

Insights into the mRNA Cleavage Mechanism by MazF, an mRNA Interferase*

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MazF is an *Escherichia coli* toxin that is highly conserved among the prokaryotes and plays an important role in growth regulation. When MazF is induced, protein synthesis is effectively inhibited. However, the mechanism of MazF action has been controversial. Here we unequivocally demonstrate that MazF is an endoribonuclease that specifically cleaves mRNAs at ACA sequences. We then demonstrate its enzymatic specificity using short RNA substrates. MazF cleaves RNA at the 5'-end of ACA sequences, yielding a 2',3'-cyclic phosphate at one side and a free 5'-OH group at the other. Using DNA-RNA chimeric substrates containing XACA, the 2'-OH group of residue X was found absolutely essential for MazF cleavage, whereas all the other residues may be deoxyriboses. Therefore, MazF exhibits exquisite site specificity and has utility as an RNA-restriction enzyme for RNA structural studies or as an mRNA interferase to regulate cell growth in prokaryotic and eukaryotic cells.

It is intriguing that almost all bacteria contain a number of seemingly suicidal genes in their genomes or on plasmids (1–4). *Escherichia coli* possesses at least five such genes: *relE* (5), *mazF* (*chpAK*) (6), *chpBK* (7, 8), *yoeB* (9, 10), and *yafQ* (9). These toxin genes are co-expressed with their cognate antitoxin genes so that the devastating effects of these toxin genes are blocked under normal growth conditions. It has been debated whether expression of these toxins cause permanent damage to the cell that leads to programmed cell death (1, 11) or whether they cause a temporary arrest of cell growth under stress conditions (12).

MazF is the most extensively characterized bacterial toxin (6, 13). The x-ray structure of the MazE-MazF (antitoxin-toxin) complex has been determined (14). Both *in vivo* and *in vitro* experiments from our laboratory clearly demonstrate that MazF functions as a sequence-specific endoribonuclease that cleaves cellular mRNAs at ACA sequences (15). However, work by other researchers led them to conclude that 1) MazF functions as a factor that exerts its endoribonuclease (or ribosomal) activity only when it associates with the ribosome and 2) its mRNA cleavage activity occurs only at a unique site at specific codons in a manner similar to the RelE toxin (16). RelE has been implied as a factor that functions as a codon-dependent endoribonuclease only when it associates with ribosomes (17, 18) or as a factor that stimulates the endoribonuclease activity

of ribosomes (19). Other *E. coli* toxins, ChpBK (16) and YoeB (9), have also been proposed to function as factors that cleave cellular mRNAs in a ribosome- and codon-dependent manner like RelE. Another report challenges the published sequence specificity of MazF, instead concluding that MazF cleaves mRNAs at NAC (N is preferentially A and U) rather than at ACA (20).

Recently, PemK (Kid), a MazF homologue encoded by plasmid R100 (21) has been shown to also function as a sequence-specific endoribonuclease involved in mRNA degradation (22). In fact, the prokaryotic kingdom contains a large family of MazF/PemK relatives. We collectively refer to this family of bacterial toxins as “mRNA interferases.” Interestingly, their utility extends beyond bacterial systems, because they are able to function in yeast and higher eukaryotes (23).

In this study, we definitively demonstrate that MazF functions as an ACA-specific endoribonuclease that functions independent of ribosomes and RNA codon context. Using short synthetic RNA or RNA-DNA chimeric substrates that contain an XACA MazF cleavage site, we show that the 2'-OH group in the X residue is absolutely essential for MazF cleavage. For cleavage, the ACA sequence may consist of deoxyriboses suggesting that MazF may use an enzymatic mechanism for the cleavage of phosphodiester linkages, which is similar to ribonuclease A (RNase A), although there is no apparent structural similarity between two enzymes. We propose that mRNA interferases are novel RNA restriction enzymes that play an important role in bacterial physiology by regulating cell growth in response to stress.

EXPERIMENTAL PROCEDURES

Chemicals and Bacterial Strains—[γ -³²P]ATP (111 TBq/mmol, Amersham Biosciences) was used for 5'-labeling of RNAs with T4 polynucleotide kinase (Biolabs, New England), and [5'-³²P]cytidine-3',5'-bisphosphate (pCp)¹ (92.5 TBq/mmol, ICN) was used for 3'-labeling with T4 RNA ligase (Biolabs). RNase A, RNase T1, and RNase T2 were purchased from Sigma. pCR®2.1-TOPO® plasmid was purchased from Invitrogen. pIN-MazG and pBAD-MazF plasmids were constructed as described previously (15). RNA and RNA-DNA chimeras listed in Table I were synthesized by Takara Bio Inc.

Primer Extension Analysis in Vivo and in Vitro—For primer extension analysis of mRNA cleavage *in vivo*, pIN-Era plasmid was transformed into *E. coli* BW25113 cells containing pBAD-MazF. The *mazG* or *era* mRNA was induced by 1 mM isopropyl 1-thio- β -D-galactopyranoside for 1 h before MazF induction. Total RNA was extracted at different time intervals as indicated in Fig. 3B. Primer extension was carried out as described previously (15). For *in vitro* primer extension analysis, the full-length *mazG* and *era* mRNAs were synthesized *in vitro* by T7 RNA polymerase from the DNA fragment containing a T7 promoter sequence and *mazG* or *era* open reading frames using RiboMAX™ T7 large scale RNA production system (Promega). A 110-base 16 S rRNA fragment (+1213 to +1322) was synthesized *in vitro* by T7 RNA polymerase from

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¹ The abbreviations used are: pCp, [5'-³²P]cytidine-3',5'-bis(phosphate); MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight.

the DNA fragment containing a T7 promoter sequence and the 16 S rRNA fragment (+1213 to +1322) using RiboMAX™ T7 large-scale RNA production system. A 151-base *mazG* RNA fragment was synthesized *in vitro* by T7 RNA polymerase from the DNA fragment containing a T7 promoter sequence and the *mazG* DNA fragment using RiboMAX™ T7 large-scale RNA production system. The resulting RNA consisted of a 71-base sequence from pET-11a and the remaining 80-base sequence within the *mazG* open reading frame from Met-1 to Gln-27. MazF(His)₆ (0.02 µg) was incubated with 1 pmol of *mazG* or *era* mRNAs in 20 µl of reaction mixture containing 10 mM Tris-HCl (pH 7.8), 1.25 mM MgCl₂, 60 mM NH₄Cl, 0.5 µl of RNase inhibitor, 1 mM dithiothreitol, 0.5 mM dNTPs, and 0.05 µM ³²P-labeled primer at 37 °C for 10 min, then reverse transcriptase (2 U) was added, and cDNA synthesis was carried out at 37 °C for 15 min. The reaction was stopped by adding the sequence 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 primers G1 (GCCAGTTCACCGGAGGATCGTC) and G2 (TGCTCTTTATCCACGGGCAG) were used for primer extension analysis of the *mazG* RNA; the primers E1 (TCAACGACAAAATCACCAGCTCA), E2 (CACGTTGTCCACTTTGTTCCACCAC), E3 (ATGACCATCTTCTCTGCGCCTCA), and E4 (GCGTTCGTCGTCGGCCCAACCGGA) were used for primer extension analysis of the *era* RNA. The primer S1 (GAGTTGCAGACTCCAATCCGGACT) was used for primer extension analysis of the 16 S rRNA fragment. The primers were 5'-labeled with [^γ-³²P]ATP using T4 polynucleotide kinase.

Cleavage of Synthetic RNA by MazF—Oligoribonucleotides were 5'-labeled with [^γ-³²P]ATP using T4 polynucleotide kinase. The MazF endoribonuclease activity was assayed in 10 µl of reaction mixture containing 10 mM Tris-HCl (pH 7.8), 0.5 µl of ribonuclease inhibitor, 0.02 µg of MazF(His)₆, and ³²P-labeled oligonucleotides. Reaction mixtures were incubated at 37 °C for 15 or 30 min and stopped by adding 4 µl of RNA loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol EF, and 15% Ficoll) or the sequence loading buffer (95% formamide, 20 mM EDTA, 0.5% bromophenol blue, and 0.05% xylene cyanol EF). The reaction mixtures were then subjected to 15% PAGE or 20% sequence gel followed by autoradiography.

Analysis of Cleavage Products—4 pmol of RB-15-1 (see Table I) was incubated with 0.02 µg of MazF(His)₆ at 37 °C for 15 min. The cleavage products were labeled with [^γ-³²P]ATP using T4 polynucleotide kinase at 37 °C for 15 min. The 5'-end labeled 3'-end product from the MazF cleavage reaction was excised from a 20% sequencing polyacrylamide gel and further purified as described previously (24). The purified 5'-end labeled 3'-end product was then digested with RNase T2 in a 10-µl reaction mixture containing 50 mM ammonium acetate (pH 5.3) and 1.5 units of the enzyme for 6 h at 37 °C. Products of the digestion were analyzed by two-dimensional thin-layer chromatography on micro-crystalline cellulose plates (Macherey-Nagel) containing a fluorescent indicator. The first dimension was performed in isobutyric acid/saturated NH₄OH/H₂O (577:38:385), and the second dimension was performed in saturated (NH₄)₂SO₄, 1 M sodium acetate, isopropyl alcohol (80:18:2). The plate was washed once by methanol at room temperature for 15 min and dried before running the second dimension. 3'-monophosphate and pAp standards were visualized by ultraviolet light. Radiolabeled products and [³²P]pCp were visualized by autoradiography (25).

Sample Preparation for MALDI-Mass Spectrometry—The saturated 3-hydroxyisobutyric acid matrix solution was prepared by dissolving 5 mg of 3-hydroxyisobutyric acid (Sigma) in 100 µl of 50 mM diammonium citrate (Sigma) containing 25% acetonitrile. 1 µl of an RNA (4 µM) sample with or without MazF(His)₆ in 10 mM Tris-HCl (pH 7.8) was spotted onto a stainless steel sample plate, and the sample was dried at room temperature. The saturated matrix solution (0.5 µl) was then spotted on the dried sample. After the sample was completely dried, mass measurements were carried out using a Voyager DE PRO MALDI-TOF mass spectrometer (Applied Biosystems).

Purification of MazF(His)₆ 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 nickel-nitrilotriacetic acid resin (Qiagen). After dissociating MazE from MazF(His)₆ in 6 M guanidine-HCl, MazF(His)₆ was reassociated by nickel-nitrilotriacetic acid resin and refolded by step-step dialysis. In text, MazF(His)₆ is referred to as MazF.

Construction of Mutants Plasmids—Site-directed mutagenesis was performed with use of pET-11a-MazG, and all mutations were confirmed by DNA sequencing.

Preparation of RNA Ladder—Partial alkaline hydrolysis of 5'-end-

labeled RNA was performed in a 10-µl reaction mixture containing 100 pmol of RNA and 0.1 N NaOH at 75 °C for 2 min. The hydrolysis was stopped by adding 40 µl of sequence loading buffer (24).

RESULTS AND DISCUSSION

Enzymatic Specificity of MazF—Previously we showed that MazF was able to cleave a 30-base RNA having an ACA sequence in the center (15). We extended this initial observation by further shortening the RNA to 15 nucleotides with the ACA sequence in the center (Fig. 1A). On the basis of our previous findings (15), MazF was able to cleave this RNA (termed RB-15-1, Table I) at site 6, site 7, or at both sites.

To examine the cleavage sites, RB-15-1 RNA was first digested with MazF, and the reaction product was then 5'-end labeled and analyzed by gel electrophoresis. A major product (Fig. 1B, lane 2) appeared at the position of the 9-base product of the RNA ladder prepared by partial alkaline hydrolysis of the ³²P 5'-end-labeled RB-15-1 RNA (lane 1), suggesting that the product was a result of cleavage at site 6. An 8-base product was also detected, which corresponded to the cleavage product at site 7. Cleavage at both sites 6 and 7 was confirmed by the identification of the 5'-end base of each 5'-end-labeled fragment after digestion with RNase T2 (which cleaves every phosphodiester bond at the 5'-side; Fig. 1, C and D). Both the 9-base and the 8-base fragments yielded pAp and pCp, respectively, indicating that MazF was able to cleave RB-15-1 RNA at both sites 6 and 7 (Fig. 1A). Notably, the same sequence in the *mazG* mRNA was preferentially cleaved at site 7 (15), whereas RB-15-1 RNA was cleaved preferentially at site 6. It appears that the hydrolysis reaction can occur at either the 5'-side or the 3'-side phosphodiester linkage of the first A residue in the ACA sequence.

The above results also indicated that MazF cleaves a phosphodiester bond at the 5'-end side yielding a free 5'-OH group on the 3'-end cleavage product and thus a 3'-phosphate (or a 2',3'-cyclic phosphate) on the 5'-end product. These data were further substantiated by MALDI-mass spectrometric analysis of MazF-digested RB-15-1 RNA. These results clearly demonstrated that MazF cleaves RB-15-1 RNA at site 6, yielding a 2',3'-cyclic phosphate and a 5'-OH group (Fig. 1E). The cleavage product at site 7 was not detectable by MALDI-mass spectrometry because of its low yield.

We synthesized three additional shorter RNA substrates, a 13-base RNA (RB-13-1), an 11-base RNA (RB-11-1), and a 7-base RNA (RB-7-1, Table I). MALDI-mass spectrometric analysis after MazF digestion of each RNA revealed that 1) each was cleaved at the 5'-side phosphodiester bond at the first A residue of the ACA sequence, and 2) all of the resulting 5'-end fragments had almost exclusively a 2',3'-cyclic phosphate at the 3'-end, and 3) all of the resulting 3'-end fragments contained a free 5'-OH group (not shown). These results clearly indicated that MazF is an endoribonuclease that cleaves a phosphodiester linkage at the 5'-side of a phosphodiester bond.

In addition to RB-11-1, RB-11-2 was also synthesized. In both RB-11-2 and RB-7-1, the U residue immediately upstream of the ACA sequence was replaced with a G residue (Table I). Both substrates were effectively cleaved by MazF (not shown), indicating that the U residue upstream of the ACA sequence is not essential for hydrolysis by MazF.

Requirement of the 2'-OH Group at Cleavage Site—Our data revealed that all of the 5'-end cleavage products contain a 2',3'-cyclic phosphate, suggesting that the 2'-OH group at the cleavage site plays an essential role for the hydrolysis reaction as in the case of RNase A. To test the role of the 2'-OH group for cleavage, we first modified the 2'-OH groups with a methyl group at the first A residue of the ACA sequence (RB-13-2, Table I), at A and C residues (RB-13-3), at the U residue

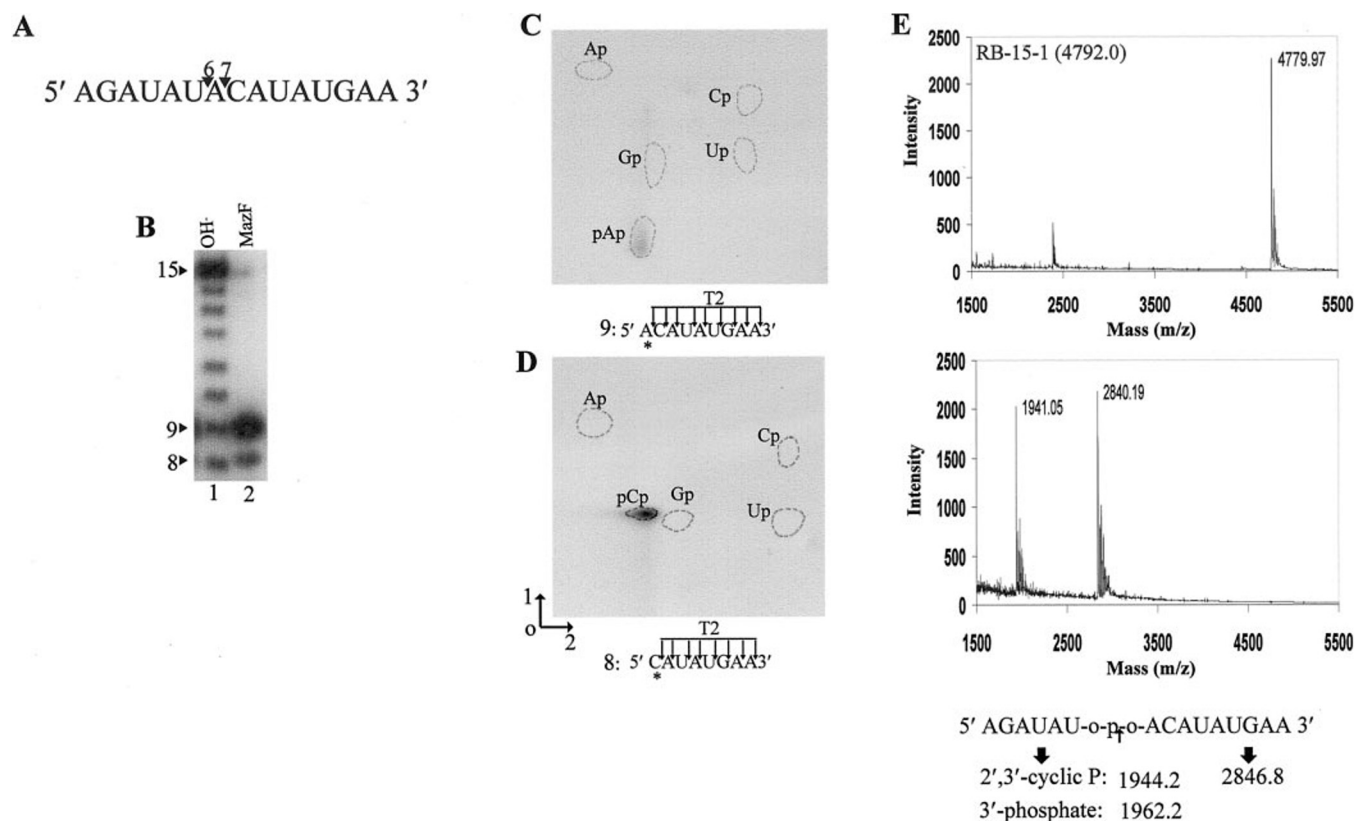


FIG. 1. The 3'- and 5'-end analysis of RNA cleavage products by MazF. A, base sequence of RB-15-1. Two MazF cleavage sites are indicated by arrowheads with numbers 6 and 7. B, the 3'-end cleavage products of RB-15-1 by MazF(His)₆. Unlabeled RB-15-1 RNA (4 pmols) was incubated with 0.02 μ g of MazF(His)₆ at 37 °C for 15 min and then the cleavage products were labeled by [γ -³²P]ATP using T4 polynucleotide kinase at 37 °C for 15 min. The ³²P-labeled 3'-products were subjected to 20% sequencing gel (with 7 M urea) (lane 2) along with the RNA ladder (lane 1), which was prepared by partial alkaline hydrolysis of the 5'-end ³²P-labeled RB-15-1. C, two-dimensional thin-layer chromatographic analysis of the 9 base 3'-end cleavage product from B. The 9-base product was labeled with [γ -³²P]ATP using T4 polynucleotide kinase, gel purified, and then incubated with RNase T2 at 37 °C for 6 h. The T2 digestion products were subjected to two-dimensional thin-layer chromatography as described under "Experimental Procedures." The position of the unlabeled 3'-monophosphate (Gp, Ap, Up, and Cp) and pAp are indicated by circles. D, two-dimensional thin-layer chromatographic analysis of the 8-base product from B. The 8-base product was treated as described for the 9-base product above. E, MALDI-mass spectrometric spectrum of RB-15-1 (4 pmol/ μ l) without and with MazF(His)₆. RB-15-1 RNA (4 pmol/ μ l) was incubated with 0.2 μ g of MazF(His)₆ at 37 °C for 15 min and then the cleavage products were analyzed by MALDI-mass spectrometry.

TABLE I
Sequences and structures of synthetic MazF substrates

MS, MALDI-mass spectrometric analysis; ∇ , cleavage site; bold, modified residues.

Name	Length	Sequence (5' \rightarrow 3')	Cleavage	MS
RB-15-1	15	AGAUAU ∇ A CAUAUGAA	+	+
RB-13-1	13	AAGUCU ∇ ACAUCAG	+	+
RB-11-1	11	AUAU ∇ ACAUAUG	+	+
RB-11-2	11	AUAG ∇ ACAUAUG	+	+
RB-7-1	7	AG ∇ ACAUA	+	+
2'-O-Methyl				
RB-13-2	13	AAGUCU ∇ A CAUCAG	+	—
RB-13-3	13	AAGUCU ∇ A CAUCAG	+	—
RB-13-4	13	AAGUC U AC AUCAG	—	—
RB-13-5	13	AAGUC U A CAUCAG	—	—
2'-Deoxy				
RB-13-6	13	AAGUCU ∇ AC AUCAG	+	—
RB-13-7	13	AAGUCU ∇ A C AUCAG	+	—
RB-13-8	13	AAGUC U AC AUCAG	—	—
RB-13-9	13	AAGUC U AC AUCAG	—	—
RNA/DNA chimeric				
RD-13-1	13	dAdAdGdTdCrU ∇ dAdCdAdTdCdAdG	+	+
RD-13-2	13	dAdAdGdTdCrG ∇ dAdCdAdTdCdAdG	+	+

immediately upstream of the first A residue (RB-13-4), and at the both U and A residues (RB-13-5). As shown in Fig. 2A, both RB-13-2 (lane 6) and RB-13-3 (lane 4) were digested, whereas both RB-13-4 (lane 10) and RB-13-5 (lane 8) were not, indicating that the 2'-OH group of the cleavage site is indeed essential. Similar results were obtained using 2'-deoxy modified

RB-13-1, RB-13-6 (UdACA), RB-13-7 (UdAdCA), RB-13-8 (dUACA), and RB-13-9 (dUdACA, Table I), underscoring the importance of the 2'-OH group of the U residue at the cleavage site (Fig. 2A, lanes 12, 14, 16, and 18, respectively).

Note that there are two cleavage products in all cases where the substrates were hydrolyzed by MazF (Fig. 2A, products a

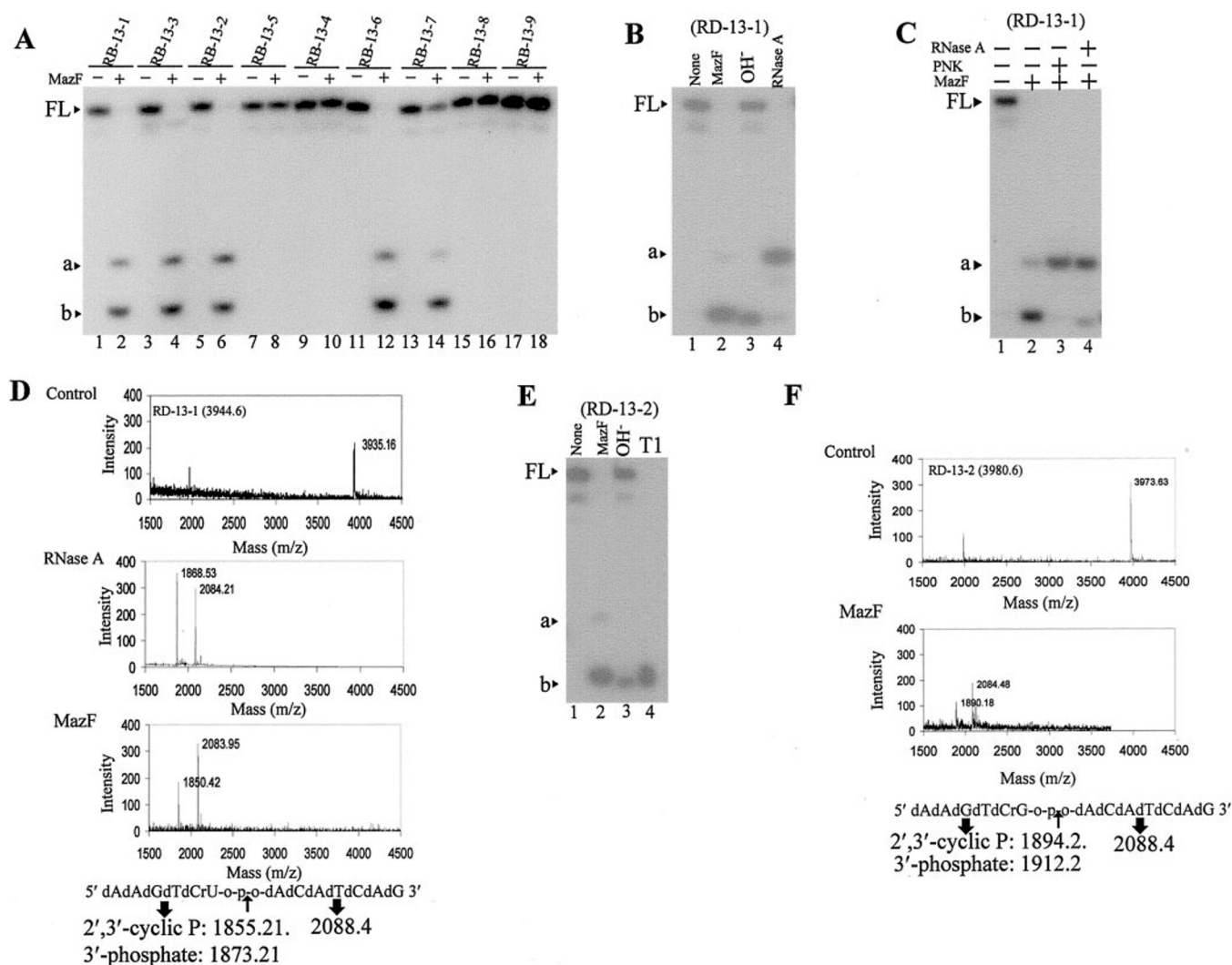


FIG. 2. Characterization of the cleavage product of various synthetic RNA substrates by MazF. A, cleavage of synthetic RNAs with different modifications at the ACA sequence by MazF(His)₆. The 13-base RNAs, RB-13-1–RB-13-9 (Table I), were labeled with [γ -³²P]ATP using T4 polynucleotide kinase. The ³²P-labeled RNA substrates (2 pmol) were incubated with 0.02 μ g of MazF(His)₆ at 37 °C for 15 min. The reaction mixtures were then subjected to 20% sequence gel electrophoresis followed by autoradiography. Lanes 1, 3, 5, 7, 9, 11, 13, 15, and 17 no MazF(His)₆. Lanes 2, 4, 6, 8, 10, 12, 14, 16, and 18, 0.02 μ g of MazF(His)₆ was added, and the mixture was incubated at 37 °C for 15 min. B, cleavage of synthetic a RNA-DNA chimera by MazF(His)₆ and RNase A. RD-13-1, was labeled with [γ -³²P]ATP using T4 polynucleotide kinase. The ³²P-labeled RD-13-1 substrate (2 pmol) was incubated with 0.11 μ g of MazF(His)₆ and RNase A at 37 °C for 15 min. The reaction mixtures were then subjected to 20% sequence gel electrophoresis followed by autoradiography. Lane 1, no MazF(His)₆; lane 2, 0.11 μ g of MazF(His)₆ was added; lane 3, partial alkaline hydrolyte of ³²P-labeled RD-13-1; lane 4, 0.11 μ g of RNase A was added. C, effect of RNase A and polynucleotide kinase (PNK) on MazF cleavage products of RD-13-1. The ³²P-labeled RD-13-1 (2 pmol) was first incubated with 0.11 μ g of MazF(His)₆ at 37 °C for 15 min and then the reaction mixtures were divided into two parts, to one RNase A (0.11 μ g) was added and to the other polynucleotide kinase (10 units) was added. The reaction mixtures were incubated at 37 °C for another 15 min. The products were subjected to 20% sequence gel electrophoresis followed by autoradiography. Lane 1, no MazF(His)₆; lane 2, 0.11 μ g of MazF(His)₆; lane 3, 10 units of polynucleotide kinase was added after the MazF(His)₆ cleavage reaction; lane 4, 0.11 μ g of RNase A was added after the MazF(His)₆ cleavage reaction. D, MALDI-mass spectrometric spectrum of a RD-13-1 with MazF(His)₆ treatment (bottom panel) and RNase A treatment (middle panel). RD-13-1 (4 pmol/ μ l) was incubated with 0.25 μ g of MazF(His)₆ (bottom panel) and RNase A (middle panel) at 37 °C for 15 min and then the cleavage products were analyzed by a MALDI-mass spectrometer. E, cleavage of RD-13-2 (Table I) by MazF(His)₆ and RNase T1. The 13-base RNA-DNA chimera, RD-13-2, was labeled with [γ -³²P]ATP using T4 polynucleotide kinase. The ³²P-labeled RD-13-2 (2 pmol) was incubated with MazF(His)₆ (0.11 μ g) or RNase T1 (0.11 μ g) at 37 °C for 15 min. The reaction mixtures were then subjected to 20% sequence gel electrophoresis followed by autoradiography. Lane 1, no MazF(His)₆; lane 2, 0.11 μ g of MazF(His)₆; lane 3, partial alkaline hydrolyte of ³²P-labeled RD-13-2; lane 4, 0.11 μ g of RNase T1. F, MALDI-mass spectrometric spectrum of RD-13-2 without (top panel) and with MazF(His)₆ treatment (bottom panel). RD-13-2 (4 pmol/ μ l) was incubated with MazF(His)₆ (0.25 μ g) at 37 °C for 15 min and then the cleavage products were analyzed by a MALDI-mass spectrometer.

and b). Product b contains a 2',3'-cyclic phosphate, whereas product a has a 3'-phosphate at the 3'-end (see the section below). The ratio of a to b varied in different experiments. However, the a/b ratio increased when the reaction was carried out with higher concentrations of MazF (not shown), suggesting that a MazF cleavage reaction of a phosphodiester linkage may occur in a similar manner as RNase A. The hydrolysis reaction by RNase A occurs in two discrete steps; in the first step, the nucleophilicity of the ribose 2'-OH plays a key role to

form a 2',3'-cyclic phosphate, in the second step, the cyclic intermediate is opened to form a 3'-phosphate.

Identification of a 2',3'-Cyclic Phosphate as an Intermediate—To test whether the 2'-OH group at the cleavage site is the only requirement for MazF, we engineered two DNA-RNA chimeric substrates, RD-13-1 and RD-13-2, based on the RB-13-1 sequence (Table I). In RD-13-2, the rU residue in RD-13-1 was replaced with a rG residue. RD-13-1 was 5'-end labeled with [γ -³²P]ATP (Fig. 2B, lane 1). MazF digestion of this substrate

A

mazG gene

1 AUGAAUCAAUCGACGUUUGCUCACUAUUUAUGCAGCGCCGCGGAUCCGGAAACGGCUGCCCGUGGGA
 72 UAAAGAGCAGACAUUUUGCCACCAUUGCGCCUACACCCUUGAAGAAACCUACGAAGUGCUGGACGCCA
 140 UCGCCCGUGAAGAUUUU ← (G1)
 156

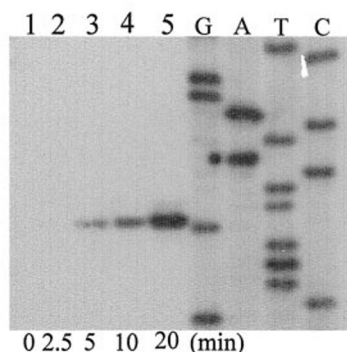
era gene

118 GCGCAGACAACUCGUCACGCAUUGUGGGGAUCCAUACUGAAGGCGCGUAUCAGGCGAUACGUCGAU
 187 ACACCGGCCUGCAUUGGAAGAAAAACGCGCCAUUAACGCCUGAUGAACAAAGCGCGAGCAGC
 253 UCUAUUGGCGA ← (E1)
 263
 288 UGAAGGCACGCGUGGACGCCGGACGACGAAAUGGUGCUACAACUGCAGGAAGGCAAAGCGCCGGUA
 358 AUCCUC ← (E2)
 362
 481 AATGTTGACACTATTGCGGCAATCGTGCGTAAGCATCUACCUGAAGCGACUCAUCACUUCCCGGAAGAUU
 551 ACAUCACGGAUCGCUCACAGCGUUUUUUGGCGUCUGAAUCAUCCGCGAAAAACUGAUGCGUUUCCUCGG
 621 CGCUGAACUGCCGUACUCCGUGACGGUGGAGAUCAAGUUUUGUCUCUAACGACGCGGUGGUUAUGACA
 692 UCAACGGUUUGAUUCUCGUUGAGCG ← (E3)
 716
 685 UAUGACAUCAACGGUUUGAUUCUCGUUGAGCGUGAAGGCGAGAAGAUGGUCAUUGGCAACAAGGGG
 755 CCAAGAUCAAAACCAUCGGGAUUGAAGCGCGUAAAGACAUGCAGGAAUUGUUGAAGCGCCUGUUCACCU
 826 GAACUGUGGGUAAAAGUGAAA ← (E4)
 846

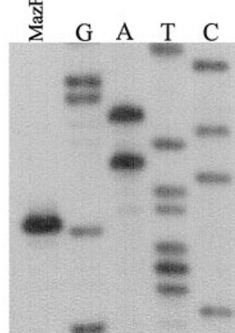
16S RNA fragment

1213 ACGACCAGGGCUACACACGUGCUACAUGGCGCAUACAAAGAGAAGCGACCUCGCGAGAGCAAGCGG
 1280 ACCUCAUAAAGUGCGUCGUAGUCCGGAUUGGAGUCUGCAACUC ←
 1322

B



C



D

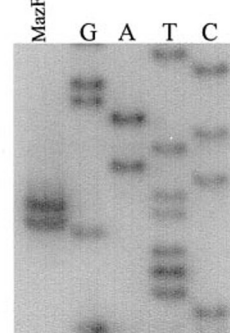


FIG. 3. Analysis of MazF cleavage sites in the *mazG* and *era* mRNAs by using *in vivo* and *in vitro* primer extension. A, the *mazG* and *era* mRNAs were induced from pIN-MazG and pIN-Era, respectively, in the presence of 1 mM isopropyl 1-thio- β -D-galactopyranoside for 1 h before MazF induction as described under "Experimental Procedures." The total RNA was extracted at each time point indicated after MazF induction (B, lanes 1–5), and a primer extension experiment was carried out with different primers indicated for each sequence. The ACA sequences cleaved by MazF are boxed by solid lines. Those ACA sequences that were cleaved very weakly or not cleaved at all are boxed in dotted lines. Base numbers are shown as taking the first base of open reading frames as 1. All of the AC sequences (excluding ACA) are underlined, and the cleavage sites are shown by the arrowheads. B, primer extension analysis of a MazF cleavage site in the *era* mRNA *in vivo*. C, primer extension analysis of a MazF cleavage site in the *era* mRNA *in vitro*. D, primer extension analysis of the same MazF cleavage site in B and C *in vitro* using a 98-base *era* mRNA fragment.

yielded a major product at position *b* (Fig. 2B) and a minor product at position *a*. The *b* product (Fig. 2B, lane 2) is identical to the product from alkaline hydrolysis (lane 3), whereas the *a* product is identical to the product from RNase A digestion. When these products were analyzed by MALDI-mass spectrometry, the mass of the 5'-end RNase A product was only 18 Daltons larger than the mass of the 5'-end MazF product (Fig. 2D). The mass of the RNase A product agreed well with the 5'-end product containing a 3'-phosphate at the 3'-end, whereas the mass of the MazF product was consistent with the 5'-end product containing a 2',3'-cyclic phosphate. These results suggest that both RNase A and MazF cleave these substrates between rU and dA. Importantly, the 3'-end products from both RNase A and MazF digestions had identical masses.

To further prove that the products cleaved by MazF contain a 2',3'-cyclic phosphate group and not a 3'-OH group, the MazF product of the 5'-end labeled RD-13-1 (Fig. 2C, lane 2) was treated either with T4 polynucleotide kinase at pH 6.0 (known to function as 3'-phosphatase under this condition (26)) (lane 3) or with RNase A (lane 4). By both treatments, product *b* in lane 2 was converted to product *a* in lanes 3 and 4. These results provided additional evidence that MazF cleavage of a phosphodiester linkage yields primarily a 2',3'-cyclic phosphate on one side and a 5'-OH group on the other side. When rU was replaced with rG (RD-13-2, Table I), MazF was still able to effectively cleave the substrate (Fig. 2E, lane 2). The products from MazF treatment migrated at the identical position to the alkaline hydrolytes (lane 3) and to RNase T1 digest. The for-

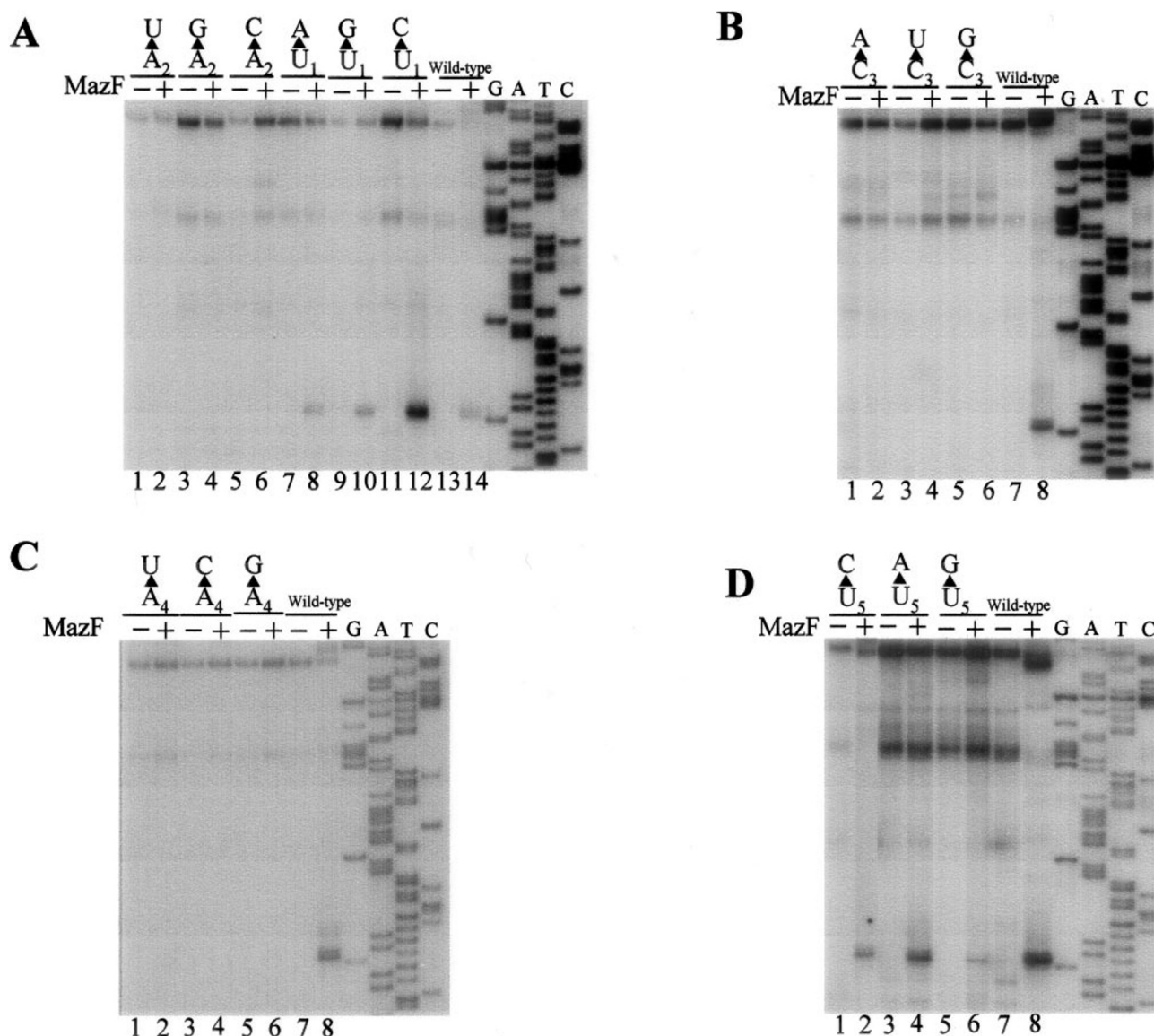


FIG. 4. Effects of mutations in the MazF cleavage site on MazF cleavage. Site-directed mutagenesis was performed with pET-11a-MazG as a DNA template. All mutations were confirmed by DNA sequence analyses. 151-base *mazG* RNA fragment was synthesized *in vitro* by T7 RNA polymerase using a RiboMAX™ T7 large-scale RNA production system. MazF(His)₆ (0.02 μg) was incubated with 1 pmol of the *mazG* RNA fragment in a 20-μl reaction mixture containing 10 mM Tris-HCl (pH 7.8), 10 mM MgCl₂, 60 mM NH₄Cl, 0.5 μl of RNase inhibitor, 1 mM dithiothreitol, 0.5 mM dNTPs, and 0.05 μM ³²P-labeled primer at 37 °C for 10 min. Reverse transcriptase (2 U) was then added, and cDNA synthesis was carried out at 37 °C for 15 min. The reaction was stopped by adding sequence 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. Primer G2 (TGCTCTTTATCCACGGGCAG) was used for primer extension analysis. The primer was 5'-end labeled with [γ-³²P]ATP using T4 polynucleotide kinase. The sequence ladder was obtained using pCR® 2.1-TOPO®-MazG as template, and the same primer used for primer extension was used.

mation of a 2',3'-cyclic phosphate at the rG residue and a 5'-OH group at the dA residue of the cleavage site was confirmed by MALDI-mass spectrometry (Fig. 2F).

Cleavage Site Shifting—Curiously, the MazF cleavage site within an RNA sequence derived from the *mazG* mRNA was shifted by 1 base upstream when RNA was shorted to 15 bases (Fig. 1). A similar shift in cleavage site was observed in the ACA sequence derived from the *era* mRNA (bases 236–238) when the cleavage conditions were altered. *In vivo* primer extension experiments revealed that this ACA sequence was cleaved between the first A and C residue (Fig. 3B). This result was also confirmed by an *in vitro* primer extension experiment using the full-length (~1 kb) *era* mRNA (Fig. 3C). However, when only a 98-base portion of the *era* mRNA was digested with MazF in an *in vitro* reaction, cleavage occurred at the phosphodiester linkage not only at the 3'-side bond but also at

the 5'-side bond of the first A residue of the ACA sequence (Fig. 3D). The reason for this cleavage site shifting is unclear. However, we speculate that an amino acid residue at the active site of MazF involved in the nucleophilic attack of the 2'-OH group of the cleavage site residue is flexible. This flexibility may enable the RNA-MazF-dimer complex to attack either the upstream 2'-OH or the downstream 2'-OH group.

Recognition Sequence of MazF—Recent *in vitro* studies conclude that MazF cleaves mRNAs between N and A residues of an NAC sequence (N is preferentially A and U) (20). Our published assertion that MazF requires ACA for its endoribonuclease activity (15) was derived from primer extension experiments with *mazG* and *era* mRNAs both *in vivo* and *in vitro* (Fig. 3A, boxed). Of all of the AC-containing sequences (Fig. 3A, underlined), only those comprising ACA were cleaved. Notably, no cleavage was observed at UACX and AACX (X is U, C, and G).

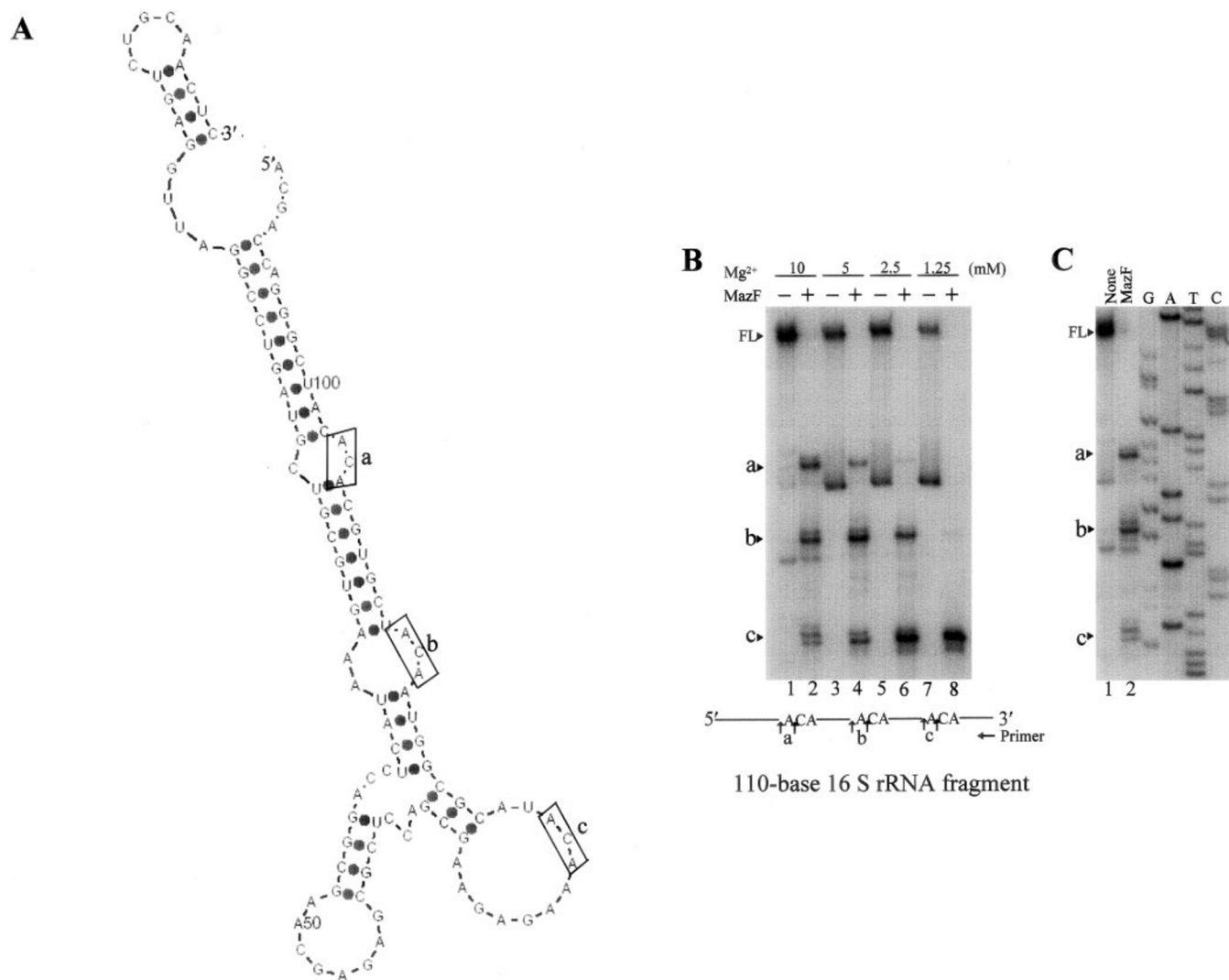


FIG. 5. Effects of Mg^{2+} on the cleavage activity of MazF. **A**, a putative secondary structure of the 110-base 16 S rRNA fragment predicted by Zuker's program. **B**, primer extension analysis of the cleavage products by MazF(His)₆ treatment of the 110-base 16 S rRNA fragment at different concentration of Mg^{2+} . The RNA fragment containing two ACA sequences (*b* and *c* in **A**) and one ACACA sequence (two overlapping ACA; *a* in **A**) was synthesized by T7 RNA polymerase *in vitro* as described under "Experimental Procedures" and used as a substrate. **Lanes 1, 3, 5, and 7**, no MazF(His)₆; **lanes 2, 4, 6, and 8**, 0.02 μ g of MazF(His)₆ was added. The concentrations of Mg^{2+} in the reaction are indicated on top of the gel. **FL**, the full-length of the RNA; the three ACA sequences are termed *a*, *b*, and *c*, respectively, as shown in **A**, and the positions of their cleavage products on the gel are indicated. **C**, cleavage site analysis by primer extension. The 110-base RNA fragment containing the ACA sequences was digested in the presence of 10 mM Mg^{2+} . The sequence ladder was obtained using pCR02.1-TOPO@-16 S as template, and the primer was used for primer extension.

To test the requirement for an ACA recognition sequence associated with the MazF endoribonuclease function, the U₁A₂C₃A₄U₅ sequence in the *mazG* mRNA was mutated to all possible bases at each position. These mutated RNAs were tested for MazF cleavage (Fig. 4). Any base changes of the ACA sequence (Fig. 4, **A**, **B**, and **C**, A₂, C₃, and A₄, respectively) completely blocked cleavage of the RNA by MazF, whereas any base changes at U₁ and U₅ residues (Fig. 4, **A** and **D**) did not block the RNA cleavage. These results revealed that the ACA sequence plays an essential role for MazF recognition of RNA substrates in support of our earlier work (15). Overall, our data conclusively establishes that MazF specifically cleaves at ACA sequences.

Effects of Mg^{2+} on MazF Cleavage—Similar to RNase A, MazF does not require Mg^{2+} for its hydrolytic reaction. Using RB-15-1 RNA as a substrate, EDTA showed little effect on MazF activity, whereas Mg^{2+} was inhibitory (not shown). The effect of Mg^{2+} was tested using a 110-base 16 S rRNA fragment, which contains two ACA (Fig. 5A, *b* and *c*) and one ACACA (Fig. 5A, *a*; two overlapping ACA) sequence. The cleav-

age at these three sites *a*, *b*, and *c*, by MazF were found to be dependent of the concentration of Mg^{2+} (Fig. 5B). At 10 mM, *a* and *b* sites were cleaved equally, but cleavage at site *c* was less efficient. At 5 mM, an apparent preferential cleavage at site *c* over *a* was observed. At 1.25 mM Mg^{2+} , cleavage at site *c* only was noted, precluding the ability to detect MazF cleavage at sites *a* and *b*. Therefore, the band intensity changes observed for site *a* and site *b* are not because of an inhibition of MazF cleavage at these sites. We suspect that cleavage at all sites was enhanced as Mg^{2+} concentrations decreased. Note that at site *a* (where two ACA sequences are overlapping) cleavage occurs only at the downstream ACA, perhaps because the cleavage site for the upstream ACA forms a stable secondary structure (Fig. 5A).

MazF as an mRNA Interferase—Gerdes and co-workers (16) reported that MazF inhibits translation by a ribosome- and codon-dependent mechanism very similar to RelE. Our data do not support their conclusion. This study and our earlier work (15) showed that MazF cleaves mRNAs in a totally codon-independent manner, because MazF cleaves mRNAs at the

same site both *in vivo* and *in vitro*. Furthermore, in this report we showed that small synthetic RNAs, even as short as 7 bases, are able to serve as MazF substrates. Interestingly, although not pointed out by Christensen *et al.* (16), distinct cleavage of the *lpp* mRNA by MazF very close to its 5'-end was evident. On the basis of the sequencing ladder provided, it clearly corresponded to the ACA sequence in the 5'-untranslated region (27). Taken together, their results and ours support the notion that MazF functions as a ribosome-independent endoribonuclease. However, we cannot exclude the possibility that MazF may be able to associate with ribosomes in the cell.

We have now unequivocally demonstrated that MazF is an endoribonuclease that specifically cleaves mRNA at ACA sequences. Furthermore, MazF was found to enzymatically function in a manner similar to RNase A in the cleavage of RNA where the 2'-OH group at the cleavage site plays a key role in the reaction. One of histidine residues in the active site of RNase A enhances the nucleophilicity of the ribose 2'-OH group of the U residue at the cleavage site resulting in the formation of a 2',3'-cyclic phosphate. It will be useful to determine which residues in the structure of the MazF dimer (14) play a role in the reaction. Although MazF does not contain a conserved histidine residue, it contains highly conserved basic residues, which may perform nucleophilic attack on the 2'-OH group. With its well defined sequence specificity, MazF essentially behaves as an RNA restriction enzyme. This property makes MazF an attractive tool for manipulation of RNA and for structural analysis. For example, it would allow for fine mapping of a RNA secondary structure (because it only cuts single-stranded RNA). The distinctive properties of MazF can also be exploited for use in organisms other than bacteria. Ongoing studies in our laboratory revealed that MazF functions as an mRNA interferase in both lower (yeast) and higher (mouse and human cells) eukaryotes, providing a exacting tool to both manipulate cell death pathways and study cell proliferation.

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