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Mg^{2+} facilitates leader peptide translation to induce riboswitch-mediated transcription termination

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We have characterized a 17-residue peptide, MgtL, which is translated specifically in high Mg^{2+} from an open reading frame (ORF) embedded in the Mg^{2+} riboswitch domain, previously identified in the 5′ leader region of Mg^{2+} transporter gene *mgtA* in *Salmonella*. We demonstrate that *mgtL* translation is required to prematurely terminate *mgtA* transcription. Abrogation of *mgtL* translation by mutation of its start codon results in transcription of the *mgtA*-coding region in high Mg^{2+}, suggesting that ribosome stalling is not required for preventing premature transcription termination. Consistently, the Mg^{2+} riboswitch responds to cytoplasmic Mg^{2+}, but not to proline or arginine, both repeatedly present in the MgtL sequence, to mediate *mgtL* translation-coupled regulation. RNA structural probing and nucleotide substitution analysis show that the riboswitch loop A region alters base pairing in response to Mg^{2+}, and favours stem-loop A1 in high Mg^{2+}, subsequently opening the ribosome-binding sequence for *mgtL* translation. Presumably, *mgtL* ORF directs translation to localize a ribosome *in cis* to act on downstream RNA in a manner similar to some upstream ORFs in prokaryotes and eukaryotes.

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Introduction

In Mg^{2+}-depleted conditions, bacteria facilitate uptake of this divalent cation by inducing synthesis of specific Mg^{2+} transporters (Snavely *et al*, 1991). The *mgtA* gene in the Gram-negative bacteria *Salmonella typhimurium* and *Escherichia coli*, which encodes a P-type ATPase to mediate Mg^{2+} influx (review see ref. Moncrief and Maguire (1999)), has served as a prototype in studies of both inducible regulation and biochemical function of genetic loci encoding Mg^{2+} transporters. It is suggested that two independent mechanisms are involved in Mg^{2+}-dependent transcriptional regulation of *mgtA*. (i) The PhoP/PhoQ two-component system responds to micromolar levels of environmental Mg^{2+} and activates transcription initiation; or to milli-molar levels of Mg^{2+} and represses transcription initiation (Garcia Vescovi *et al*, 1996). (ii) Once transcription is initiated, the 5′ untranslated region (here, called the 5′ leader region or 5′LR) of nascent *mgtA* transcripts functions as an alternative Mg^{2+}-sensing system. If the Mg^{2+} concentration increases in the bacterial cytoplasm, the latter system interrupts *mgtA* transcription before it is extended to the downstream coding region (Cromie *et al*, 2006).

In *Salmonella*, the *mgtA* transcript initiated from the QJ;PhoP-activated promoter contains a 264-nt 5′LR (Lejona *et al*, 2003), which contains a *cis*-acting regulatory element responsive to Mg^{2+}. This element is similar to other riboswitches that are able to interact with a small molecule, normally a specific metabolite, and modify the RNA structures through stem-loop switching, subsequently exerting their regulatory effects (review see ref. Tucker and Breaker (2005)). A structural probing of the 5′LR showed that a high Mg^{2+} condition (3.5 mM) induced regions containing nucleotides 56–125 and 136–159 to form stem-loops A and B (see ref. Cromie *et al* (2006) and illustrated in Figure 1A). Whereas, a low Mg^{2+} condition (0.35 mM) caused a stem-loop switching via alternative base pairing between nucleotides 118–125 located in the right arm of stem A, and 140–147 in the left half of stem-loop B, resulting in formation of stem-loop C. Stem-loop B is a prerequisite for initiating premature termination of *mgtA* transcription mediated by the *mgtA* Mg^{2+} riboswitch (Cromie *et al*, 2006). The stem-loop C, favoured in low Mg^{2+}, may have prevented formation of stem-loop B, and thus allowed *mgtA* transcription to be extended into the coding region (Cromie *et al*, 2006). The *E. coli mgtA* 5′LR also responds to Mg^{2+} similarly as its *Salmonella* homologue (Cromie *et al*, 2006). A truncated RNA representing the *mgtA* 5′LR was characterized from *E. coli* cells (Kawano *et al*, 2005), providing direct evidence for premature termination of *mgtA* transcription *in vivo*. Notably, the truncated transcripts are different in length from *in vivo* (~240-nt; Kawano *et al*, 2005) and *in vitro* (220-nt; Cromie *et al*, 2006) samples. As the *mgtA* 5′LR does not have sequences consistent with a Rho-independent terminator, the 220-nt transcript is unlikely a product generated *in vitro* through transcription termination, but a product from the strong pausing of the RNA polymerase in high Mg^{2+}. The mechanism of termination or pausing, however, is not known. It is possible that *mgtA* transcription is paused at nucleotide 220, probably by an RNA conformation induced in high Mg^{2+}, and subsequently terminated near nucleotide 240 *in vivo* by additional cellular components.

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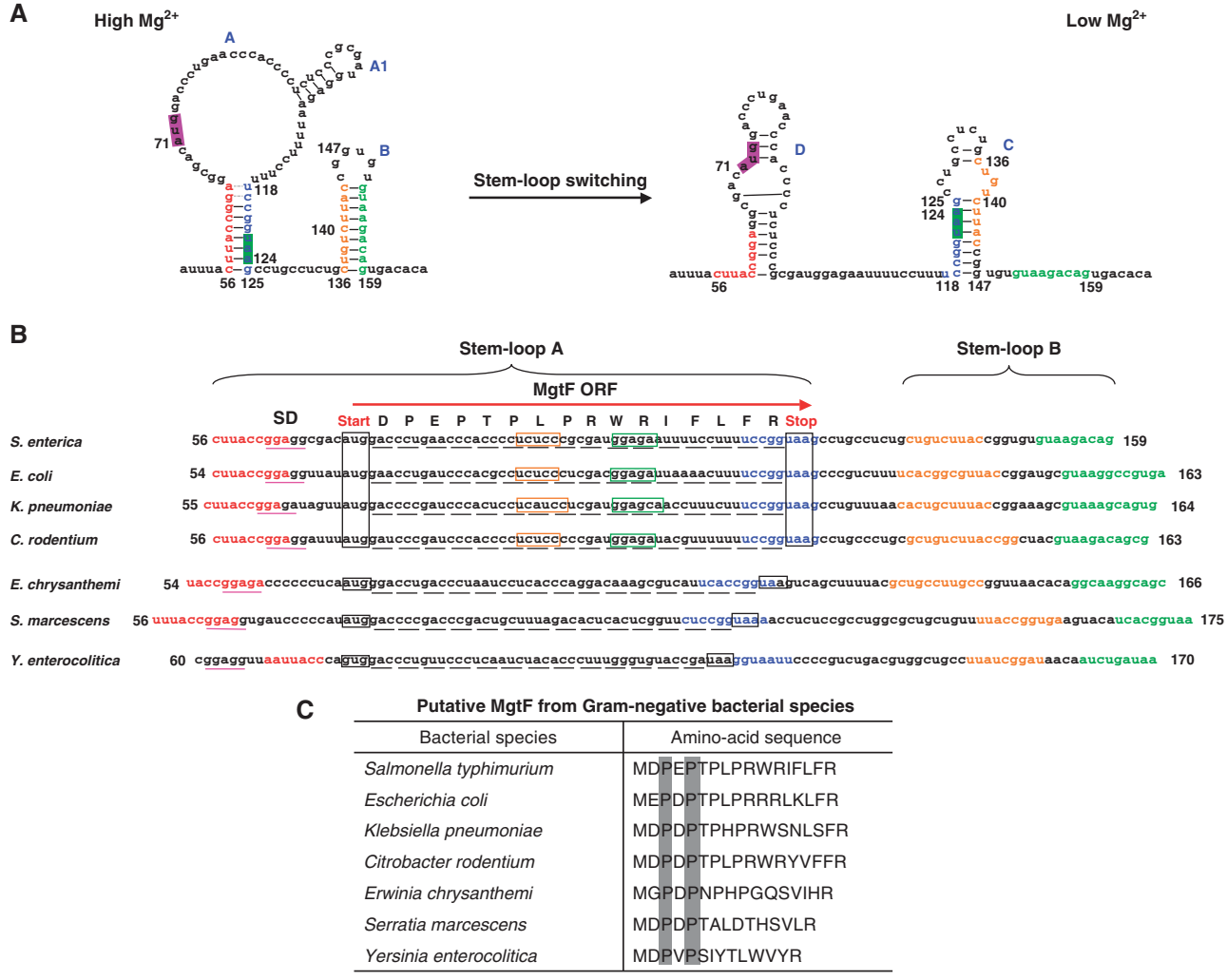


C

Putative MgtF from Gram-negative bacterial species

Bacterial species	Amino-acid sequence
Salmonella typhimurium	MDPEPTPLPRWRIFLFR
Escherichia coli	MEPDPTPLPRRRLKLFR
Klebsiella pneumoniae	MDPDPTPHPRWSNLSFR
Citrobacter rodentium	MDPDPTPLPRWRYVFFR
Erwinia chrysanthemi	MGPDPNPHPGQSVIHR
Serratia marcescens	MDPDPTALDTHSVLR
Yersinia enterocolitica	MDPVPSIYTLWVYR

Figure 1 Prediction of the MgtL leader peptide encoded by the 5′LR of *mgtA* homologues in Gram-negative bacteria. (**A**) A schematic representation of the Mg^{2+} riboswitch domain in *Salmonella mgtA* 5′LR (5′LR). Coloured letters are the nucleotide sequences involved in the stem-loop switching in different Mg^{2+} concentrations. Numbering represents the positions of nucleotides in the *mgtA* 5′LR. Uppercase letters are the stem-loop structures formed in different Mg^{2+} concentrations. Highlighted sequences are the start and stop codons of the *mgtL* open reading frame (ORF). The dotted lines indicate that base pairing is possible, however, our data does not support it. (**B**) Sequence alignment of the stem-loops A and B in the 5′LR region of the *mgtA* gene. Sequences in colour and underlined in red are stem-loop structures A and B, and the ribosome-binding sites (SD). Three-letter sequences black framed, and underlined in black are start and stop codons, and codons in the *mgtL* ORF. Orange and green frames are the sequences to form stem A1. Amino-acid residues in the MgtL peptide from *Salmonella* are shown. Numbering represents the positions of nucleotides in the *mgtA* 5′LR. (**C**) MgtL peptides predicted from Gram-negative bacterial species. Highlighted residues are conserved in these MgtL peptide sequences.

Stem-loop A is critical for Mg^{2+} sensing in the riboswitch because Mg^{2+}-promoted conformational changes in stem-loops B and C depend on the presence of the stem-loop A sequence (Cromie *et al*, 2006). As the stem-loop A region is transcribed, it might trap the 5′LR RNA into distinct structures depending on the Mg^{2+} concentration that ultimately determine whether transcription is prematurely terminated. Interestingly, a transition mutation in loop A, that substituted nucleotide 98 from C to U, resulted in uncharacteristic expression of *Salmonella mgtA* in high Mg^{2+} concentrations (O'Connor *et al*, 2009). While the significance of stem-loop A has been implicated, it remained unknown what regulatory element embedded in this region contributed to the 5′LR function.

Importantly, previous results suggested that additional cellular factors could have a role in transcriptional regulation of *mgtA* via the 5′LR. (i) When transcribed in *E. coli*, *mgtA* transcript from *Salmonella* was degraded more in a high Mg^{2+} condition in an RNase E-dependent manner (Spinelli *et al*, 2008). Mutations at the 5′LR eliminated the degradation, suggesting that this nuclease degrades the *mgtA* mRNA by targeting the 5′LR. (ii) A transcriptional regulator, Rob, can bind to the *Salmonella* sequence: 5′-accgccaTaattgccacaaa-3′, which includes the PhoP-dependent transcription start (shown in uppercase) (Barchiesi *et al*, 2008). When overexpressed, Rob initiates transcription from nucleotide 44 of the 5′LR in a PhoP/PhoQ-independent manner.

An Mg^{2+}-responsive RNA element was also characterized in Gram-positive bacteria. The 5′LR of the Mg^{2+} transporter gene, *mgtE* from *Bacillus subtilis*, harbours a metal-sensing domain (M box), which is able to bind Mg^{2+} and enhance formation of the downstream Rho-independent terminator

structure (Dann *et al*, 2007). Interestingly, these regulatory RNAs from Gram-negative and Gram-positive bacteria do not share homologous sequences, suggesting that they employ different mechanisms to sense Mg^{2+} and mediate transcription regulation.

In this study, we identify a novel component that controls the regulatory function of the Mg^{2+} riboswitch in *mgtA*. Our results demonstrate that the stem-loop A region in the 5′LR comprises a translational unit, which encodes the 17-residue peptide, MgtL, in *Salmonella*. We show that Mg^{2+} facilitates modification of the stem-loop A conformation through stem switching to allow *mgtL* translation from the integral open reading frame (ORF), resulting in premature termination of *mgtA* transcription. This mechanism seems to be adopted by the MgtA-type Mg^{2+} transporters in many other Gram-negative species, providing an example in which a small molecule ligand stimulates regulatory function of its cognate riboswitch to initiate premature termination of transcription by coupling translation of a leader peptide. During the submission of our manuscript, a publication became available online which reported an ORF encoding a putative peptide MgtL in the *Salmonella mgtA* 5′LR presumably responsive to proline (Park *et al*, 2010). While the presence of the *mgtL* ORF is undisputed, our model of the Mg^{2+}-dependent/proline-independent *mgtL* translation via a novel stem-loop switch does not support their conclusions.

Results

An ORF embedded in the stem-loop A region of the mgtA 5′LR

To study the regulatory function of stem-loop A, we analysed the phylogenetically conserved sequences from several Gram-negative species that harbour *mgtA* homologues, including *S. typhimurium, E. coli, Klebsiella pneumoniae, Citrobacter rodentium, Erwinia chrysanthemi*, *Serratia marcescens,* and *Yersinia enterocolitica*. These stem-loop A sequences greatly varied, but contained three highly conserved regions that could form an ORF: (i) a consensus sequence for ribosome-binding (SD) located upstream of a start codon, (ii) a start codon, AUG (GUG in *Yersinia*), and (iii) a stop codon, UAA, lying in the same reading frame (Figure 1B). In *Salmonella*, this ORF encodes a 17-residue peptide (referred to as MgtL, hereafter) from the start codon ^{71}AUG^{73}, which is located 4-nt downstream of a putative SD sequence ^{62}GGAGG^{66}, to the stop codon ^{122}UAA^{124} (Figure 1B). MgtL homologues can also be predicted from stem-loop A sequences of other Gram-negative species (Figure 1B). MgtL in *E. coli*, *K. pneumoniae*, and *C. rodentium* are also 17-residue peptides sharing high identity with that in *Salmonella* (70.6, 76.5, and 64.7%, respectively, Figure 1C). On the other hand, MgtL from *E. chrysanthemi*, *S. marcescens*, and *Y. enterocolitica* are shorter peptides, and merely share proline residues at positions 3 and 5 (amino acid residues highlighted in Figure 1C), and arginine at the C terminus, as with the peptides from other species. Regardless of the varied sequence and length in these species, the stop codon UAA is always located at the end of the right arm of stem A (Figure 1B, except *Y. enterocolitica* whose UAA is located just before the right arm). The right arm is the switching sequence in the riboswitch structure, that base pairs with alternative sequences to form stem-loop C in low Mg^{2+} and stem-loop A in high Mg^{2+} (Cromie *et al*, 2006). We presume that this architectural design of the *mgtL* ORF is important for the regulatory function of the *mgtA* 5′LR. Similar to our observation, an 18 codon ORF, predicted to encode a peptide whose suggested sequence is the same as MgtL, was identified from *Salmonella mgtA* 5′LR in a recent study (Park *et al*, 2010).

Characterization of MgtL peptide encoded by the stem-loop A sequence in Salmonella mgtA 5′LR

The MgtL peptide is probably either highly unstable or produced at very low levels in the conditions used in this study. We were unable to detect MgtL peptide expressed from the 5′LR in the chromosomal location *in vivo* using western blot. Therefore, we constructed a plasmid, pYS1475, which carries the full-length *mgtA* 5′LR with an inserted 21-nt sequence encoding the FLAG-epitope to generate MgtL tagged by FLAG at the N terminus (hereafter MgtL–FLAG; Figure 2A). In this plasmid, the P_{lac1-6} promoter (Liu *et al*, 2004), which is independent of Mg^{2+} and the PhoP/PhoQ system (Cromie *et al*, 2006; Kong *et al*, 2008), initiates transcription of the 5′LR and a downstream *lacZ* gene. Notably, the Rob regulator does not control this transcription because the Rob-binding site is partially deleted in this plasmid (data not shown). β-Galactosidase activity in wild-type *Salmonella* harbouring pYS1475 and its parent plasmid pYS1010 (i.e., P_{lac1-6}-*mgtA* 5′LR-*lacZ*) (Cromie *et al*, 2006) grown in N minimal medium (Snavely *et al*, 1989) supplemented with 0.01 mM (low) Mg^{2+} are 13.3- and 18-fold higher than those with 10 mM (high) Mg^{2+}, respectively. This suggests that the engineered *mgtA* 5′LR responds similarly to Mg^{2+} as wild-type 5′LR. Because the *Salmonella* 5′LR can also function in *E. coli* (β-galactosidase activity from MC4100 harbouring pYS1010 grown in low Mg^{2+} is ~15-fold higher than in high Mg^{2+}), we introduced pYS1475 into an *E. coli* Maxicell mutant, CSR603. MgtL–FLAG was produced in UV-irradiated bacterial cells in which protein synthesis directed by chromosomal loci, but not by plasmid, was generally inhibited due to extensive degradation of the chromosomal DNA (Sancar *et al*, 1979). Affinity chromatography was carried out to isolate MgtL–FLAG (MW 3164 da) from bacterial cultures grown in low and high Mg^{2+}. The peptide sample was separated through electrophoresis and a band was detected from the bacterial cells grown in high Mg^{2+} (Figure 2B), which migrated to a position slightly slower than a control peptide, magainin 2 (MW 2465 da). However, this peptide could not be detected from the bacterial cells grown in low Mg^{2+}, suggesting that MgtL–FLAG is synthesized only in high Mg^{2+}. We then carried out a parallel experiment using a plasmid, pYS1475–A71C, which carries an A–C substitution at nucleotide 71 of the 5′LR that changes ^{71}AUG^{73} to ^{71}CUG^{73}, resulting in deletion of the start codon. The MgtL–FLAG peptide could not be detected from the cells harbouring this plasmid grown in low and high Mg^{2+} (Figure 2B). Furthermore, when MgtL was overexpressed in a *Salmonella* wild type harbouring an IPTG-inducible plasmid pUHE-*mgtL*, we were able to detect an *m/z* 2171.38 peak in a MALDI-TOF mass spectrum analysis from an eluent derived from bacteria cells grown in the presence of IPTG and 10 mM Mg^{2+} (Figure 2C, bottom). This peak is specific because it could not be detected from a wild-type cell lysate harbouring control vector (Figure 2C, top).

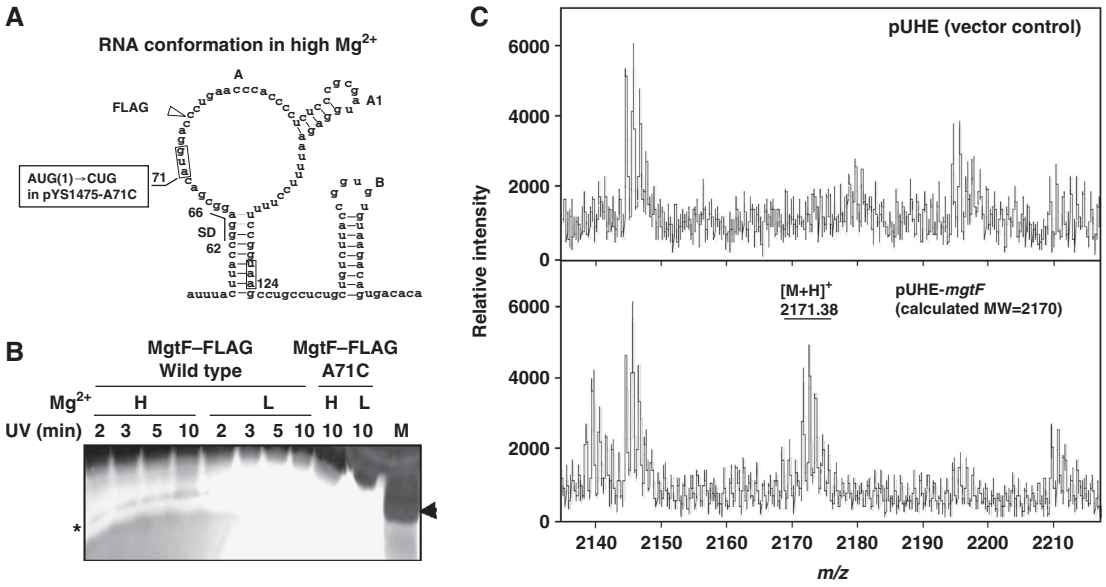


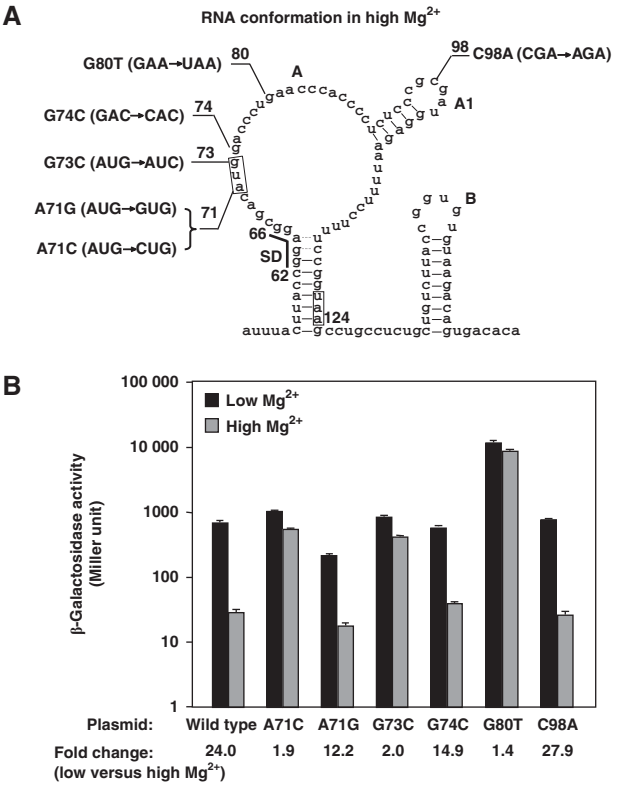
Figure 2 Characterization of the MgtL leader peptide encoded by the 5′LR of *Salmonella mgtA* gene. (**A**) A schematic representation of the FLAG insertion site in plasmid pYS1475 containing *Salmonella mgtA* 5′LR. A site-directed substitution is marked in the small frame, and (1) is the mutated start codon in the MgtL sequence. (**B**) Silver staining of *Salmonella* MgtL peptides. Peptide preparations were derived from *E. coli* Maxicell mutant (CSR603) harbouring pYS1475 and pYS1475-A71C. Bacterial cultures were subjected to UV irradiation (50 J/m^2) for 2, 3, 5, and 10 min to enhance MgtL–FLAG synthesis. H and L represent N medium supplemented with 10 and 0.01 mM Mg^{2+}, respectively. Arrow indicates the position of magainin 2. Asterisk represents MgtL–FLAG bands. (**C**) MALDI-TOF mass spectrum analysis of MgtL from *Salmonella* harbouring vector pUHE (top) and pUHE-*mgtL* (bottom) grown for 4 h in N medium with 0.5 mM IPTG. *m/z* represents the mass-to-charge ratio, and MgtL peptides carry one positive charge.

Premature termination of Salmonella mgtA transcription in high Mg^{2+} is coupled to mgtL translation initiation

The observation that *mgtL* translation and premature termination of *mgtA* transcription both occur in the 5′LR in high Mg^{2+} suggests that these convergent phenomena are coordinated in response to Mg^{2+}. Thus, we hypothesize that MgtL synthesis is a prerequisite for the premature termination of *mgtA* transcription. We constructed a set of pYS1010 derivatives with site-directed substitutions inside the *mgtL*-coding region (Figure 3A and C), and determined *lacZ* expression in *Salmonella* wild-type cells harbouring these plasmids. In contrast to the result from parental pYS1010, *lacZ* expression in cells carrying pYS1010-A71C, in which MgtL could not be synthesized due to disruption of the start codon (Figure 2A and B) remained activated in high Mg^{2+} because β-galactosidase activity was only 1.9-fold lower in high Mg^{2+} than in low Mg^{2+} (Figure 3B). On the other hand, *lacZ* transcription from cells harbouring pYS1010-A71G, which also carried a substitution at nucleotide 71, but changed ^{71}AUG^{73} to another start codon ^{71}GUG^{73} (Figure 3A and C), was repressed in high Mg^{2+} because β-galactosidase activity was 12.2-fold lower when grown in high Mg^{2+} than in low Mg^{2+} (Figure 3B). To further determine the importance of the start codon, we tested pYS1010-G73C in which the start codon was disrupted by a substitution at nucleotide 73 (Figure 3A and C). Comparable to pYS1010-A71C, β-galactosidase activity from cells with pYS1010-G73C was only two-fold lower in high Mg^{2+} than in low Mg^{2+} (Figure 3B). Apparently, if *mgtL* fails to be translated, high Mg^{2+} is not sufficient to prematurely terminate *mgtA* transcription. The 5′LR in another plasmid, pYS1010-G74C, which contains a substitution at nucleotide G74 that changes the second amino acid from Asp to His without interfering with *mgtL* translation (Figure 3A and C) remained responsive to Mg^{2+} because β-galactosidase activity in high Mg^{2+} was 14.9-fold lower than in low Mg^{2+} (Figure 3B). Collectively, these observations demonstrate that *mgtL* translation is essential for the premature termination of *mgtA* transcription in high Mg^{2+}.

Disruption of mgtL translation elongation prevents the premature termination of mgtA transcription in high Mg^{2+}

We created a stop codon within the *mgtL* ORF to determine whether interference of its translation elongation could inhibit premature termination of *mgtA* transcription in high Mg^{2+}. The plasmid, pYS1010-G80T, harbours a substitution that replaces the fourth codon, ^{80}GAA^{82} (Glu), with stop codon ^{80}UAA^{82} (Figure 3A and C), in which *mgtL* translation should be stopped prematurely. β-Galactosidase activity from cells harbouring this plasmid in high Mg^{2+} was only 1.4-fold lower than that in low Mg^{2+} (Figure 3B), indicating that *mgtA* transcription could not be prematurely terminated due to the nonsense point mutation. Furthermore, a recent study showed that a substitution, C98U, in the *mgtA* 5′LR resulted in *mgtA* expression in high Mg^{2+} (O'Connor *et al*, 2009). This mutation changes the tenth codon, ^{98}CGA^{100} (Arg), to a stop codon ^{98}UGA^{100}, thus causing a premature stop of *mgtL* translation at a codon far downstream of ^{80}GAA^{82} (fourth codon). We constructed a plasmid pYS1010-C98A, which carried a substitution at the same nucleotide, C98, and generated a silent mutation (Figure 3A and C) and found that β-galactosidase activity was 27.9-fold lower in high Mg^{2+} than in low Mg^{2+} (Figure 3B), indicating that the 5′LR carrying a substitution of C98A, unlike C98U, remained responsive to Mg^{2+}. With these results and the observation



C

Substitution in pYS1010	Characteristic of substitution	Effect on premature termination of *mgtA* transcription in high Mg^{2+}
Wild type	NA	Termination
A71C	Change start codon to Leu codon	No termination
A71G	Change start codon to another start codon	Termination
G73C	Change start codon to Ile codon	No termination
G74C	Change Asp-2 codon to His codon	Termination
G80T	Change Glu-4 codon to stop codon	No termination
C98A	Arg-10 codon, no change	Termination

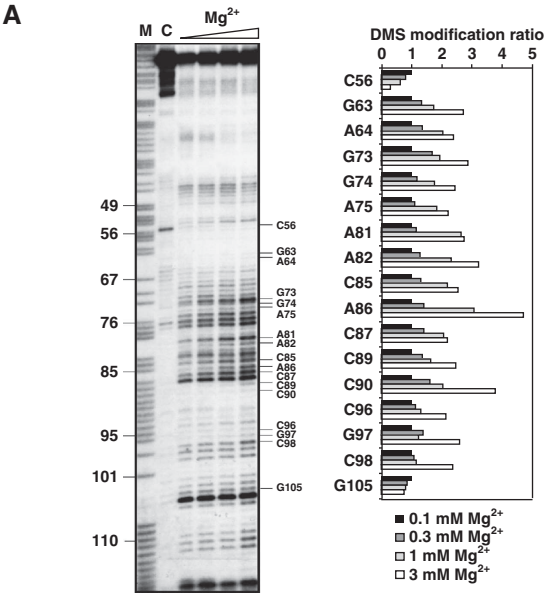
Figure 3 Genetic evidence demonstrates that MgtL translation is coupled to premature termination of *mgtA* transcription in the *mgtA* 5′LR. (**A**) Illustration of the substituted nucleotides in pYS1010 derivatives. Numbering represents the positions of the nucleotides in the *Salmonella mgtA* 5′LR. Framed sequences represent the start and stop codons. The SD sequence is underlined. (**B**) β-Galactosidase activity was determined in *Salmonella* wild-type 14028s, which harboured wild-type plasmid, pYS1010, or one of the substituted derivatives shown in (**A**). Bacteria were grown for 4 h in N medium supplemented with 0.01 mM (low) or 10 mM (high) Mg^{2+}. Fold change was determined by β-galactosidase activity from low Mg^{2+} divided by activity from high Mg^{2+}. Assays were conducted in triplicate. Error bars correspond to the standard deviation. (**C**) Effect of the substitutions of the *mgtL* ORF on premature termination of *mgtA* transcription based on the results from (**B**).

that the full-length MgtL peptide is detected specifically in high Mg^{2+} (Figure 2B), we propose that *mgtL* translation should be completed in high Mg^{2+} to prematurely terminate *mgtA* transcription.

Mg^{2+} concentration modulates a stem switching within stem-loop A that determines conformation of the ribosome-binding site for mgtL translation

We synthesized the full-length 264-nt *mgtA* 5′LR and probed the stem-loop A structure in different Mg^{2+} conditions using dimethyl sulphate (DMS) which modifies adenosine, cytidine, and guanosine when located in single-stranded regions. A primer extension assay, in which the reverse transcription reaction is disrupted at the modified nucleotides in RNA templates, showed that ^{62}GGAGG^{66}, proposed to be the SD sequence here (Figure 1B), was located in a double-stranded region in low Mg^{2+}, however, in a single-stranded region in high Mg^{2+} (Figure 4A). The nucleotides G63 and A64 in the SD sequence were modified 2.7- and 2.4-fold more in high Mg^{2+} (3 mM) than in low Mg^{2+} (0.1 mM), respectively (Figure 4A), indicating their locations in a single-stranded region in high Mg^{2+} regardless of simulated base pairs (Cromie *et al*, 2006). Furthermore, G73, G74, C85, A86, C96, and G97 were modified 2.9-, 2.4-, 2.6-, 4.7-, 2.1-, and 2.6-fold more in high Mg^{2+} than in low Mg^{2+}, respectively, implying that they are base paired or protected in a stem (named D, Figure 1A) formed in low Mg^{2+}. Based on these observations, we proposed that the sequence, ^{91}UCUCC^{95} (named anti-SD), can form part of stem D by base pairing with the SD sequence in low Mg^{2+}; and can alternatively form stem A1 with the sequence, ^{102}GGAGA^{106} (named anti-anti-SD), in high Mg^{2+} in which the SD site is accessible for *mgtL* translation (summarized in Figure 6). Consistent with this, substitution of the anti-SD sequence in stem A1 with ^{91}AGAGG^{95} enhanced premature transcription termination regardless of Mg^{2+} because β-galactosidase activity in a wild-type strain harbouring this substituted plasmid (pYS1010-A1-sub) grown in low Mg^{2+} was as low as that in wild-type strain harbouring the wild-type plasmid grown in high Mg^{2+} (Figure 4B, also see ref. Cromie *et al* (2006)). To determine the role of the anti-SD sequence, we used DMS to map the full-length RNA carrying this substituted sequence. We found that G63 and A64 in the SD sequence was modified regardless of Mg^{2+} at a similar level as wild type in high Mg^{2+} (Figure 4C), indicating that without the anti-SD sequence, the SD sequence remained single stranded in low and high Mg^{2+}, causing constitutive repression of *mgtA* transcription likely due to the continuous translation of *mgtL*. Introduction of a second substitution to pYS1010-A1-sub, which replaced ^{102}GGAGA^{106} with ^{102}CCUCU^{106} to form pYS1010-A1-rev, complemented the first substitution by creating a modified stem-loop A1 and restored a wild-type-like response to Mg^{2+} (Figure 4B, also see ref. Cromie *et al* (2006)). It is likely that ^{102}CCUCU^{106} forms a new anti-SD sequence that base pairs with the SD sequence, thus resulting in inhibition of *mgtL* translation similar to wild type in low Mg^{2+}; whereas the ^{91}AGAGG^{95} becomes a new anti-anti-SD sequence in the double substituted 5′LR and turns on *mgtL* translation in high Mg^{2+}. This was supported by a DMS probing assay using this double substituted full-length RNA in which, like wild-type RNA, the nucleotides G63 and A64 in the SD sequence were protected from DMS modification in low Mg^{2+} (Figure 4C). Additional mapping of the full-length wild-type RNA with RNase T1, which cleaves unpaired G residues, revealed that high Mg^{2+} facilitates the accessibility of this nuclease to G65 and G66 located in the SD sequence because they were cleaved 3.4-fold more in high Mg^{2+} than in low Mg^{2+} (Supplementary Figure S2), suggesting that the SD site was localized in a single-stranded region in high Mg^{2+} making it more accessible. In contrast, G105 in the anti-anti-SD sequence was cleaved 3.7-fold more in low Mg^{2+} than in high Mg^{2+}, implying that it should be located

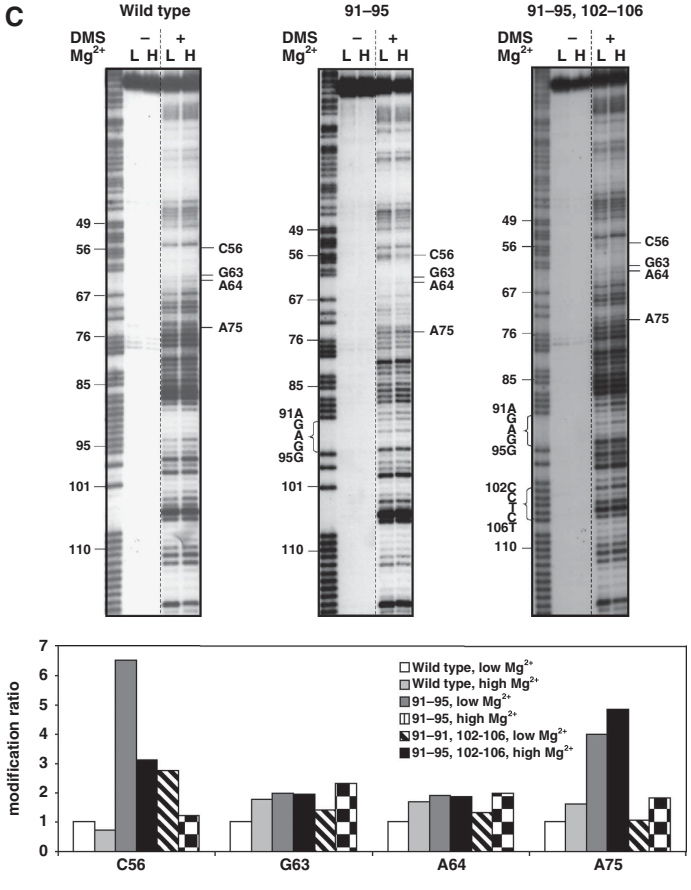
in double-stranded region by base paring with the anti-SD sequence in high Mg^{2+}, however, located in a single-stranded region when the anti-SD sequence is switched to form stem-loop D in low Mg^{2+} (Figure 6). Collectively, these results provide evidence that Mg^{2+} controls the accessibility of the *mgtL* SD sequence via a stem-loop switching that determines formation of stem-loop A1 and D by which it modulates *mgtL* translation.



B

		β-Galactosidase activity (Miller unit)	
		Low Mg^{2+}	High Mg^{2+}
pYS1010 (wild type)	62 ggaggcgacauggacccugaacccaccccucucccgcgauggaga 106	764±27	26±2
pYS1010-A1-sub (91–95)	----------agagg----------	43±3	31±2
pYS1010-A1-rev (91–95, 102–106)	----------agagg------ccucu	681±39	37±3

Stem-loop A1



Similar stem-switching domains that determine the accessibility of the SD site for *mgtL* translation were also found in additional species, such as *E. coli*, *K. pneumoniae*, and *C. rodentium* (Figure 1B). It remains to be investigated how the SD site is modulated in *E. chrysanthemi*, *S. marcescens*, and *Y. enterocolitica*.

Investigation of premature termination of mgtA transcription influenced by amino acids conserved in the MgtL sequence

To determine whether the four Pro residues in *Salmonella* MgtL sequence (Figure 1B) are involved in *mgtL* translation-coupled transcriptional regulation of *mgtA*, we constructed four plasmids pYS1010-C77G, pYS1010-C83G, pYS1010-C89G, and pYS1010-C95G which harbour single substitutions at nucleotides 77, 83, 89, and 95, respectively, and therefore substitute the individual Pro codons to Ala codons. β-Galactosidase activity from wild-type cells with the substituted plasmids remained responsive to Mg^{2+} similar to the wild-type plasmid (Figure 5A). In addition, we constructed a plasmid that replaced the Pro-3 and Pro-5 codons, which are conserved in the *mgtA* 5′LRs from various enteric bacteria (listed in Figure 1C), with Ala and found that this double substitution still retained an Mg^{2+} response similar to the wild-type plasmid. Furthermore, there are three Arg residues in the *Salmonella* MgtL sequence (Figure 1B). Therefore, we assayed the function of the 5′LR by depleting the amino acids from the medium in proline and arginine auxotrophs. It was presumed that reduction of the cytoplasmic concentration of proline or arginine may slow down or prematurely stop *mgtL* translation due to a possible ribosome stalling and subsequent inhibition of premature termination of *mgtA* transcription. However, β-galactosidase activity from the auxotrophs (constructed using one-step gene disruption strategy; Datsenko and Wanner, 2000) carrying pYS1010 grown with low and high Mg^{2+}, and 0 and 10 mM proline (for *proC* mutant, Deutch *et al*, 1982; YS14029) or 10 mM arginine (for *argA* mutant, Marvil and Leisinger, 1977; YS12014) was similar to that from wild-type strain harbouring pYS1010 (Figure 5B), suggesting that starvation of these amino acids does not affect the 5′LR-mediated premature termination. Similar to the results from *Salmonella*, β-galactosidase activity from *E. coli* proline auxotrophic mutants, *proB* (Smith *et al*, 1984) and *proC*, as well as arginine auxotrophic mutants, *argB* (Parsot *et al*, 1988) and *argE* (Meinnel *et al*, 1992), harbouring pYS1010 was similar to wild type in each Mg^{2+} condition regardless of the proline or arginine concentration (Supplementary Figure S3D).

A recent study compared the level of *mgtA* mRNA transcribed from its native promoter and proposed that the 5′LR could respond to cytoplasmic concentration of proline (Park *et al*, 2010). On the contrary, we found that in high Mg^{2+} without supplemented proline the 5′LR-dependent *mgtA* transcription in the *proC* mutant was similar to that in wild type (Figure 5B), although the cytoplasmic proline level was much higher in the *Salmonella* wild type than in the *proC* mutant (~3.7 and 2.4 mmol/g protein, respectively, Figure 5C). When *proC* mutant was grown in high Mg^{2+} with supplemented proline (10 mM), which significantly raised the cytoplasmic proline concentration (from ~2.4 to 3.4 mmol/g protein), β-galactosidase activity remained similar to that without proline (Figure 5B). These results indicate that intracellular proline does not influence 5′LR-dependent *mgtA* expression. Noteworthy, the proline levels in wild type grown in high Mg^{2+} are actually lower than that grown in low Mg^{2+}, ruling out the possibility that high Mg^{2+} facilitates premature transcription termination by raising the cytoplasmic proline levels to enhance *mgtL* translation.

Furthermore, we repeated our assays using the conditions in a recent study investigating the proline effect on *mgtA* transcription (Park *et al*, 2010). In a time (i.e., 15 min) sufficient to see an ~7-fold Mg^{2+}-dependent response of the 5′LR-dependent *mgtA* transcription in wild-type and *proC* strains, a proline effect was not observed. In fact, the cytoplasmic proline concentration was lower in the *proC* mutant than the wild type in all the tested conditions. Previous results indicate that supplementing proline reduces *mgtA*-coding mRNA levels within 15 min, we found that this time period is too short for the accumulation of cytoplasmic proline because supplementation of proline cannot significantly increase the cytoplasmic concentration (Supplementary Figure S3C). Thus, we prolonged the incubation for 2 h and found that β-galactosidase activity in the *proC* mutant supplemented with proline had similar expression levels as wild type grown in low Mg^{2+} without proline supplemented, while the *proC* mutant expression without proline was significantly reduced (Supplementary Figure S3A) due to the significant reduction of colony forming units (CFUs) (Supplementary Figure S3B). Although the cytoplasmic proline levels from cultures grown in low Mg^{2+} with proline supplemented are lower in the *proC* mutant (3.7 mmol/g protein) than the wild type (6.0 mmol/g protein), the β-galactosidase activity from both remained similar (Supplementary Figure S3A). In addition, we constructed two pYS1010 derivatives harbouring substituted Pro codons at three (5, 7, 9) and four (3, 5, 7, 9) Pro residues, similar to the introduced substitutions at the chromosomal *mgtA* 5′LR in the recent study in which they report a loss

Figure 4 Mg^{2+} modifies the secondary structure of stem-loop A in the full-length 264-nt *mgtA* 5′LR. (**A**) Primer extension of DMS-treated stem-loop A following incubation with 0.1, 0.3, 1, and 3 mM Mg^{2+}. In both (**A**) and (**C**), 6% polyacrylamide gel was used to separate the products. Lane M corresponds to Maxam–Gilbert reaction using DNA fragment amplified from pYS1010 with primers 220 and ^{32}P-labeled 201. Lane C corresponds to a reaction with untreated RNA. Quantification in both (**A**) and (**C**) was conducted using Quantity One software (Bio-Rad). After all bands in a lane were normalized by an unchanged band at different Mg^{2+}, the DMS modification ratio was calculated and shown on the right of (**A**) by comparing with the corresponding band from the sample incubated with 0.1 mM Mg^{2+}. The *x*-axis represents the DMS modification ratio at a nucleotide as calculated while its position is shown on the *y*-axis. (**B**) The *mgtA* 5′LR with substitution at 91–95 does not respond to Mg^{2+} and the transcription is significantly reduced. The SD and stem-loop A1 sequences of plasmid pYS1010 and derivatives with the substitutions are shown. Numbering represents position of nucleotide in the *mgtA* 5′LR. β-Galactosidase activity was determined in *Salmonella* wild-type 14028s, which harboured wild-type plasmid pYS1010 or derivatives. Bacteria were grown for 4 h in N medium supplemented with 0.01 mM (low) or 10 mM (high) Mg^{2+}. (**C**) DMS modification of the full-length 264-nt 5′LR with wild-type or substituted sequences shown in (**B**). The RNA was incubated with 0.1 mM (L) and 3 mM (H) Mg^{2+} before DMS treatment and primer extension. After all bands in a lane were normalized by an unchanged band at different Mg^{2+}, the DMS modification ratio was calculated and shown on the bottom of (**C**) by comparing with the corresponding band from wild-type sample incubated with 0.1 mM Mg^{2+}.

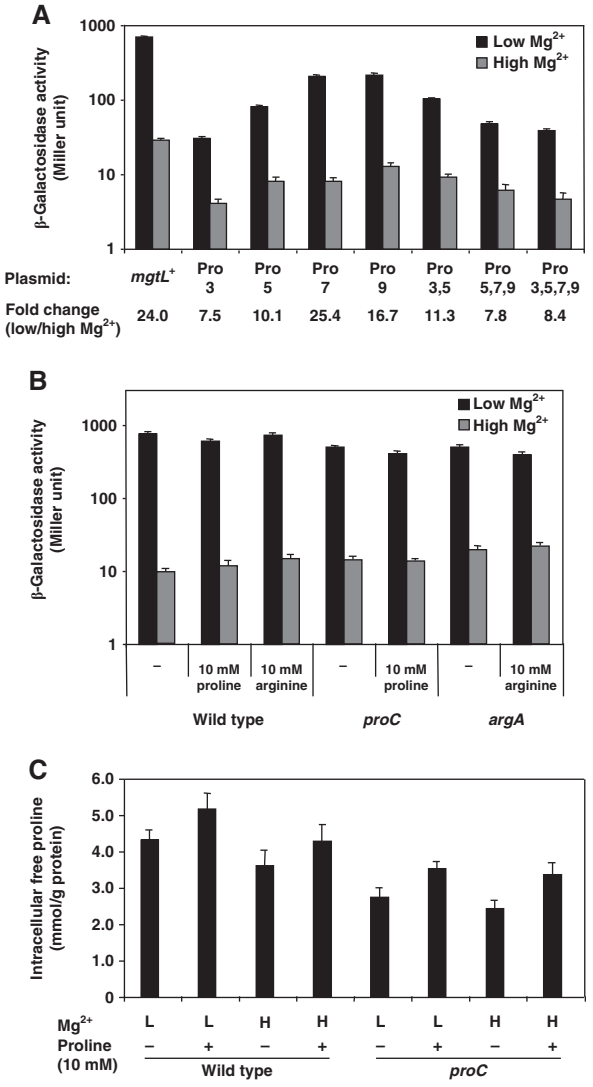


Figure 5 Clarification of the effect of amino acids on premature termination of *mgtA* transcription. (**A**) β-Galactosidase activity was determined in *Salmonella* wild-type 14028s, which harboured wild-type plasmid, pYS1010, or one of the derivatives with substituted Pro codons. Bacteria were grown for 4 h in N medium supplemented with 0.01 mM (low) or 10 mM (high) Mg^{2+}. Fold change was determined by β-galactosidase activity from low Mg^{2+} divided by activity from high Mg^{2+}. (**B**) β-Galactosidase activity determined in 14028s, *proC* mutant (YS14029), and *argA* mutant (YS12014) harbouring pYS1010. β-Galactosidase activity was determined from two groups: one is 14028s and *proC* grown for 4 h in N medium supplemented with 0 or 10 mM proline, and 0.01 mM (low) or 10 mM (high) Mg^{2+}; and the other is 14028s and *argA* grown for 4 h in N medium supplemented with 0 or 10 mM arginine, and 0.01 mM (low) or 10 mM (high) Mg^{2+}. (**C**) Intracellular proline concentration determined in 14028s and *proC* grown as described in (**B**). Assays were conducted in triplicate. Error bars correspond to the standard deviation.

in its ability to respond to proline (Park *et al*, 2010). Although β-galactosidase activity from the *proC* mutant harbouring these plasmids was generally lower, overall, the 5′LR activity was similar in wild-type and *proC* mutant strains harbouring wild-type, triple-substituted, and tetra-substituted plasmids in the conditions (Figure 5A; Supplementary Figure S3A). Taken together, our data does not support the conclusion that the 5′LR responds to proline.

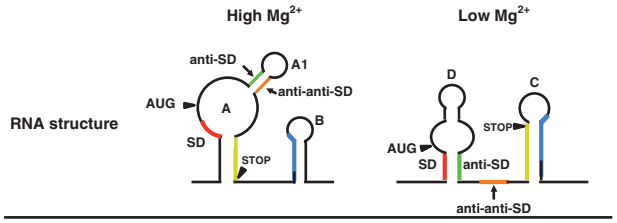
Investigation of the mgtL translation-coupled premature termination of mgtA transcription in vitro

Full-length *mgtA* 5′LR is sufficient to direct *mgtL* translation *in vivo* (Figure 2C), but not *in vitro* (data not shown). To explore the possibility that *mgtL* may be translated from a transcribing *mgtA* 5′LR, we carried out a transcription–translation-coupled *in vitro* reaction using DNA fragments in which the P_{lac1-6} promoter controls the transcription initiation of the *mgtA* 5′LR. We detected the full-length transcript (264-nt) and the truncated transcript (220-nt), which was produced at higher levels in high Mg^{2+} (Supplementary Figure S1A) similarly as a previous *in vitro* transcription result (Cromie *et al*, 2006). However, we could not detect the MgtL peptide in this reaction that could detect a truncated LacZ (~14.8 kDa) in low and high Mg^{2+} at similar protein levels using a control DNA fragment with the P_{lac1-6}-promoted 390-bp *lacZ* coding region (Supplementary Figure S1B). A template containing the P_{lac1-6}-promoted *mgtA* 5′LR followed by the 390-bp *lacZ* coding region could direct synthesis of truncated LacZ at a lower amount from the reaction without supplemented EDTA (i.e., high Mg^{2+}) than that with EDTA (i.e., low Mg^{2+}), yet, MgtL could not be detected (Supplementary Figure S1B). The 5′LR carrying mutation in the anti-SD sequence (A1-sub, 91–95), which resulted in constitutive *mgtL* translation *in vivo*, could produce truncated transcripts, *in vitro,* in high Mg^{2+}, similar to wild type (Supplementary Figure S1A). Thus, it is likely that the *in vitro* system allows the 5′LR to mediate pausing of the downstream *lacZ* transcription in high Mg^{2+}, independent of *mgtL* translation. As MgtL peptide could not be detected from *in vitro* reactions, it is very likely that translation of *mgtL* requires additional cellular factors.

Discussion

A Mg^{2+}-dependent translation of leader peptide within a riboswitch is coupled to the premature termination of transcription

This study presents an example in which a translational unit comprises an integral part of a riboswitch to mediate premature termination of transcription. An ORF is characterized within the 5′LR of the *Salmonella mgtA* gene, which encodes a 17-residue peptide, MgtL. The *mgtL* ORF is embedded in stem-loop A region, which has been regarded as the Mg^{2+}-sensing domain in the Mg^{2+} riboswitch (Cromie *et al*, 2006). We found that this translation occurs specifically in high Mg^{2+}, a condition known to facilitate premature termination of *mgtA* transcription, thus indicating that high Mg^{2+} should stimulate *mgtL* translation that triggers this transcription termination. A typical model of translation-coupled transcription attenuation is the 5′LR that harbours a coding region for the 14-residue TrpL in the *E. coli trp* operon, which includes two tandem tryptophan codons. Lack of tryptophan caused the TrpL-translating ribosome to stall due to the uncharged $tRNA^{Trp}$ at the 5′LR, which triggers modification of the leader RNA to eliminate a downstream terminator structure (a review see Henkin and Yanofsky (2002)). Different from *trp* regulation (i) *mgtA* transcription will not be prematurely terminated in high Mg^{2+} if *mgtL* translation is unable to be initiated due to start codon mutations, in which no ribosome can enter the *mgtL* ORF region (Figure 3A and B); and (ii) cytoplasmic levels of amino acids present repeatedly in the



Ribosome binding	YES	NO
mgtL translation	YES	NO
Premature termination of *mgtA* transcription	YES	NO

Figure 6 Illustration of the *mgtA* 5′LR conformational changes that influence *mgtL* translation. In high Mg^{2+}, the SD site is accessible (in loop A), while the anti-SD and anti-anti-SD sequences are base paired (in stem A1), resulting in *mgtL* translation and premature termination of *mgtA* transcription once translation is completed. In low Mg^{2+}, the SD sequence is base paired to the anti-SD sequence (in stem D) inhibiting *mgtL* translation.

MgtL sequence, such as proline and arginine, are irrelevant to the regulatory activity of the *mgtA* 5′LR because premature transcription termination takes place similarly in wild-type and isogenic auxotrophs (Figure 5A–C; Supplementary Figure S3A, C, and D). These observations rule out the possibility that a stalled ribosome would be required to induce a specific RNA conformation in order to prevent premature transcription termination. The *mgtL* translation has only been detected *in vivo* so far, possibly because this intricate process requires additional cellular component(s) in the translation system. An ongoing project was initiated to identify chromosomal loci that are required for *mgtL* translation in high Mg^{2+} that has led to the identification of a mutant that abrogates premature termination of *mgtA* transcription in high Mg^{2+} (data not shown).

Mg^{2+} induces a conformation change in the stem-loop A RNA region that determines initiation of mgtL translation

Alternative strategy must be adapted for regulation of *mgtL* translation since ribosome stalling does not appear to prematurely terminate translation. Our results suggest that *mgtL* translation is not constitutive, but Mg^{2+} dependent (Figure 2B and C). *mgtL* translation unlikely modulates the *mgtA* 5′LR stem switching because, *in vitro*, Mg^{2+} is sufficient to induce formation of stem-loops A and B (in high concentrations); or alternative stem-loop C (Cromie *et al*, 2006), and likely stem-loop D (in low concentrations). According to the model in Figure 6, we conclude that, in low Mg^{2+}, the SD sequence, ^{62}GGAGG^{66}, is base paired with the anti-SD sequence, ^{91}UCUCC^{95}, to form stem-loop D and prevent access of the ribosome, thus inhibiting translation of MgtL and subsequent premature termination of *mgtA* transcription. However, in high Mg^{2+}, along with the formation of stem-loop A, the anti-SD sequence base pairs with the anti-anti-SD sequence, ^{102}GGAGA^{106}, to form stem-loop A1, thus opening the SD site for *mgtL* translation. Stem-loop D cannot be formed in low Mg^{2+} when the anti-SD sequence is substituted, which leaves the SD site single stranded even in low Mg^{2+} (Figure 4C), resulting in *mgtL* translation and significantly reduced *mgtA* transcription (Figure 4B, and also ref. Cromie *et al* (2006)). Our model suggests that it is the Mg^{2+} signal that determines the *mgtL* translation, not the *mgtL* translation that leads to the Mg^{2+} sensing. That is to say, *mgtL* translation is an intermediate step that relays the Mg^{2+} signal from the Mg^{2+}-sensor domain to the far downstream region in which the premature termination of *mgtA* transcription takes place.

A possible role of mgtL translation in the Mg^{2+} riboswitch function

Premature termination of *mgtA* transcription is abrogated in high Mg^{2+} if the ribosome is either absent due to the lack of *mgtL* translation initiation, or is released early by a nonsense mutation in the *mgtL* ORF at the fourth codon (Figure 3B) or even at the tenth and thirteenth codons located downstream of the four Pro codons (O'Connor *et al*, 2009; Park *et al*, 2010). Thus, it is plausible that completion of *mgtL* translation is a key step that determines premature termination in high Mg^{2+}. This is probably the reason why MgtL peptide can only be detected from bacterial cells grown in high Mg^{2+} (Figure 2B). In addition, *mgtA* transcription cannot be prematurely terminated if the stem-loop B structure is disrupted (Cromie *et al*, 2006), suggesting that both *mgtL* translation and stem-loop B formation are essential for premature transcription termination. However, it is unlikely that *mgtL* translation is required for formation of stem-loop B because it can be induced in high Mg^{2+} *in vitro* without any protein component (Cromie *et al*, 2006). Accordingly, these two regulatory elements likely exert their effects simultaneously. Two possible mechanisms might facilitate premature transcription termination when *mgtL* translation is completed: (i) *mgtL* translation may bring the ribosome to a position that facilitates termination of *mgtA* transcription. This is in a manner comparable to the function of some eukaryotic upstream ORFs (uORF) in which the post-translation release of the ribosome subunit(s) can cause destabilization of the downstream coding region (Vilela and McCarthy, 2003). Thus, the *mgtL* ORF sequence may provide a 'track' that, once opened in high Mg^{2+}, allows a ribosome to reach a region to the *mgtL* stop codon or a far downstream and enables premature transcription termination. This is why the ribosome has to be introduced by the *mgtL* translation *in cis* because a ribosome supplemented in an *in vitro* transcription system could not facilitate premature transcription termination in low Mg^{2+} (Supplementary Figure S1C). The premature termination of transcription takes place at a region far downstream of the *mgtL* stop codon (Kawano *et al*, 2005; Cromie *et al*, 2006). It has been suggested that high Mg^{2+} reduced the 5′LR *mgtA* transcript stability in an RNase E-dependent manner (Spinelli *et al*, 2008). Furthermore, it is shown that RNase E cleaves the ferric uptake regulator *fur* mRNA when the ribosome cannot initiate translation of the upstream ORF due to its SD site base paired with a *trans*-acting regulatory RNA RyhB (Veæerek *et al*, 2007). We propose that, in high Mg^{2+} when the *cis*-acting anti-SD sequence is unpaired to its target, the SD site of *mgtL*, translation confers a novel function to bring a ribosome on site, probably to interact with stem-loop B, subsequently facilitating RNase E to bind and degrade *mgtA* 5′LR. (ii) The MgtL peptide could function as a *trans*-acting factor and regulate transcription of the *mgtA* downstream region. However, the translation machinery, but not the MgtL peptide itself, most likely has a role in premature termination of *mgtA*

transcription because the MgtL sequence from various species is not well conserved (Figure 1C), and because MgtL synthesized *in trans* from a plasmid cannot prematurely terminate *mgtA* transcription in *mgtL* translation initiation mutants (i.e., A71C and G73C) in high Mg^{2+} (data not shown).

Our conclusions that the Mg^{2+}-dependent synthesis of MgtL is required for premature termination of *mgtA* transcription contradicts a recent report in which they found that *mgtL* translation was Mg^{2+} independent (Park *et al*, 2010). Their conclusions, however, are misleading because (i) their engineered *mgtL–lacZ* fusion contained a deleted *mgtL* stop codon, which disrupted the stem A required for formation of the Mg^{2+}-sensing domain of the 5′LR. (ii) Their 'low Mg^{2+}' condition used to test the *mgtL–lacZ* fusion was indeed a high Mg^{2+} condition (Cromie *et al*, 2006; Cromie and Groisman, 2010) and therefore could not be distinguished by the 5′LR whose Mg^{2+} sensing had been disrupted anyway. (iii) Different from the low-copy number plasmid (pYS1010) that we used to study regulatory function of the *mgtA* 5′LR in which transcription is only regulated by the 5′LR, they determined *mgtA* transcription, particularly its response to proline, from its chromosomal locus, which, in addition to the 5′LR (Cromie *et al*, 2006), is regulated by at least two independent promoters controlled by PhoP, in response to the extracytoplasmic Mg^{2+} (Garcia Vescovi *et al*, 1996) and Rob (Barchiesi *et al*, 2008). It is shown that when the 5′LR is located in its native chromosomal location, it appears to have an additional regulatory function because the C98T substitution in the 5′LR unexpectedly led to constitutive *mgtA* transcription even in high Mg^{2+} (O'Connor *et al*, 2009), in which the transcription initiation is supposed to be repressed by the PhoP/PhoQ system. This result cannot be explained by the premature termination of *mgtL* translation which takes place after transcription is initiated, simply because transcription initiation does not occur. (iv) The regulatory activity of Rob might be changed in altered nutrient conditions they used, such as proline, which should mediate transcription initiated from nucleotide 44 of the 5′LR (Barchiesi *et al*, 2008). Their real-time PCR assay can measure the transcripts of the *mgtA*-coding region, but not the 5′LR due to a primer (Park *et al*, 2010) which corresponds to nucleotides 7–31 of the 5′LR absent in Rob-stimulated transcripts, resulting in biased ratios of the 5′LR to other RNAs.

In summary, our results suggest a model in which *mgtL* translation should function as an intermediate to transduce the Mg^{2+} signal from the upstream signal sensing domain to the far downstream effector domain, thus providing an integral component for the riboswitch function to facilitate premature termination of transcription, such as in *mgtA*, while the Rho-independent terminator and ribozyme structures seem to be absent.

Materials and methods

Bacterial strains and growth conditions

All *Salmonella enterica* serovar Typhimurium strains were derived from the wild-type strain 14028s. Bacteria were grown at 37°C in Luria–Bertani broth or N minimal medium (Snavely *et al*, 1989), pH 7.4, supplemented with 0.1% casamino acids and 38 mM glycerol. When necessary, antibiotics were added at final concentrations of 50 μg/ml for ampicillin and 20 μg/ml for chloramphenicol. *E. coli* DH5α and DH5α T1 (Invitrogen) were used as host for the preparation of plasmid DNA. χ2680 (*recA uvrA phr-1*) was an *E. coli* Maxicell mutant CSR603 (Sancar and Rupert, 1978) used to express MgtL–FLAG from plasmid pYS1475. Amino-acid auxotrophic mutants *proB*, *proC*, *argB*, and *argE* were derived from an *E. coli* wild-type BW25113 in the Keio Knockout Collection (Baba *et al*, 2006). Oligonucleotides used in this study are described in Table I.

β-Galactosidase assay

β-Galactosidase assays were carried out in triplicate using a VERSAmax plate reader (Molecular Device) and the activity (Miller unit) was determined as described (Miller, 1972). Data correspond to three independent assays conducted in duplicate, and all values are mean ± s.d.

Table I Primers used in this study

No.	Sequence (from 5′ to 3′)
201	AGGTAATCCCTCCGCGCCG
220	TAATACGACTCACTATAGTAATTGCCACAAAACTTATGG
241	GGCCTGCTTCTCGCCGAAACGTTTGG
298	GCGTCGACCTTTACACTTTAAGCTTTTTATGTTTATGTTGT GTGGATAATTGCCACAAAACTTATGGATTTATGC
299	CCGCTCGAGGTAATCCCTCCGCGCCGAAGTCAGGCG
768	TCGAATAATAATTCACTAGTGGGGCGCGCACATATGAATA TCCTCCTTAG
769	GATAAGCGCAGCGCCATCAGGCCCCCCTTG GTGTAGGCTGGAGCTGCTTC
794	TCTCCATCGCGGGAGAGGGGTGGGTTCAGGCTTGTCATCG TCGTCCTTGTAGTCCATGTCGCCTCCGGT
811	CCCGTGGCGTGACGCTGATGGTGATGAAAACATATGAATAT CCTCCTTAG
812	CCATTGCCCTTCGCTTGAGTAAAGTTACTCGTGTAGGCTGG AGCTGCTTC
903	CCGGATCCTAATTGCCACAAAACTTATG
904	CCCAAGCTTAGGTAATCCCTCCGCGCCGAAG
912	TACTTACCGGAGGCGACCTGGACTACAAGGACGAC
913	GTCGCCTCCGGTAAGTA
1154	AACCCACCCCTCTCC
1243	ATGTCGCCTCCGGTAAG
1244	CTTACCGGAGGCGACATCGACCCTGAACCCACCCCTCTCC
1245	CTTACCGGAGGCGACATGCACCCTGAACCCACCCCTCTCC
1361	CGGGAGAGGGGTGGGTTCAG
1362	GAACCCACCCCTCTCCCGAGATGGAGAATTTTCCTTTTCC
1553	ACTTACCGGAGGCGACCTGGACCCTGAACCCAC
1554	ACTTACCGGAGGCGACGTGGACCCTGAACCCAC
1555	GGGAGAGGGGTGGGTTAAGGGTCCATGTCGCCTC
1557	GGGAGAGGGGTGGGTTCAGCGTCCATGTCGCCTCCG
1559	TTCAGGGTCCATGTCGCCTC
1560	GCGACATGGACCCTGAAGCCACCCCTCTCCCGCGAT
1562	GCGACATGGACCCTGAACCCACCGCTCTCCCGCGATGGA GAAT
1564	GCGACATGGACCCTGAACCCACCCCTCTCGCGCGATGGA GAATTTTCCTT
1578	GTCCATGTCGCCTCCGGTAAG
1579	CGGAGGCGACATGGACGCTGAAGCCACCCCTCTCCCGC GATG
1600	GGGGTGGGTTCAGGGTCC
1601	GACCCTGAACCCACCCCAGAGGCGCGATGGAGAATTTTCC
1602	GACCCTGAACCCACCCCAGAGGCGCGATCCTCTATTTTC CTTTTCCGGTAAGCC
1607	CCATAACACACAAACATAGGGAGTGACGAGCATATGAAT ATCCTCCTTAG
1608	CGAAGTGGCGGCATGACGTCCAGCCGGGCTGTGTAGGC TGGAGCTGCTTC
1609	TTCAGGGTCCATGTCGCC
1610	GGCGACATGGACCCTGAAACCACCCATCTCCTGCGATGG AGAATTTTCC
1611	GTCCATGTCGCCTCCGGTAAG
1612	ACCGGAGGCGACATGGACCTTGAAACCACCCATCTCCTG CGATGGAGAATTTTCC
1613	TATTCATTAAGGTAATCCCTCCGCGCCG
1614	TATTCATTATTCATCAACATTAAATGTGAGC

Preparation and analysis of MgtL peptide

The MgtL–FLAG peptide was expressed in bacteria as follows: an *E. coli* Maxicell mutant CSR603 (χ2680) (*recA uvrA phr-1*), carrying plasmid pYS1475 or pYS1475-A71C was grown in N medium with 0.01 and 10 mM Mg^{2+} at 37°C to mid-log phase. After subjecting the culture to UV irradiation (50 J/m^2), cultures were further incubated with shaking for 20 h. Then, bacterial cells were harvested by centrifugation and resuspended in phosphate-buffered saline (PBS) buffer, and opened by ultrasonication. Cell lysates with a same amount of total protein (protein concentration was determined using the BCA Protein Assay Kit from Pierce) were loaded onto columns each containing 2 ml Red anti-FLAG affinity gel (Sigma). The peptide was eluted with 0.1 M glycine HCl at pH 3.5 and lyophilized. The sample was treated with SDS loading buffer, separated in SDS–PAGE (18% total acrylamide–bisacrylamide monomer, acrylamide:bisacrylamide = 15:1) and then visualized by silver staining using Color Silver Stain Kit (Pierce). The overexpressed MgtL peptide from *Salmonella* harbouring pUHE-*mgtL* was determined as follows: *Salmonella* wild-type 14028s carrying pUHE-*mgtL* was grown with shaking in N medium with 10 mM Mg^{2+} and 0.5 mM lPTG at 37°C for 4 h. Bacterial cells were harvested by centrifugation and resuspended in PBS buffer, and opened by ultrasonication. Cell lysate was ultracentrifuged at 38 000 r.p.m. for 1 h, and the supernatant was passed through a Microcon YM-3 filter (cutoff, 3000 da; Millipore). The pass-through was lyophilized, resuspended in PBS, desalted using ZipTip C18 (Millipore), and analysed by a Bruker Ultraflex MALDI-TOF-MS operated in the positive ion reflector mode.

Enzymatic and chemical probing of the mgtA 5′ leader RNA structure

The *mgtA* full-length 5′LR RNA was synthesized with T7 RNA Polymerase (New England Biolabs) using PCR product as template. The PCR product was generated using wild-type plasmid pYS1010 or derivatives and primers 220 and 201. Probing of the *mgtA* 5′LR RNA with DMS and RNase T1 was carried out as described in a previous study (Cromie *et al*, 2006).

Intracellular proline quantification

Two kinds of growth conditions were used: (i) Bacteria were grown overnight in 2 ml of an N minimal medium (38 mM glycerol, 0.1% casamino acid, and 10 mM $MgCl_2$). Bacteria were harvested, washed in N medium without $MgCl_2$, and resuspended in 2 ml of the same medium. The cell suspension was used to inoculate 15 ml of medium containing 10 mM $MgCl_2$ and 10 mM proline, 10 mM $MgCl_2$ and no proline, 0.01 mM $MgCl_2$ and 10 mM proline, or 0.01 mM $MgCl_2$ and no proline (1:50 dilution). Bacteria were grown for 4 h and harvested to determine β-galactosidase activity, CFUs, and intracellular proline concentration. (ii) Bacteria were grown overnight in 2 ml of a modified N medium (0.2% glucose, 10 mM $MgCl_2$, and 1 mM proline). The overnight culture was used to inoculate 60 ml of the same medium (1:50 dilution) and grown for 3 h at 37°C with shaking. The harvested cells were washed in this modified N medium containing 0.5 mM $MgCl_2$ and grown in 60 ml of the same medium with 1 mM proline for 1 h. The harvested bacteria were washed in the modified N medium containing 0.5 mM $MgCl_2$ and suspended in 0.6 ml of the same medium. This cell suspension was used to inoculate 15 ml of medium containing 0.5 mM $MgCl_2$ and 1 mM proline, 0.5 mM $MgCl_2$ and no proline, no $MgCl_2$ and 1 mM proline, or no $MgCl_2$ and no proline (1:100 dilution). Bacteria were grown for 15 min and 2 h and harvested to determine β-galactosidase activity, CFU, and intracellular proline concentration. For the intracellular proline determination, bacterial cells ($\sim 5 \times 10^9$) were washed in cold PBS, suspended in 0.25 ml of PBS, and disrupted by sonication. The lysate was centrifuged (30 000 *g*, 15 min) and protein concentration in supernatant was determined using the BCA Protein Assay Kit (Pierce). Then the proteins were precipitated from the supernatant using trichloroacetate, followed by centrifugation at 30 000 *g* for 15 min. The intracellular proline concentration was determined by measuring the remaining supernatant solution as described (Bates *et al*, 1973). Briefly, 0.2 ml of the supernatant was incubated with 0.2 ml of acid-ninhydrin (0.25 g ninhydrin dissolved in 6 ml glacial acetic acid and 4 ml 6 M phosphoric acid) and 0.2 ml of glacial acetic acid for 1 h at 100°C. The tubes were then transferred to an ice bath to stop the reaction and the mixtures were extracted with 0.8 ml of toluene. The toluene phase was separated and the absorbance was read at 520 nm with a spectrophotometer. The absorbance was converted to the proline concentration by comparing with a standard curve plotted with a set of known concentrations of proline (Supplementary Figure S4), and normalized by the total protein concentration above.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Conflict of interest

The authors declare that they have no conflict of interest.

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