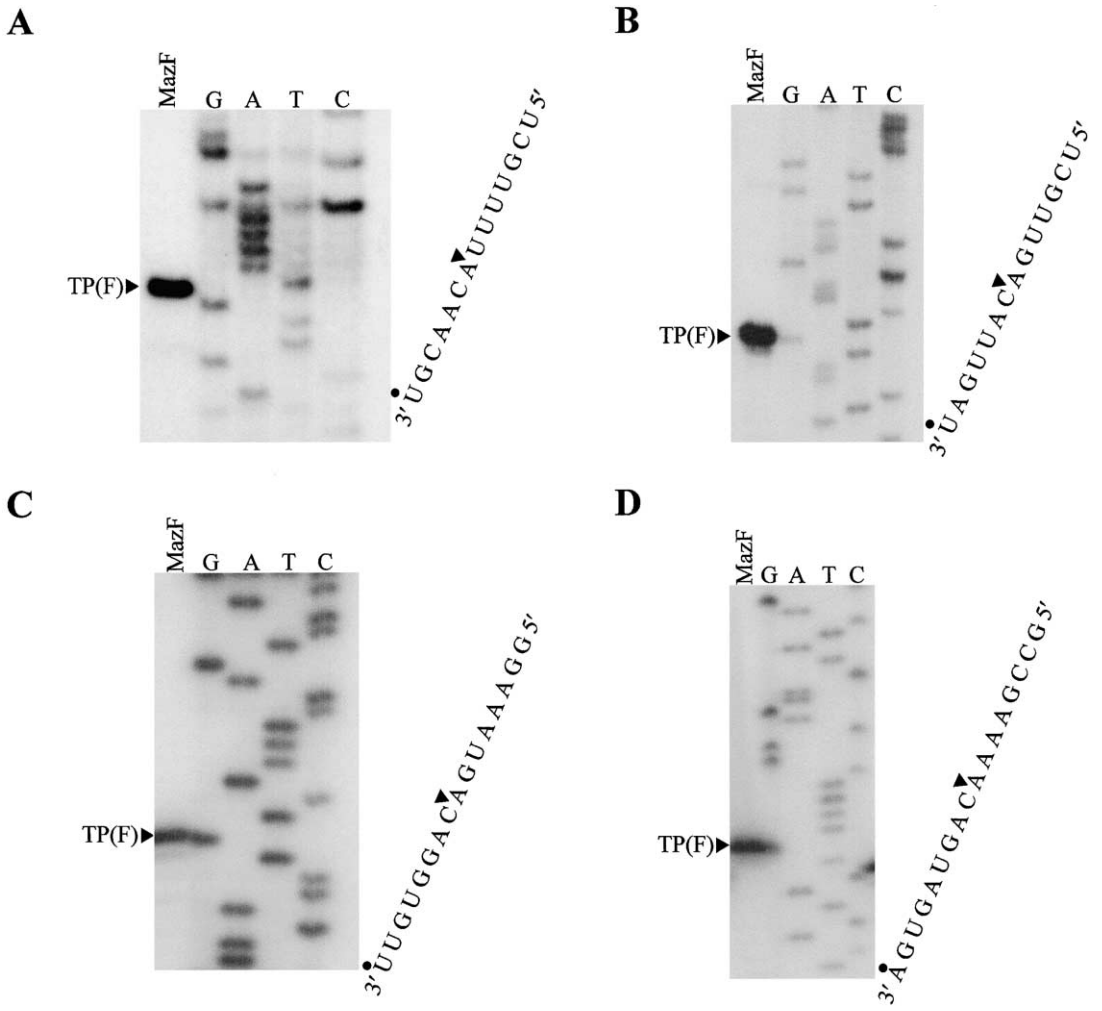

Figure 4. Primer Extension Analyses of MazF Cleavage Sites in the *lacZ* mRNA, the *yeeW* mRNA, and the *mazG* mRNA

(A) Primer extension analysis of MazF cleavage site in the *lacZ* mRNA. The experiment was carried out as described in the Experimental Procedures. The sequence ladder was obtained using pINZ (Etchegaray and Inouye, 1999) as a template and the same primer used for primer extension.

(B) Primer extension of MazF cleavage site in the *yeeW* mRNA. The sequence ladder was obtained with pET-17b-yeeW as template using the same primer as used for primer extension.

(C and D) Primer extension of MazF cleavage sites in the *mazG* mRNA. Two new MazF cleavage sites in the ORF of the *mazG* mRNA were determined in addition to the site in Figure 3A. For each site a 27 base primer was designed 9 and 11 bp downstream of the ACA sequence for (C) and (D), respectively. Each sequence ladder was obtained using the same primer as used for primer extension. The complementary RNA sequence to the DNA sequence ladder around each MazF cleavage site is shown at the right-hand side, and the cleavage site is shown by an arrow.

bases in length using a synthetic a 15 base RNA as standard (data not shown). When the RNA was annealed with an antisense DNA before the addition of MazF(His)$_6$, the RNA cleavage was inhibited (Figure 6A, lanes 3–7). A similar result was obtained when the substrate RNA was hybridized with an antisense RNA (Figure 6B). These

Table 1. mRNA Sequences around the MazF Cleavage Sites

Gene Name																			
yeeW	G	U	C	G	U	U	G		A	*	C	A	U	U	G	A	U	G	G
lacZ	U	C	G	U	U	U	U	*	A		C	A	A	C	G	U	C	G	U
mazG	G	A	G	A	U	A	U		A	*	C	A	U	A	U	G	A	A	U
	G	C	C	G	A	A	A		A	*	C	A	G	U	A	G	U	G	A
	G	G	A	A	A	U	G		A	*	C	A	G	G	U	G	U	U	G

The mRNA sequences around the MazF cleavage sites (indicated by an asterisk) in the *mazG* mRNA (from Figures 3A, 4C, and 4D), the *lacZ* mRNA (Figure 4A), and the *yeeW* mRNA (Figure 4B) are shown. The conserved ACA sequences are underlined.

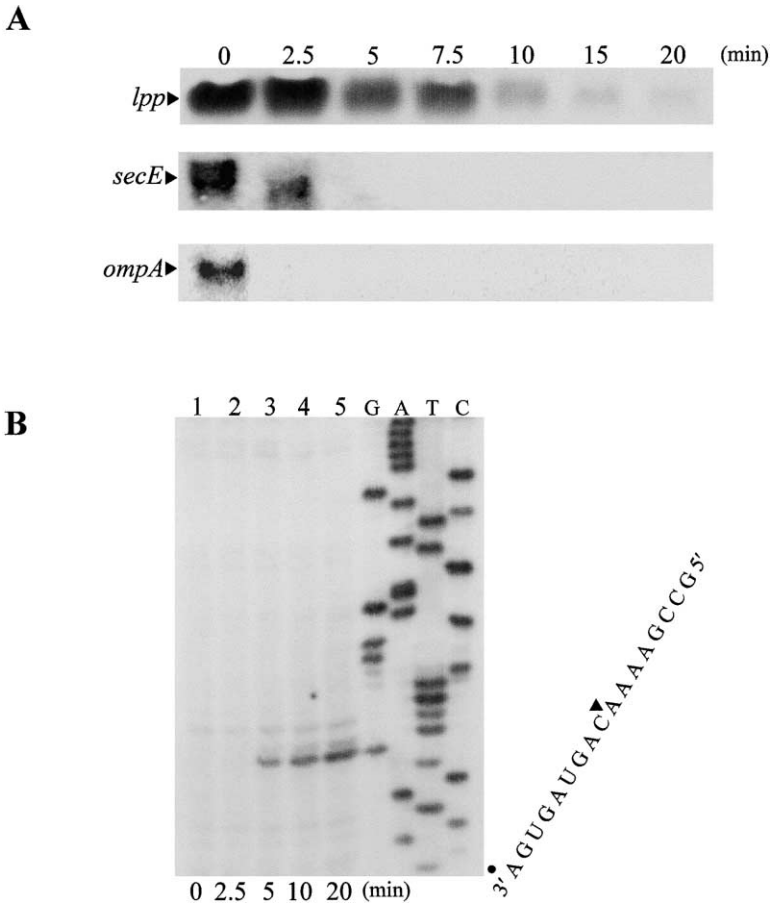

Figure 5. Effect of MazF on Cellular mRNAs In Vivo

(A) Total cellular RNA was extracted from *E. coli* BW25113 cells containing pBAD-MazF at various time points as indicated after the addition of arabinose and subjected to Northern blot analysis using radiolabeled *lpp*, *secE*, and *ompA* ORF DNA as probes.

(B) Primer extension analysis of a MazF cleavage site in the *mazG* mRNA in vivo. The *mazG* mRNA was produced from pIN-MazG in the presence of 1 mM IPTG as described in Experimental Procedures. The total RNA was extracted at each time point indicated (lanes 1–5), and primer extension experiment was carried out with the same primer as used in Figure 4D. The complementary RNA sequence to the DNA sequence ladder around the MazF cleavage site is shown at the right-hand side, and the cleavage site is shown by an arrow.

results indicate that MazF cannot cleave the ACA sequence in RNA/DNA or RNA/RNA duplex. We also found that MazF cannot cleave single-stranded DNA having the same base sequence as the RNA substrate (data not shown). Thus we conclude that MazF is an endoribonuclease highly specific to single-stranded RNA.

Discussion

Sequence-Specific Endoribonuclease

In the present paper, we demonstrate that MazF is an endoribonuclease interfering mRNA function by specifically cleaving cellular mRNA at the ACA triplet sequence, independent of ribosomes, which elucidates the mechanism of the MazF toxicity in the *mazEF* addiction module. We demonstrate that the ACA sequence in the RNA/DNA or RNA/RNA duplex cannot be cleaved, indicating that the ACA sequences within double-stranded duplex structures cannot be digested by MazF as several other ribonucleases such as A and T1. Interestingly, both 16S and 23S rRNA in the cell were protected from MazF cleavage even if they contained a large number of the ACA sequences, but both rRNAs become degraded in vitro by MazF once they were treated by phenol extraction (data not shown). Therefore the observed resistance of rRNA in vivo against MazF cleavage appears to be due to the protection by ribosomal proteins. It is important to note that the preincubation of the *E. coli* cell-free system with MazF before adding MazE and plasmid DNA did not significantly affect the capacity of the protein synthesis of the cell-free system (Figure 2C). This further supports the notion that the primary cellular target of MazF is mRNA.

The mRNA stability against the MazF function is quite different depending upon mRNAs. The *lpp* mRNA was significant stable against the MazF induction (Figure 5A). We found that three out of four ACA sequences are located in the proposed secondary structures of the *lpp* mRNA (Nakamura and Inouye, 1979). The remaining ACA sequence is located close to the 5′-end of the *lpp* mRNA so that only a 4 base fragment is removed from the 5′-end. The mRNA resistance to MazF-mediated degradation may also be related to the efficiency of translation initiation, as an mRNA having a better translation initiation efficiency is likely to be more protected by ribosomes bound to the mRNA. The observed stability of the *lpp* mRNA against MazF degradation may also be due to its high translation efficiency, as the *lpp* product, lipoprotein, is the most abundant protein in *E. coli*.

Interaction between MazF and RNA

It is important to note that MazF is functionally distinct from RelE, which cannot act as an endoribonuclease by itself (Christensen and Gerdes, 2003; Pedersen et al., 2003). RelE assists mRNA cleavage in a codon-specific manner positioned at the ribosome A site. On the other hand, MazF is able to cleave mRNA independent of ribosomes in a more sequence-specific manner than

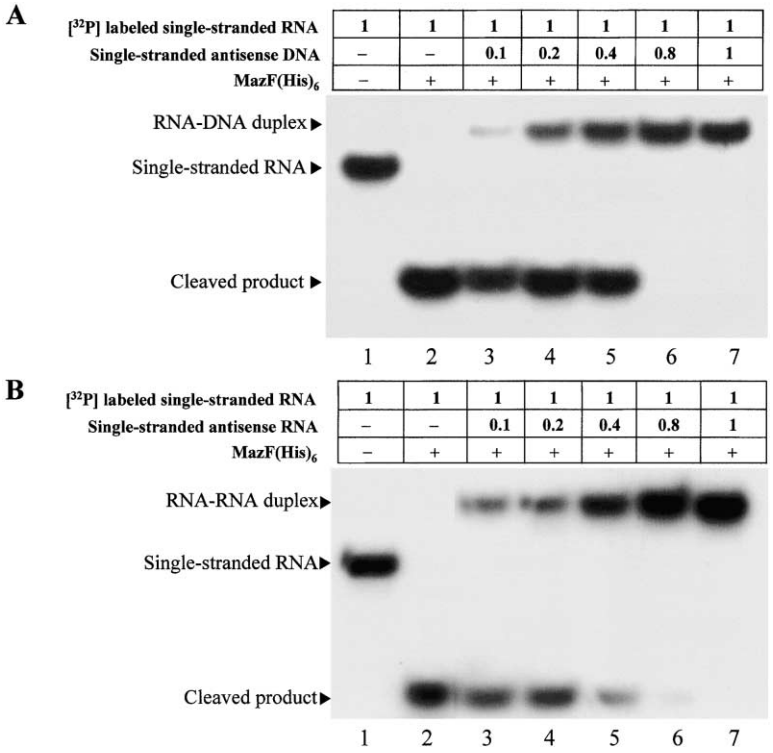

Figure 6. MazF Cannot Cleave at the ACA Sequence in RNA/DNA or RNA/RNA Duplex

A 30 base RNA identical to the region around the MazF cleavage site in the *mazG* mRNA in Figure 3A was synthesized with ACA in the center. It was labeled at 5′-end with T4 polynucleotide kinase using [γ-^{32}P]ATP. ^{32}P-labeled RNA (1 pmol) was annealed with various amounts of 30 base antisense DNA (A) or 30 base antisense RNA (B), and then incubated with 0.1 μg MazF(His)$_6$ in the reaction mixture (10 μl) at 37°C for 30 min. The reaction products were analyzed by 15% native PAGE followed by autoradiography.

RelE. Furthermore, MazF is able to cleave *mazG* mRNA at an ACA sequence overlapping two adjacent codons both in vitro and in vivo, demonstrating that mRNA cleavage by MazF is independent of the reading frame of an ORF (Figures 4D and 5B).

Recently the crystal structure of the MazF-MazE complex was determined (Kamada et al., 2003; de la Cueva-Mendez, 2003). In the crystal structure, MazE and MazF formed a 2:4 heterohexmer composed of alternating MazE and MazF homodimers ($MazF_2$-$MazE_2$-$MazF_2$). Interestingly, the C-terminal region of MazE is unstructured extending over the cleft formed between two MazF molecules in the MazF homodimer. It is interesting to note that the unstructured C-terminal region of MazE is highly negatively charged. It is tempting to speculate that this C-terminal MazE extension may mimic the single-stranded RNA structure to bind to a MazF dimer disrupting its RNA-substrate binding site to block MazF endoribunuclease function. Correspondingly, there are highly conserved basic residues in MazF homologs (Kamada et al., 2003). Indeed two mutant MazF proteins, R29S and R86G, lost the endoribonuclease activity (data not shown). We have recently demonstrated that MazF exists as a dimer in solution in the absence of MazE and that in the presence of MazE, it forms a stable 1:2 MazE-MazF complex with MazE (Zhang et al., 2003). Consistently, MazE effectively inhibited MazF endoribonuclease activity and released the MazF-mediated inhibition of the protein synthesis in cell-free systems (Figures 2 and 3D). The structural determination of a MazF-RNA complex is now crucial for our understanding of MazF function as an ACA-specific endoribonuclease.

Although computer analysis of the *E. coli* genome reveals that *E. coli* genes contain an average 11 ACA sequences per gene, it is intriguing to examine whether there are any *E. coli* mRNAs that are resistant to cleavage by MazF either by having no ACA sequences or by having uncleaved ACA sequences protected by secondary structures or other means. MazF itself contains surprisingly many ACA sequences, a total of 9 within the *mazF* ORF, among which 4 ACA sequences are clustered in the middle of the ORF. We found that the *mazF* mRNA was indeed cleaved by MazF in vitro (data not shown). This suggests that *mazF* expression may be negatively autoregulated by its own gene product.

mRNA Interference

In the present paper, we revealed a new mechanism for mRNA interference that involves a sequence-specific endoribonuclease, MazF. It is possible that there are a number of MazF-like endoribonucleases having different sequence specificities that remain to be identified. MazF amino acid sequences are highly conserved in a wide range of prokaryotic organisms. We speculate that MazF homologs from other bacteria may have different triplet sequence specificities. We recently found that PemK, a MazF homolog encoding by *E. coli* plasmid R100 is also an endoribonuclease, which cleaves mRNA with different sequence specificity (unpublished data). While the present paper was under review, a paper was published, which claims that MazF (ChpAK) inhibits translation by a mechanism very similar to that of *E. coli* RelE (Christensen et al., 2003). Our results from both in vitro and in vivo experiments do not support that conclusion as MazF cleaves mRNA totally independently of ribosomes. The reason for this discrepancy is not known at present.

There is an intriguing possibility that this new mecha-

nism of disrupting mRNA function by MazF-like endoribonucleases, as demonstrated for *E. coli* in the present study, may also pertain to eukaryotes. This would have numerous implications for the cellular physiology of many, if not all, living organisms. Furthermore, it is interesting to see if a highly sequence-specific MazF-like endoribonucleases may be used as therapeutic tools for human diseases.

Experimental Procedures

Strains and Plasmids

E. coli BL21(DE3), BW25113 (Δ*araBAD*) (Datsenko and Wanner, 2000) and MRE600 (Swaney et al., 1998) were used. Plasmid pET-21cc-MazEF was constructed from pET-21cc (Novagen) to coexpress MazE and MazF(His)$_6$ under a T7 promoter, using the Shine-Dalgarno (SD) sequence from the *mazEF* operon. Plasmid pET-28a-MazE was constructed from pET-28a (Novagen) to express (His)$_6$MazE. pBAD-MazF was constructed from pBAD (Guzman et al., 1995) to tightly regulate *mazF* expression by the addition of arabinose (0.2%).

Assay of Protein and DNA and RNA Synthesis in Toluene-Treated Cells

A 50-ml culture of *E. coli* BW25113 containing pBAD-MazF plasmid was grown at 37°C in glycerol-M9 medium. When the OD_{600} of the culture reached 0.6, arabinose was added to a final concentration of 0.2%. After incubation at 37°C for 10 min, the cells were treated with 1% toluene (Halegoua et al., 1976a, 1976b). Using toluene-treated cells, protein synthesis was carried out with ^{35}S-methionine as described previously (Halegoua et al., 1976a, 1976b). The toluene-treated cells were washed once with 0.05 M potassium phosphate buffer (pH 7.4) at room temperature, and then resuspended into the same buffer to examine DNA synthesis using [α-^{32}P]dTTP as described previously (Moses and Richardson, 1970). For assaying RNA synthesis, the toluene-treated cells were washed once with 0.05 M Tris-HCl buffer (pH 7.5) at room temperature, and then resuspended into the same buffer to measure [α-^{32}P]UTP incorporation into RNA as described previously (Peterson et al., 1971).

Assay of In Vivo Protein Synthesis

E. coli BW25113 cells containing pBAD-MazF were grown in glycerol-M9 medium without methionine. When the OD_{600} of the culture reached 0.6, the culture was divided into two equal parts. To one part, arabinose was added to a final concentration of 0.2%, and to the second part, water was added. At different time intervals as indicated in Figure 1D, 1 ml of the culture was taken into a test tube containing 10 μCi ^{35}S-methionine, and the mixture was incubated for 1 min at 37°C. 50 μl of the reaction mixture was then applied to a filter paper disk (Whatman 3 mm, 2.3 cm diameter). Filters were treated in 5% TCA solution as described previously (Hirashima and Inouye, 1973) and radioactivity was determined with a liquid scintillation counter. The other 500 μl of the reaction mixture was put into a chilled test tube containing 25 μl of 100% TCA solution and 100 μg/ml nonradioactive methionine. The mixture was then kept in an ice bath for 60 min. The pellets were collected by centrifugation and dissolved in 50 μl SDS-PAGE loading buffer by incubating the mixture in a boiling water bath for 30 min. After removing insoluble materials, the supernatant (10 μl) was analyzed by SDS-PAGE followed by autoradiography.

Purification of MazF(His)$_6$ and (His)$_6$MazE Proteins

MazF(His)$_6$ tagged at the C-terminal end was purified from strain BL21(DE3) carrying pET-21cc-MazEF. The complex of MazF(His)$_6$ and MazE was first trapped on Ni-NTA resin. After dissociating MazE from MazF(His)$_6$ in 5 M guanidine-HCl, MazF(His)$_6$ was retrapped by Ni-NTA resin and refolded by step-step dialysis. (His)$_6$MazE tagged at the N-terminal end was purified from strain BL21(DE3) carrying pET-28a-MazE with use of Ni-NTA resin.

Effect of MazF on Protein Synthesis in Prokaryotic and Eukaryotic Cell-Free Systems

Prokaryotic cell-free protein synthesis was carried out with *E. coli* T7 S30 extract system (Promega). The reaction mixture consisted of 10 μl of S30 premix, 7.5 μl of S30 extract, and 2.5 μl of an amino acid mixture (1 mM each of all amino acids but methionine), 1 μl (10 μCi) of ^{35}S-methionine, and different amounts of MazF(His)$_6$ and (His)$_6$MazE in a final volume of 24 μl. The reaction mixture was preincubated for 10 min at 37°C and then the assay was started by adding 1 μl of pET-11a-MazG plasmid-DNA (0.16 μg/μl) (Zhang and Inouye, 2002). The reaction was performed for 1 hr at 37°C, and proteins were then precipitated with acetone and analyzed by SDS-PAGE. Eukaryotic cell-free protein synthesis was carried out with rabbit reticulocyte lysates system TNT T7 Quick for PCR DNA (Promega). A DNA fragment encoding a human protein under a T7 promoter was used as template for mRNA transcription. The reaction was performed for 1 hr at 37°C, and proteins were then precipitated with acetone and analyzed by SDS-PAGE.

Preparation of *E. coli* 70S Ribosomes

70S ribosomes were prepared from *E. coli* MRE 600 as described previously (Aoki et al., 2002; Du and Babitzke, 1998; Hesterkamp et al., 1997). The final 70S ribosome fraction was suspended in buffer A [10 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 60 mM NH_4Cl, and 6 mM 2-mercaptoethanol] and stored at −80°C.

Primer Extension Inhibition (Toeprinting) Assays

Toeprinting was carried out as described previously (Moll and Blasi, 2002) with minor modifications. The mixture for primer-template annealing containing the *mazG* mRNA and ^{32}P-end-labeled DNA primer complementary to base 60 to 80 of the *mazG* mRNA was incubated at 65°C for 5 min, and then cooled slowly to room temperature. The ribosome binding mixture contained 2 μl of 10× buffer [100 mM Tris-HCl (pH 7.8) containing 100 mM $MgCl_2$, 600 mM NH_4Cl, and 10 mM DTT], different amounts of MazF(His)$_6$, 0.375 mM dNTP, 0.5 μM 70S ribosomal subunits, 2.5 μM tRNA^{fMet}, and 2 μl of the annealing mixture in a final volume of 20 μl. The final mRNA concentration was 0.05 μM. This ribosome binding mixture was incubated at 37°C for 10 min, and then reverse transcriptase (2 U) was added. cDNA synthesis was carried out at 37°C for 15 min. The reaction was stopped by adding 12 μl of the sequencing loading buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol EF). The sample was incubated at 90°C for 5 min prior to electrophoresis on a 6% polyacrylamide sequencing gel. The *mazG* mRNA was synthesized in vitro from a 175 bp DNA fragment containing a T7 promoter using T7 RNA polymerase. The DNA fragment containing a T7 promoter and a part of the *mazG* ORF was obtained by PCR amplification using pET-11a-MazG plasmid (Zhang and Inouye, 2002) as DNA template. The RNA transcript from this DNA fragment by T7 RNA polymerase consisted of 151 bases, of which the region from +1 to +71 was derived from pET-11a and the region from +72 to +151 was from the *mazG* ORF encoding the region from Met1 to Gln27. The *lacZ* and *yeeW* mRNAs were used for the toeprinting experiments without the addition of 70S ribosomes. The *lacZ* and *yeeW* mRNAs were synthesized in vitro by T7 RNA polymerase from the DNA fragments containing a T7 promoter sequence. The DNA fragments for the *lacZ* and *yeeW* mRNAs were amplified by PCR using appropriate primers using pINZ (Etchegaray and Inouye, 1999) and pET17b-YeeW (unpublished data) as templates, respectively. The 5′-end primers for both cases contained the T7 promoter sequence. The RNA thus produced by T7 RNA polymerase from the *lacZ* DNA fragment consisted of 254 bases, of which the 3′-end 72 bases were derived from the *lacZ* gene corresponding to the N-terminal 24 amino acid residues. The RNA from the *yeeW* DNA fragment consisted of 175 bases, of which the 3′-end 113 bases were from the *yeeW* gene.

Toeprinting of the *mazG* mRNA after Phenol Extraction

The experiment was carried out in the same way as described above except that 70S ribosomes and tRNA^{fMet} were omitted. The reaction mixtures were phenol-extracted to remove proteins before primer extension.

Construction of Mutant Plasmids

Site-directed mutagenesis was performed with pET-11a-MazG plasmid as DNA template. The mutations were confirmed by DNA sequence analysis.

Northern and Primer Extension Analyses

E. coli BW25113 containing pBAD-MazF was grown at 37°C in glycerol-M9 medium. When the OD_{600} value reached 0.8, arabinose was added to a final concentration of 0.2%. At different intervals as indicated in Figure 5A, total RNA was isolated using the hot-phenol method as described previously (Sarmientos et al., 1983). Northern blot analysis was carried out as described previously (Baker and Mackie, 2003). For primer extension analysis of mRNA cleavage in vivo, pIN-MazG plasmid was transformed into *E. coli* BW25113 cells containing pBAD-MazF. The *mazG* mRNA was induced by 1 mM IPTG for 30 min before MazF induction. Total RNA was isolated at different time intervals as indicated in Figure 5B. Primer extension was carried out using the same primer as in Figure 4D.

Cleavage of Synthesized RNA by MazF

The 30 base RNA 5′-UAAGAAGGAGAUAUACAUAUGAAUCAAAUC-3′, antisense RNA 5′-GAUUUGAUUCAUAUGUAUAUCUCCUUC UUA-3′, and the antisense DNA 5′-GATTTGATTCATATGTATATCT CCTTCTTA-3′ were commercially synthesized. The 30 base RNA was 5′-end labeled by T4 polynucleotide kinase using [γ-^{32}P]ATP. The concentration of the labeled 30 base RNA was 1 pmol/μl. The ^{32}P-labeled 30 base RNA was mixed with antisense DNA or antisense RNA in different ratios (see Figure 6). The mixtures were denatured at 70°C for 3 min, followed by annealing by gradual cooling to room temperature. The cleavage reaction mixture contained 1 μl 10× buffer (100 mM Tris-HCl [pH 7.8], 100 mM $MgCl_2$, 600 mM NH_4Cl, and 60 mM 2-mercaptoethanol), 0.1 μg MazF(His)$_6$, 2 μl ^{32}P-labeled RNA (1 pmol) or ^{32}P-labeled RNA/antisense DNA or ^{32}P-labeled RNA/antisense RNA mixtures. The final volume was adjusted to 10 μl by adding H_2O treated with DEPC. After incubating at 37°C for 30 min, the reaction was stopped by incubating the reaction mixture in a boiling-water bath for 2.5 min. The reaction mixtures were then subjected to 15% PAGE followed by autoradiography.

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