Genetic Analysis of Riboswitch-mediated Transcriptional Regulation Responding to Mn\(^{2+}\) in Salmonella*

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Background: Divalent cation binding to riboswitch RNAs regulates expression of their transporter genes in bacteria.

Results: Mn\(^{2+}\) interacts with Salmonella riboswitches characterized from Mn\(^{2+}\) transporter mntH and Mg\(^{2+}\) transporter mgtA to modulate transcription of the downstream coding region.

Conclusion: Specific riboswitches control gene expression in response to Mn\(^{2+}\) in bacteria.

Significance: This is the discovery of a Mn\(^{2+}\) riboswitch.

Riboswitches are a class of cis-acting regulatory RNAs normally characterized from the 5’-UTR of bacterial transcripts that bind a specific ligand to regulate expression of associated genes by forming alternative conformations. Here, we present a riboswitch that contributes to transcriptional regulation through sensing Mn\(^{2+}\) in Salmonella typhimurium. We characterized a 5’-UTR (UTR1) from the mntH locus encoding a Mn\(^{2+}\) transporter, which forms a Rho-independent terminator to implement transcription termination with a high Mn\(^{2+}\) selectivity both in vivo and in vitro. Nucleotide substitutions that cause disruption of the terminator interfere with the regulatory function of UTR1. RNA probing analyses outlined a specific UTR1 conformation that favors the terminator structure in Mn\(^{2+}\)-replete condition. Switch sequence GUCAUG can alternatively base pair duplicated hexanucleotide CAUAGC to form either a pseudoknot or terminator stem. Mn\(^{2+}\), but not Mg\(^{2+}\), and Ca\(^{2+}\), can enhance cleavage at specific nucleotides in UTR1. We conclude that UTR1 is a riboswitch that senses cytoplasmic Mn\(^{2+}\) and therefore participates in Mn\(^{2+}\)-responsive mntH regulation in Salmonella. This riboswitch domain is also conserved in several Gram-negative enteric bacteria, indicating that this Mn\(^{2+}\)-responsive mechanism could have broader implications in bacterial gene expression. Additionally, a high level of cytoplasmic Mn\(^{2+}\) can down-regulate transcription of the Salmonella Mg\(^{2+}\) transporter mgtA locus in a Mg\(^{2+}\) riboswitch-dependent manner. On the other hand, these two types of cation riboswitches do not share similarity at the primary or secondary structural levels. Taken together, characterization of Mn\(^{2+}\)-responsive riboswitches should expand the scope of RNA regulatory elements in response to inorganic ions.

Manganese is a redox-active metal, and the manganese ion Mn\(^{2+}\) plays a pivotal role in both eukaryotic and prokaryotic organisms as a required or preferred cofactor in many metalloenzymes including DNA and RNA polymerases, kinases, and various redox enzymes (1, 2). The bacterial pathogen Borrelia burgdorferi even utilizes manganese to bypass host defense by eliminating the need for iron (3). Various metal divalent ion transporters are able to mediate the uptake of Mn\(^{2+}\), thus maintaining the Mn\(^{2+}\) cytoplasmic concentration. In eukaryotic cells, two natural resistance-associated macrophage proteins, Nramp1 and Nramp2, are characterized as divalent cation transporters (reviewed in Refs. 4 and 5). Nramp1, particularly, is a proton-divalent cation antiporter mediating the uptake of Mn\(^{2+}\), Co\(^{2+}\), Fe\(^{3+}\), Zn\(^{2+}\), and others (6). This transporter, expressed exclusively in macrophages, is regarded as a host resistance factor against different intracellular pathogens probably by depleting divalent cations from these bacteria (for reviews see Refs. 4, 7, and 8). Consistently, lack of Nramp1 causes an inability of the murine macrophage to destroy intracellular Salmonella enterica serovar typhimurium, Leishmania donovani, and Mycobacterium bovis (9).

As reported from several studies, the intracellular Mn\(^{2+}\) level of bacteria is detected at an order of 0.01 mM; however, expression of Mn\(^{2+}\) transporters can raise this level by more than 10-fold (0.2–0.3 mM) and readily to the millimolar range under specific conditions (1, 10–12). Many bacteria develop an Nramp1-dependent mechanism to transport Mn\(^{2+}\), as the mntH gene, which encodes an Nramp1 homolog, has been characterized in both Gram-positive and Gram-negative bacteria (13). In Escherichia coli, MntH mediates uptake of Mn\(^{2+}\) and several transition metal divalent ions including Cd\(^{2+}\), Co\(^{2+}\), Fe\(^{3+}\), and Zn\(^{2+}\) (14). Additionally, the sitABCD loci in Salmonella encode a member of the ABC-type ATPase superfamily that mediates Mn\(^{2+}\) transport (12). Both MntH and SitABCD are highly selective for Mn\(^{2+}\) over other divalent cations. It seems that SitABCD is mostly active under alkaline pH conditions (12).

Mn\(^{2+}\) uptake is important for virulence in pathogenic bacteria. A Salmonella strain harboring mutations at both mntH and sitABCD loci exhibited an avirulent phenotype in a mouse infection model (15). On the other hand, Mn\(^{2+}\) overload causes cytotoxicity regardless of its biological importance (16). Bacteria establish Mn\(^{2+}\) homeostasis mainly by modulating expression of the Mn\(^{2+}\) transporters. A transcriptional repressor,

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Salmonella Riboswitches Responding to Mn\(^{2+}\)

MntR, plays a major role in regulating mntH expression in *Bacillus subtilis*. Mn\(^{2+}\) can bind to MntR to facilitate its binding to the mntH promoter via a palindromic sequence, 5′-TTCGCCCTAAGGAAAC-3′, resulting in repression of mntH transcription (17). Transcriptional regulators with low identity (~30%) with *B. subtilis* MntR have also been characterized in many Gram-negative bacteria (18, 19). In *E. coli* and *Salmonella*, binding to Mn\(^{2+}\) facilitates these MntR proteins to interact with a different palindromic sequence termed MntR-box, 5′-AAACATAGCAGGCTATGT-3′, thus implementing repression of mntH transcription (18, 19). The mntH transcription is also partially repressed by Fe\(^{2+}\) via a global iron regulator, Fur, which specifically binds Fe\(^{2+}\) and targets a Fur-binding site in the mntH promoter (11, 20). Importantly, inactivation of Fur disrupts Fe\(^{2+}\)-dependent repression of mntH transcription but retains Mn\(^{2+}\)-dependent repression (19). Besides the negative regulation, mntH transcription is activated through the H\(_2\)O\(_2\)-sensing regulator OxyR, which binds to the OxyR-binding site in the promoter (11, 19). It is known that Fe\(^{2+}\), but not Mn\(^{2+}\), has a high reactivity with peroxide, which generates the reactive hydroxyl radical through a Fe\(^{2+}\)-mediated Fenton reaction. Because Mn\(^{2+}\) is regarded as an antioxidant to counter the effect of Fe\(^{2+}\), facilitation of Mn\(^{2+}\) import in response to oxidative stress may allow Mn\(^{2+}\) to replace Fe\(^{2+}\) in some metalloenzymes to prevent protein damage caused by reactive oxygen species (21).

The 5′-untranslated region (5′-UTR) of particular bacterial genes can exert a regulatory effect on either transcription elongation to the downstream region or translation initiation of the open reading frame (ORF). Many of these 5′-UTRs are riboswitches that bind a specific signal molecule, thus forming an alternative conformation via switching between mutually exclusive base pairs to modulate transcription or translation of the downstream region (for recent reviews see Refs. 22–24). The signal molecules, which are mainly metabolites present in the cytoplasm, interact with the ligand-binding domain (or aptamer) of the riboswitches. Most commonly, the riboswitch domain in a nascent transcript can cause a transcription termination by forming an intrinsic transcription terminator (22, 23, 25). It has been shown that inorganic ions such as Mg\(^{2+}\) and F\(-\) can serve as ligands to interact with specific riboswitches (26–28). It is generally believed that inorganic cations play an important role in neutralizing negatively charged phosphate groups that come into close proximity in the transition states of RNA folding (for a recent review see Ref. 29). Essentially, Mg\(^{2+}\) contributes to the folding of all large RNAs (29). Meanwhile, particular RNA molecules display high specificity to interact with Mg\(^{2+}\). The 5′-UTR of two Mg\(^{2+}\)-transporter genes, *mgtA* from *Salmonella* and *mgtE* from *Bacillus*, responds to Mg\(^{2+}\) through a riboswitch domain regardless of having no homology at the primary or secondary structure, which subsequently facilitates a transcription termination of the downstream coding region (26, 27). It has been shown that six Mg\(^{2+}\) ions residing in the *mgtE* riboswitch stabilize the conformation with a Rho-independent terminator, thus facilitating *mgtE* transcription termination (27). Also, a regulatory mechanism that controls transcription termination is involved in the mgtA Mg\(^{2+}\) riboswitch function via a Rho-dependent terminator (30) as an open reading frame encoding a 17-residue leader peptide that is translated within the 5′-leader region (LR)\(^{2+}\) (31, 32).

In this article, we have provided evidence of *Salmonella* response to Mn\(^{2+}\) through divalent cation riboswitches. By conducting *in vivo* gene expression assays, RNA structural probing, mutational analysis, and *in vitro* transcription experiments, we have established that a 5′-UTR of the mntH mRNA functions as a regulatory element by sensing Mn\(^{2+}\) to determine whether transcription reads through into the mntH coding region or stops within the 5′-UTR. We also have demonstrated that the Mg\(^{2+}\) riboswitch can exert its regulatory effect by responding to Mn\(^{2+}\) in a manner similar to Mg\(^{2+}\).

**MATERIALS AND METHODS**

**Bacterial Strains, Growth Conditions, and Oligonucleotides**—All *S. enterica* serovar *typhimurium* strains were derived from the wild-type strain ATCC14028s and are listed in Table 1. Bacteria were grown at 37 °C in Luria–Bertani (LB) broth or in N minimal medium (33), pH 7.4, supplemented with 0.1% casamino acids and 38 mM glycerol. MnCl\(_2\) and MgCl\(_2\) were added to the required concentrations. When necessary, antibiotics were added at final concentrations of 50 μg/ml for ampicillin, 20 μg/ml for chloramphenicol, and 50 μg/ml for kanamycin. *E. coli* DH5α and BL21-Gold (DE3) were used as hosts for the preparation of plasmid DNA and protein production, respectively. Oligonucleotides used in this study are described in Table 2.

**Construction of Strains with Chromosomal Deletions, lac Fusions, FLAG Fusions, and Site-directed Mutations**—Strains harboring deletions and FLAG fusion were generated as described previously (34). If needed, the antibiotic resistance cassette was removed using plasmid pCP20. Deletion of the mntH, mntR, and fur genes was carried out using primer pairs 144 and 145, 1253 and 1254, and 1262 and 1263, respectively, to amplify the kanamycin resistance cassette (Km\(^{5}\)) from plasmid pKD4 and integrate the resulting PCR product into the chromosome. A lac gene was integrated in the deleted chromosomal mntH location using plasmid pKG137, which was inserted into the FLP recombination target sequence generated after the Km\(^{5}\) cassette was removed (35). mntH-lac strain which contained substitution of the mntH 5′-UTR, stem-R[mut], was constructed using primer pair 145 and 1575 to amplify the PCR product from the chromosomal DNA of the ΔmntH:Km strain; the product was electroporated into wild-type cells harboring pKD46, and then Km\(^{5}\) colonies were selected, and substitution was confirmed. Construction of the strain harboring a chromosomal copy of the mntH-FLAG (C terminus) fusion was carried out using primer pair 7 and 8 to amplify the kanamycin resistance cassette (Km\(^{5}\)) from plasmid pKD4 and integrate the resulting PCR product into the chromosome of a *Salmonella* strain that carried a chromosomal corA-FLAG fusion (our laboratory collection).

**Plasmid Construction**—All plasmids used in this study are listed in Table 1. pYS1300 was constructed using PCR fragments containing mntH full-length UTR1 generated with primer pair 1307 and 1308 and wild-type 14028s chromosomal

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2 The abbreviations used are: LR, leader region; DMS, dimethyl sulfate.
**TABLE 1**

**Bacterial strains and plasmids used in this study**

| Strain or plasmid | Description | Reference or source |
|-------------------|-------------|---------------------|
| *S. enterica serovar typhimurium* | | |
| 14028s | Wild type | ATCC |
| YS13290 | ΔmntH-lacZY | This work |
| YS13291 | ΔmntH-lacZY ΔmntR | This work |
| YS14063 | ΔmntH-lacZY stem- R[mut] | This work |
| YS14065 | ΔmntH-lacZY stem- R[mut] ΔmntR | This work |
| YS14261 | ΔmntR Δfur | This work |
| YS10211 | mntH-FLAG corA-FLAG | This work |

**E. coli**

| | | |
| DH5α | F- supE44 ΔlacU169 (d80 lacZΔM15) thi-1 relA1 | New England Labs |
| BL21-Gold (DE3) | E. coli B F- ompT hsdS B (rB 1 -dcm) | Agilent Technologies |

**Plasmids**

| pKD4 | repB, γAp, FRT Km<sup>+</sup> | Ref. 34 |
| pKD46 | rep<sup>Mrec48</sup> Ap<sup>+</sup> ParcBAD γβ exo | Ref. 34 |
| pCP20 | rep<sup>Mrec48</sup> Ap<sup>+</sup> Cm<sup>+</sup> cI857 Ap<sup>+</sup> | Ref. 34 |
| pKg137 | repB, γAp FRT lacZY | Ref. 35 |
| pACYC184 | pET28a | New England Labs |
| pUHE21-2lac<sup>4</sup> | | |
| pYS1000 | | |
| pYS1010 | rep<sup>Plac</sup> Ap<sup>+</sup> lacP<sup>+</sup> | Ref. 36 |
| pYS1011 | rep<sup>Plac</sup> Cm<sup>+</sup> Plac-lacZ | Ref. 36 |
| pYS1017 | | |
| pYS1100 | mgA 5′-LR lacZ<sub>hu</sub> | This work |
| pYS1300 | mgA 5′-LR lacZ<sub>hu</sub> Stem-loop B<sup>−</sup> | This work |
| pYS1301 | mgA 5′-LR lacZ<sub>hu</sub> Wild-type lacZ<sub>hu</sub> | This work |
| pYS1331 | mgA 5′-LR lacZ<sub>hu</sub> Stem-[mut] lacZ<sub>hu</sub> | This work |
| pET28a | | |
| pYS1466 | | |
| pYS1008 | | |
| pYS1013 | rep<sup>Plac</sup> Ap<sup>+</sup> lacP<sup>+</sup> mntH-FLAG (D34E) | This work |
| pYS1014 | rep<sup>Plac</sup> Ap<sup>+</sup> lacP<sup>+</sup> mntH-FLAG (E102D) | This work |
| pYS1020 | rep<sup>Plac</sup> Ap<sup>+</sup> lacP<sup>+</sup> corA | This work |

DNA as template. These fragments were digested with PstI and XhoI and then ligated between the PstI and XhoI sites of pYS1000 (36). Derivatives of pYS1000 with nucleotide substitutions were constructed using the GeneTailor site-directed mutagenesis system (Invitrogen) with Platinum Taq Polymerase High Fidelity (Invitrogen) and pYS1300 DNA and the following primer pairs: for pYS1301, 1341 and 1342; and for pYS1331, 1543 and 1544. pYS1466 was constructed using PCR fragments containing the mntR coding region generated with primer pair 1630 and 1631 and 14028s chromosomal DNA as template; these were digested with NdeI and XhoI and then ligated between the NdeI and XhoI sites of pET28a (EMD Biosciences). pYS1008 (a FLAG epitope in the C terminus of MntH) was constructed as follows. A PCR fragment containing the mntH coding region with its Shine-Dalgarno sequence that was generated with a primair pair 12 and 13 and 14028s chromosomal DNA as template was digested with BamHI and HindIII and cloned into pUHE21–2lac<sup>4</sup> that had been digested with the same enzymes. Derivatives of pYS1008 with nucleotide substitutions were constructed through site-directed mutagenesis (described above) using the following primer pairs: 20 and 21 for pYS1013 (D34E) and 23 and 24 for pYS1014 (E102D). pYS1020 was constructed using a PCR fragment containing the corA coding region with its Shine-Dalgarno sequence that was generated with primer pair 105 and 106 and 14028s chromosomal DNA as template was digested with BamHI and HindIII and cloned into pUHE21–2lac<sup>4</sup>, that had been digested with the same enzymes. The pYS1010 derivative pYS1011, with nucleotide substitutions at stem-loop B, was constructed through site-directed mutagenesis using primer pair 17 and 18.

In *Vitro* Transcription Assays Using *E. coli* RNA Polymerase Holoenzyme—Linear DNA templates containing the P<sub>lac1–6</sub> promoter region, the mntH full-length UTR, and the first 62 nucleotides (nt) of the lacZ ORF were generated by PCR from pYS1300 and its derivatives using primers 241 and 1302. The template harboring the full-length mgtA 5′-UTR was amplified using plasmid pYS1010 DNA and primers 201 and 248. A control template containing the P<sub>lac1–6</sub> promoter and the first 151 nt of the lacZ gene was amplified from pYS1000 (36) using primers 241 and 565. *In vitro* transcription was carried out as described (37). Briefly, 1 unit of *E. coli* RNA polymerase α<sup>70</sup> holoenzyme (Epicientre) was incubated with 0.5 μg of template DNA in 35 μl of transcription buffer, which contained 100 mM Tris·HCl, pH 8.0, 100 mM NaCl, 0.2 mM EDTA, 0.2 mM DTT, 50 μg/ml BSA, and 0.35 mM Mg<sup>2+</sup>, at 37 °C for 30 min to form open complexes. RNA synthesis was initiated by adding 15 μl of NTP mixture, which contained 0.32 mM ATP, CTP, and GTP, 0.1 mM UTP, and 2 μCi of [α-<sup>32</sup>P]UTP (PerkinElmer Life Sciences). After a 10-min incubation at 37 °C, transcripts were precipitated with 5 μl of 3 M sodium acetate, pH 5.5, and 150 μl of ethanol, separated in a 6% denaturing polyacrylamide gel electrophoresis, and detected by autoradiography. When necessary, Mn<sup>2+</sup>, Mg<sup>2+</sup>, and Ca<sup>2+</sup> were added to the required concentrations. The length of transcripts was determined by using a DNA sequencing ladder generated from a PCR product amplified with primers 241 and <sup>32</sup>P-labeled 1302 from pYS1300 (for mntH UTR1) or primers 248 and <sup>32</sup>P-labeled 201 (for mgtA 5′-LR), and degraded using the Maxam-Gilbert sequencing reaction.

**Primer Extension**—Bacteria were grown to mid-exponential phase (A<sub>600 nm</sub> of 0.4–0.6) in 50 ml of N minimal medium, pH 7.4, containing 10 mM MgCl<sub>2</sub> and 0 or 0.05 mM MnCl<sub>2</sub>, and total RNA was isolated from the harvested bacterial cells using the SV total RNA isolation system (Promega) according to the manufacturer’s specifications as described previously (38). The primer extension assay was performed in a 25-μl reaction with 10 μg of total RNA, <sup>32</sup>P-labeled primer 1301 (complementary to the 31–50 nt of the mntH ORF), 100 units of M-MuLV reverse transcriptase (Promega), and 1× reaction buffer and incubated at 42 °C for 2 h. The cDNA were synthesized and resuspended in 10 μl of H<sub>2</sub>O after precipitation with ethanol. Samples (3 μl) were analyzed by 6% denaturing
polyacrylamide gel electrophoresis by comparison with a DNA ladder amplified from chromosome with primers 1256 and 32P-labeled 1301 and generated by Maxam-Gilbert reaction.

Isolation of MntR-His<sub>6</sub>—E. coli BL21-Gold (DE3) harboring plasmid pET28a-mntR (pYS1466) was grown at 37 °C with shaking to an OD<sub>600</sub> of 0.5 in 500 ml of LB medium; then IPTG (final concentration, 1 mM) was added, and the bacteria were incubated for 2 h. Cells were harvested, washed with PBS once, resuspended in 10 ml of PBS, and opened by sonication. The whole cell lysate was used for MntR-His<sub>6</sub> purification by mixing with His-Select nickel affinity gel (Sigma) following the instructions of the manufacturer. A pure MntR-His<sub>6</sub> sample was tested with His-Select nickel affinity gel (Sigma) following the instructions of the manufacturer.

DNase I Protection Assays—DNase I protection assays were carried out using DNA fragments amplified by PCR using 14028s chromosomal DNA as template. Prior to the PCR, primer 1583 was labeled with T4 polynucleotide kinase and [γ-32P]ATP. The mntH promoter region was synthesized with primers 1582 and 32P-labeled 1583. Approximately 25 pmol of labeled DNA and 0, 100, or 200 pmol of the MntR-His<sub>6</sub> protein were used in a 100-μl reaction. 0.05 mM Mn<sup>2+</sup> was added to the reaction, and the samples were incubated for 2 h at 37 °C. Samples (3 μl) were analyzed by 6% denaturing polyacrylamide gel electrophoresis by comparison with a DNA ladder generated from the PCR product that was amplified as template and primers 1312 and 1355. A DNA sequencing ladder was generated from the PCR product that was amplified as template and primers 1312 and 1355 using T7 promoter were generated using pYS1300 and its derivatives as templates. The PCR products harboring the degraded products as templates. The PCR products harboring the T7 RiboMax large scale RNA production system (Promega) according to the manufacturer’s instructions using PCR-generated products as templates. The PCR products harboring the T7 promoter were generated using pYS1300 and its derivatives as template and primers 1312 and 1355. A DNA sequencing ladder was generated from the PCR product that was amplified using primer 1312 and 32P-labeled 1355 and degraded using the Maxam-Gilbert reaction. Chemical modification of the RNA with dimethyl sulfate (DMS) was carried out as follows. 1 μl of 17.5 mM MgCl<sub>2</sub> was added and incubated at 37 °C for 30 min. The positions of radioactive DNA fragments in the gels were detected by autoradiography.

Structural Probing of the mntH UTR1 RNA Structure—An RNA containing full-length mntH UTR1 was synthesized with a T7 RiboMax large scale RNA production system (Promega) according to the manufacturer’s instructions using PCR-generated products as templates. The PCR products harboring the T7 promoter were generated using pYS1300 and its derivatives as template and primers 1312 and 1355. A DNA sequencing ladder was generated from the PCR product that was amplified using primer 1312 and 32P-labeled 1355 and degraded using the Maxam-Gilbert reaction.
When the specificity of UTR1 to cationic ions was clarified, Mg$^{2+}$ in the solution was substituted by Mn$^{2+}$, Mg$^{2+}$, or Ca$^{2+}$ at the required concentrations. 2 µl of DMS (Acros) was added, and the reaction mixture was incubated at 37°C for 5 min. Then, 20 µl of 3 M sodium acetate, pH 5.2, and 600 µl of cold ethanol were added, and the tube was kept at –80°C for 15 min. The RNA was precipitated by centrifugation at 14,000 rpm for 15 min, and the pellet was washed with 75% ethanol and air-dried. The products were reverse-transcribed using M-MLV reverse transcriptase (Promega) and 32P-labeled primer 1355 upstream of the mntH. Determination of Mn$^{2+}$-facilitated Cleavage of the UTR1 RNA—A cleavage reaction of RNA molecules induced by Mn$^{2+}$ was carried out as follows. One µl of RNA solution (corresponding to 11 µg) was mixed with 153.5 µl of H$_2$O, incubated at 95°C for 5 min, cooled on ice for 30 s, and placed at room temperature for 5 min. 40 µl of 1 M HEPES, pH 8.0, and 5 µl of 2 M KCl were added and incubated at 90°C for 1 min. 2 µl of DMS (Acros) was added, and the reaction mixture was incubated at 90°C for 30 s. The RNA was precipitated and reverse-transcribed.

RESULTS AND DISCUSSION

A MntR-independent Mechanism Contributing to Mn$^{2+}$-responsive mntH Transcription in Salmonella—0.05 mM Mn$^{2+}$ was able to turn off mntH transcription in Salmonella because β-galactosidase activity was reduced to an undetectable level in a mntH-lacZ strain grown in N minimal medium supplemented with Mn$^{2+}$ (Fig. 1). In a mutant harboring deletion of the regulator mntR gene, mntH transcription could still be repressed 2.8-fold by Mn$^{2+}$ although it was not turned off as in the wild type (Fig. 1). This observation implies that Salmonella may regulate Mn$^{2+}$-responsive mntH transcription through a MntR-independent mechanism. When a chromosomal region localized upstream of the mntH ORF and overlapping a previously characterized MntR-binding site (referred to as MntR-box; see Ref. 18) was substituted with an alternative sequence (i.e. stem-R[mut]), this MntR-independent regulation was eliminated because mntH transcription could no longer be repressed by Mn$^{2+}$ and therefore remained similarly activated regardless of Mn$^{2+}$ in a mntR stem-R[mut] mutant (Fig. 1). On the other hand, mntH transcription could still be repressed significantly by Mn$^{2+}$ in a stem-R[mut] mutant, implying that MntR could exert its effect by interacting with a region other than the MntR-box, in the mntH promoter (Fig. 1).

Mn$^{2+}$ Down-regulates mntH Transcription from Two Different Initiation Sites—To gain insights into this regulation, we characterized the promoter region of mntH gene by mapping its transcription initiation site. We carried out primer extension and detected two products from the wild-type strain grown in a low Mn$^{2+}$ condition (Fig. 2A, lane 1). Therefore, mntH transcription is initiated from two locations, which correspond to adenosines located 76 and 18 nt upstream of the mntH ORF, respectively (Fig. 2, A and C, referred to as +1 and +1’ hereinafter). Mn$^{2+}$ (0.05 mM) added to the wild-type culture reduced the level of both transcripts initiated from +1 and +1’ by 5.1- and 7.4-fold, respectively (Fig. 2A, lane 2), suggesting that Mn$^{2+}$ down-regulates the transcription initiated from both starts. We examined the MntR-binding site in the mntH promoter by carrying out DNase I footprinting and found that MntR protein could protect two regions (Fig. 2B) (referred to as R1 and R2, respectively). R1 is located in the −21 and −2 nt upstream of +1, which is protected by MntR mainly in the presence of Mn$^{2+}$ (Fig. 2B, lanes 4–6), whereas R2 is located in the −18’ and −1’ nt upstream of +1’, which is protected under the conditions we tested regardless of Mn$^{2+}$ (Fig. 2B, lanes 1–3 and 4–6). Thus, MntR most likely exerts its inhibitory effect by binding to individual −10 regions upstream of +1 and +1’, respectively (illustrated in Fig. 2C). Consistent with previous results, R2 partially overlaps the MntR-box demonstrated previously (18). A pentanucleotide motif, CAAAG, is shared by the R1 and R2 sequences and is highly conserved in the mntH promoter of E. coli and Shigella (Fig. 2C), and thus it most likely
represents a consensus sequence for MntR binding in these Gram-negative bacteria.

Regulatory Role of the 5’-Untranslated Region from Transcription Start +1 in Response to Mn^{2+}—We examined whether the mntH 5’-UTR could play a role in MntR-independent regulation. Transcription initiated from +1 and +1’ will produce a long 76-nt 5’-UTR (termed UTR1 hereinafter) and a short 18-nt 5’-UTR, respectively. We investigated the regulatory role of the UTR1 by constructing a plasmid (pYS1300) that carried full-length UTR1-lacZ transcriptional fusion under the control of P_{lac1–6} (i.e. a promoter independent of IPTG) (40). P_{lac1–6} is insensitive to Mn^{2+} because the level of β-galactosidase from wild type harboring parental pYS1000, which carries a P_{lac1–6}-controlled lacZ gene (26), is similar regardless of Mn^{2+} (Fig. 3A). β-Galactosidase activity from wild type harboring pYS1300 is 2.6-fold lower in the medium supplemented with Mn^{2+} than without (Fig. 3A). It is still reduced by about 2.6-fold in an mntR fur mutant harboring pYS1300 by supple-

FIGURE 2. Mn^{2+}-responsive mntH transcription is initiated from two start sites, both controlled by MntR. A, mapping transcription start site of Salmonella mntH gene. Primer extension products were generated using primer 1301, and total RNA was isolated from wild-type strain 14028s grown in N-minimal medium, pH 7.4, with 0 (−) and 0.05 mM (+) Mn^{2+}, respectively. M corresponds to a DNA ladder derived from a Maxam-Gilbert A–G reaction. Arrows indicate the transcription start nucleotides (uppercase letters) labeled as +1 and +1’. B, DNase I footprinting analysis of the mntH promoter with increasing amounts of MntR-His protein (0, 50, 100, and 200 pmol) and with 0 (−) and 0.05 mM (+) Mn^{2+}. Solid vertical lines correspond to the R1 and R2 regions protected by the MntR protein. M corresponds to the DNA ladder derived from a Maxam-Gilbert A–G reaction. Numbering in the R1 and R2 sequences are from +1 and +1’, respectively. C, sequence alignment of the upstream region of mntH ORF from chromosome of S. typhimurium (STM), E. coli (ECO), and Shigella flexneri (SFL). The horizontal lines are the previously characterized binding sites for OxyR, Fur, and MntR. The boxed sequences are the MntR protected sequences, R1 (the one overlapping the OxyR binding region) and R2 (the one overlapping the MntR-box). The bold uppercase letters with arrows are the transcription starts, +1 and +1’. The light highlighted sequences are the consensus sequences of the MntR-binding site. The dark highlighted sequence is the putative −10 corresponding to +1. The bold italic letters represent a palindromic sequence in the MntR-box. Underlined sequences represent the mntH start codon. Numbering in the sequence is from the start codon.
activity from wild type harboring pYS1300 was reduced by supplementing 0.05 mM Mn^{2+}, but not Mg^{2+} or Ca^{2+} (Fig. 3C), indicating that these ions could not replace Mn^{2+} to repress transcription through UTR1. To determine whether the UTR1 would respond to a higher concentration of Mg^{2+}, we tested a high (H) (10 mm) and low (L) (0.01 mm) Mg^{2+}, respectively, which were used to determine the regulatory activity of the Salmonella Mg^{2+} riboswitch, i.e. the mgtA 5’-LR (26). Indeed, β-galactosidase activity from wild type harboring pYS1010 (a plasmid that carried a P_\text{lac}–6controlled lacZ fusion with mgtA 5’-LR; also see Ref. 26) grown in N medium with 10 mm Mg^{2+} was 56-fold lower than that with 0.01 mm Mg^{2+} (see Fig. 3D, right panel, Mg^{2+} L/H column). However, the ratio of the β-galactosidase activity from wild type harboring pYS1300 grown in high and low Mg^{2+} was ~1-fold (Fig. 3D, left panel, Mg^{2+} L/H column), indicating that Mg^{2+}, even at a high level, had no effect on UTR1. Furthermore, when Mg^{2+} uptake was enhanced by overexpressing a Salmonella Mg^{2+} transporter, corA, mgtA 5’-LR-controlled transcription was repressed because β-galactosidase activity from wild type harboring pYS1010 and pYS1020 (P_\text{lac}-controlled corA gene) grown in low Mg^{2+} with IPTG (0.5 mm) was ~25-fold lower than that without IPTG (Fig. 3D, right panel, IPTG −/+ column). However, β-galactosidase activity from wild type harboring pYS1300 and pYS1020 remained similar in low Mg^{2+} regardless of IPTG (Fig. 3D, left panel, IPTG −/+ column). Taking these results together, we concluded that the UTR1 is highly specific to Mn^{2+}.

An Intrinsic Rho-independent Terminator within the UTR1—We observed that UTR1 nucleotides 46–65 formed a stem-loop (termed T hereinafter; Fig. 3B, middle panel) when two complementary segments, 46CAUAGC51 (i.e. stem-L) and 56GCUAUG61 (poly(U)), were base-paired to each other and followed by 62UUUU65 (poly(U)). Hence, it is likely a Rho-independent terminator that is responsible for the regulatory function of UTR1. According to this structure, the stem-R[mut] substitution actually changed complementary bases at the right arm of this stem-loop (Fig. 3B, right panel), thus explaining why it caused disruption of the MntR-independent regulation (Fig. 1). We constructed two pYS1300 derivatives, pYS1301 and pYS1331, in which UTR1 contained sequences of stem-R[mut] and stem-L[mut] (Fig. 3B, left panel), respectively, and reconfirmed that disruption of this terminator impaired the regulatory activity of UTR1, because β-galactosidase activity remained at levels similar to the wild-type strain or mntR fur double mutant carrying these plasmids grown in the medium regardless of Mn^{2+}; this gave rise to a ratio change of ~1-fold with and without supplementing Mn^{2+} (Fig. 3A, last four columns) and was also similar to the wild-type strain carrying pYS1300 grown without Mn^{2+} (data not shown). The UTR1 sequence is highly conserved in E. coli and Shigella, and therefore, this Mn^{2+}-responsive regulation is also most likely employed in these enteric bacteria (Fig. 2C). On the other hand, the short (18 nt) 5’-UTR transcribed from the +1’ start is unlikely to mediate a premature termination because it contains only three nucleotides from the region of the intrinsic terminator (Fig. 2C).
UTR1 Independently Facilitates Transcription Termination—We carried out an in vitro transcription assay in which a linear DNA template amplified from pYS1300 was used to produce a 165-nt runoff RNA containing full-length 76-nt UTR1, an upstream 9-nt linker sequence, and a downstream 80-nt lacZ coding region (Fig. 4A). In this in vitro system, the only protein component supplemented was E. coli RNA polymerase/σ^70 holoenzyme, thus ruling out the influence from any cellular regulatory factors. The only product detected in the Mn^2+/σ^70-free condition was the runoff transcript (Fig. 4B, lane 1, designated R), indicating that no terminator structure was formed to stop transcription before it read through the template. Mn^2+ (0.2 mM) significantly enhanced overall transcription and facilitated production of the 72–77-nt terminated transcripts (Fig. 4B, lane 2, designated T). These terminated transcripts contained 63–68-nt UTR1 fragments, indicating that Mn^2+ induced the terminator structure to cause transcription termination occurring from the second nucleotide of the polyuridine sequence (Fig. 4A, red arrowheads). Mn^2+, but no other cations tested, also enhanced production of additional truncated transcripts appearing mainly as 110- and 111-nt bands (Fig. 4B, lane 2, designated U), probably by pausing transcription at nucleotides 7 or 8 of the lacZ sequence (indicated in Fig. 4A by pink arrowheads). Thus, these truncated products should most likely be regarded as runoff transcripts as well because they stopped downstream of the UTR1. As a result, the level of runoff transcripts was reduced to 31% of the total transcripts through the Rho-independent termination in the presence of 0.2 mM Mn^2+/σ^70 (the percentage was calculated by using the formula \[ \frac{R}{R+T+U} \] × 100 after bands were quantified using Quantity One software (Bio-Rad). The M in B and C is a ladder prepared from a Maxam-Gilbert A+G reaction.

FIGURE 4. The UTR1 is sufficient to mediate premature transcription termination in vitro. A, Illustration of the in vitro transcription template with UTR1 wild-type sequence. The boxed sequences in red and blue are a partial P^lac1–6 promoter and mntH UTR1. The +1 corresponds to the transcription start for P^lac1–6. The bold uppercase letter A is the +1′ start. The red arrows indicate the terminator stem sequence. The lacZ fragment is underlined. The red arrowheads indicate the positions where the transcription stops by the terminator. The pink arrowheads indicate the positions where transcription is terminated/interrupted within the lacZ coding region. Blue numbers indicate is the position of nucleotides from +1. B, in vitro transcription of wild-type template without any supplemental divalent cation (−) or 0.2 mM Mn^2+, Mg^2+, and Ca^2+, respectively. Arrows indicate products labeled by their length from reactions. C, in vitro transcription of templates coding wild type, substituted stem-L[mut], and stem-R[mut] UTR1 in the presence of 0, 0.1, and 0.2 mM Mn^2+. A control template (lacZ) contains the first 151 lacZ nucleotides. To determine a Mn^2+-responsive effect through the Rho-independent termination, the percentage of runoff RNAs (R and U) versus all transcripts (R, T, and U) was calculated by the formula \[ \frac{R}{R+T} \] versus \[ \frac{R+U}{R+T+U} \] × 100 after bands were quantified using Quantity One software (Bio-Rad). The M in B and C is a ladder prepared from a Maxam-Gilbert A+G reaction.
nated products than Mn$^{2+}$. This observation is consistent with the in vivo expression assay (Fig. 3C), thus suggesting that UTR1 has a high specificity in response to Mn$^{2+}$. This transcription termination was dependent on Mn$^{2+}$ concentration, because the level of runoff RNA dropped to 48% of the total transcripts when 0.1 mM Mn$^{2+}$ was supplemented (Fig. 4C, lane 2) and was further reduced to 27% when the Mn$^{2+}$ concentration was raised to 0.2 mM (see Fig. 4C, lane 3, similar to the result in Fig. 4B, lane 2). However, it was most interrupted when the stem-R[mut] UTR1 was transcribed because the runoff transcript in Mn$^{2+}$-containing reactions represented almost all (99%) of the total products (Fig. 4C, lanes 8 and 9). Furthermore, the UTR1 transcription using terminator-insensitive T7 RNA polymerase could only produce the runoff transcript regardless of Mn$^{2+}$ (data not shown), confirming that the terminator is essential for transcription termination at the UTR1 63–68-nt region.

Interestingly, the 110- and 111-nt truncated products were still dependent on the UTR1 because they appeared in the Mn$^{2+}$-containing reactions with both the wild type and the substituted templates (Fig. 4, B, lane 2, and C, lanes 2 and 3, 5 and 6, and 8 and 9) and because there were no truncated bands representing termination at nucleotides 7 and 8 of lacZ observed in Mn$^{-}$-containing reactions using a template from pYS1000 that carried $P_{lac-}$-lacZ (the runoff transcript is 179 nt; see Fig. 4C, lanes 10–12). We postulated that the UTR1 might interact with this downstream region in the presence of Mn$^{2+}$ to pause transcription in a terminator-independent manner. Surprisingly, we did not observe the predicted $+1'-$transcript, i.e. a 98-nt band, in these in vitro reactions (Fig. 4, B and C), although the $+1'$-site is located within the cloned UTR1 (indicated by upper case A, Fig. 4A). This was probably because additional regulators, which are absent in our in vitro system, are required to initiate transcription from this start.

The UTR1 Favors a Terminator Conformation in the Presence of Mn$^{2+}$—Mn$^{2+}$ most likely induces a conformational modification of UTR1, by which this 5'-UTR formed the terminator structure to facilitate premature transcription termination. We predicted two UTR1 conformations mainly through canonical base-pairing (Fig. 5A). Structure 1, designated S1, possesses stem-loops A, B, and C. Based on our results, this conformation, which lacks the terminator stem-loop (T), most likely represent a structure favored in low Mn$^{2+}$, thus allowing transcription to pass through UTR1. On the contrary, structure 2 (S2), which contains stem-loops D, E, and T, is likely a conformation favored in high Mn$^{2+}$.

We carried out structural mapping using full-length UTR1 RNAs folded in a buffer supplemented without (−Mn) (Fig. 5B, −) or with 0.35 mM Mn$^{2+}$ (+Mn) (Fig. 5B, +). The folded RNA was treated with DMS, which modifies RNA by methylating unpaired adenosines, cytidines, and guanosines. We tested wild-type RNA and observed that nucleotides A$^{52}$, A$^{53}$, A$^{54}$, and G$^{55}$ are paired in stem $R$ sequence56GCUAUG61 (Fig. 5). Instead, the 46CAUAGC51 sequence, which is predicted to be paired in stem $R$ in S2, appears to be modified significantly by DMS (Fig. 5A, lanes 4, 5, and 9), thereby suggesting that the alternative CAUAGC sequence 46CAUAGC51 may allow the alternative conformation of UTR1 in the thermodynamically favored conformation regardless of Mn$^{2+}$ (Fig. 5C). An in vitro transcription assay using the stem-L[mut] template showed that the runoff RNA could be modified less in Mn$^{2+}$-treated RNA (Fig. 5, lanes 4 and 3, upper panel, and quantified data in lower panel). These results are consistent with the predicted conformation in which they are located in the terminator loop $T$ formed in S2 and thus are likely unpaired and modified more by DMS in +Mn (i.e. high Mn$^{2+}$).

On the other hand, A$^{52}$, A$^{53}$, A$^{54}$, and G$^{55}$ are paired in stem C formed in S1, and indeed, they are modified less by DMS in −Mn (i.e. low Mn$^{2+}$). Contrastingly, A$^{49}$ and G$^{50}$ were predicted to be located in terminator stem $T$ and paired in S2 but unpaired in a single-strand region of S1; thus they are likely modified less by DMS in +Mn than in −Mn. Consistently, our results showed that A$^{49}$ and G$^{50}$ were modified 4.7- and 7.2-fold less, respectively, in Mn$^{2+}$-treated RNA than in Mn$^{2+}$-free RNA (Fig. 5B, lanes 4 and 3).

Unlike the wild-type RNA, DMS modified A$^{52}$, A$^{53}$, A$^{54}$, and G$^{55}$ from stem-R[mut] UTR1 poorly but similarly in both −Mn and +Mn (Fig. 5B, lanes 6 and 7). This suggests that Mn$^{2+}$ could not induce stem switching in this substituted UTR1, and we predicted that these nucleotides would remain paired within stem-loop $C$ regardless of the Mn$^{2+}$ concentration (Fig. 5C). We reasoned that this conformation was thermodynamically favored in this substituted UTR1 because the free energy reduction in stem-loop $C'$ ($\Delta G = -7.7$ Kcal/mol, estimated using the Mfold tool) was greater than that in another conformation ($\Delta G = -3.8$ Kcal/mol), which could only form stem-loops $D$ and $E$ in wild-type S2 ($\Delta G = -9.6$ Kcal/mol) but not $T$ ($\Delta G = -5.8$ Kcal/mol).

UTR1 Forms Terminator Structure through a Mn$^{2+}$ Riboswitch—We found two CAUAGC sequences in UTR1, representing nucleotides 34–39 and 46–51 and alternatively base-pairing stem-$R$ sequence 56GCUAUG61 (Fig. 5A, sequences in dashed-line boxes in red and blue, respectively). We proposed that switching their base-pairing would contribute to the regulatory function of UTR1. In S1, the 34CAUAGC51 sequence is located in loop $C$, meanwhile forming a pseudoknot through pairs with stem-$R$ sequence and thus playing a role as an terminator by preventing the formation of a terminator structure (Fig. 5A). Instead, the 46CAUAGC51 sequence, i.e. stem-$L$, base-pairs with stem-$R$ in S2 to form the terminator (Fig. 5A). Consistently, RNA probing results showed that the stem-$R$ sequence remained paired and could not be modified by DMS in either Mn$^{2+}$-free or Mn$^{2+}$-treated RNAs (Fig. 5B, lanes 3 and 4). The conformational change is also shown by nucleotide A$^{29}$, which is predicted to be paired in stem $A$ in S1, is switched to become unpaired in loop $E$ in S2, and is modified 3.1-fold more in Mn$^{2+}$-treated RNA than Mn$^{2+}$-free RNA. On the contrary, C$^{21}$ and A$^{22}$, which appear to be unpaired in loop A in S1, but paired in stem $E$ in S2, are modified 7.2- and 7.4-fold less, respectively, in Mn$^{2+}$-treated RNA than Mn$^{2+}$-free RNA (Fig. 5B, lanes 4 and 3). On the other hand, C$^{21}$ and A$^{22}$ in stem-$R$[mut] UTR1 were modified significantly by DMS in both low and high Mn$^{2+}$, and the level of modification was similar to that in Mn$^{2+}$-free wild-type RNA; meanwhile, A$^{29}$ remained unmodified under the Mn$^{2+}$ concentrations tested (Fig. 5B, lanes 6 and 7). This demonstrates that loss of the riboswitch due to inability to form stem-loop $T$ is a reason to keep stem-$R$[mut] UTR1 in the thermodynamically favorable conformation regardless of Mn$^{2+}$ (Fig. 5C). An in vitro transcription assay using the stem-L[mut] template showed that the runoff RNA could be reduced to 92 and 88% in 0.1 and 0.2 mM Mn$^{2+}$, respectively (Fig. 4C, lanes 5 and 6). We postulate that lack of the stem-$L$ sequence 46CAUAGC51 may allow the alternative CAUAGC sequence, i.e. 34CAUAGC51, to base pair the stem-$R$ sequence.
56GCUAUG61 (the same base-pairing occurred in the pseudoknot structure of S1 (Fig. 5A)), resulting in the formation of an alternative stem-loop followed by the poly(U) sequence 62UUUU65. Thus, this structure could be an alternative terminator and facilitate termination of stem-L[mut] transcription, despite its functioning less efficiently than stem-loop T (88% of the runoff RNA in the mutant versus 27% in the wild type from reactions containing 0.2 mM Mn2+/H11001; Fig. 4C, lanes 6 and 3). On the other hand, this alternative terminator could not be formed in vivo, probably for some unknown reason, because lacZ expression remained similar to the strains carrying pYS1331 grown in the medium regardless of Mn2+/ (Fig. 3A). Unlike the stem-L[mut] UTR1, elimination of 56GCUAUG61 resulted in the inability of the stem-R[mut] UTR1 to form either terminator structure and thus could not mediate transcription termination in vivo and in vitro (Figs. 3A and 4C).

It is worth pointing out that Mg2+ was supplemented as a component in the RNA structural mapping reaction of Fig. 5B for the sake of the UTR1 transcription in vitro, which requires this cation. Thus, we carried out additional structural mapping using only one given divalent cation to fold wild-type UTR1 RNA. 0.01 mM Mn2+/H11001 (referred to as low Mn2+/H11001 hereinafter), also at a level analogous to the cytoplasmic concentration under the physiological condition (1), caused the UTR1 conformation to resemble that of S1, because the same set of nucleotides was modified similarly by DMS (Fig. 5D, lane 3, arrowheads). Concomitantly, the addition of 0.35

FIGURE 5. RNA structural probing of UTR1. A, schematic representation of the predicted secondary structure of the 76-nt mntH UTR1 from S. enterica serovar typhimurium. Left, S1 conformation, which is postulated to form in low Mn2+. Right, S2 conformation, which is predicted to form in high Mn2+. Sequences in blue and red represent nucleotides that showed stronger modification by DMS in Mn2+-free and Mn2+-supplemented reactions through detection, respectively. Asterisks in red and pink represent nucleotides cleaved more after wild-type and stem-R[mut] UTR1 were incubated with Mn2+, respectively. Dashed line boxes in red and light blue represent repeated CAUAGC sequences that base pair alternatively with 56GCUAUG61. Pink lines indicate a pseudoknot formed between 54CAUAGC and 56GCUAUG. Numbering represents the location from the +1 transcription site. The sequences highlighted in blue and yellow were the substitutions in stem-L[mut] and stem-R[mut], respectively. B, DMS treatment of the full-length UTR1 RNA. Wild-type and stem-R[mut] UTR1 were incubated with 0 (−) or 0.35 mM (+) Mn2+. M corresponds to a ladder prepared from a Maxam-Gilbert A+G reaction. U is the untreated sample. The blue and red arrowheads indicate nucleotides in wild-type UTR1 that are modified by more Mn2+-free and Mn2+-supplemented reactions, respectively. The numbers represent the nucleotides located in the UTR1. The corresponding modification ratio (−Mn2+/+Mn2+) is calculated and shown in the right panel. C, the predicted secondary structure of substituted stem-R[mut] UTR1, which was similar to wild-type S1 except for the pseudoknot. D, comparative DMS analysis of the UTR1 RNA treated with Mn2+, Mg2+, and Ca2+, respectively. Each reaction was supplemented with only one of the given cations and carried out similar to those shown in B. Mn2+ was added to 0.01 mM (L) and 0.35 mM (H), respectively; Mg2+ was added to 0.35 mM (L) and 3.5 mM (H), respectively; and Ca2+ was added to 0.01 mM (L) and 0.35 mM (H), respectively.
Salmonella Riboswitches Responding to Mn\(^{2+}\)

mm Mn\(^{2+}\) (a concentration presumably falling in the range accumulated in the cytoplasm when Mn\(^{2+}\) uptake was enhanced; see Ref. 1) caused C\(^{21}\), A\(^{22}\), A\(^{39}\), and G\(^{50}\) to be modified less by DMS and A\(^{29}\), A\(^{52}\), A\(^{53}\), A\(^{54}\), and G\(^{55}\) to be modified more (Fig. 5, B and D, lanes 4), indicating that this high Mn\(^{2+}\) level likely enhances UTR1 to form the same S2 structure in Mg\(^{2+}\)-depleted and Mg\(^{2+}\)-containing conditions.

We also compared modifications of UTR1 treated by Mn\(^{2+}\) as well as Mg\(^{2+}\) and Ca\(^{2+}\). We chose 0.35 and 3.5 mm Mg\(^{2+}\) to investigate the UTR1 folding; these were used previously as low and high Mg\(^{2+}\) conditions in structural mapping of the mgtA 5′-LR, respectively (26). In comparison to the stem-loop T region in S1 and S2 (Fig. 5A), A\(^{52}\), A\(^{53}\), A\(^{54}\), and G\(^{55}\) were paired in stem C formed in S1 and unpaired in loop T formed in S2, were indeed modified less in low Mg\(^{2+}\) than in high Mg\(^{2+}\) (Fig. 5D, lanes 5 and 6). On the other hand, A\(^{49}\) and G\(^{50}\), which resided in 49CAUAGC\(^{51}\) (stem-L), were modified more in high Mg\(^{2+}\) than in low Mg\(^{2+}\), suggesting that they stayed in a single-strand region in high Mg\(^{2+}\). This contrasted with the Mn\(^{2+}\)-treated reaction (Fig. 5D, lane 4), in which this switching sequence formed stem T (i.e. paired) in S2 (Fig. 5A). Thus, we postulated that Mg\(^{2+}\) is unlikely to facilitate the formation of an intact terminator structure. In addition, we mapped the conformation of UTR1 treated with 0.01 mm (i.e. low) and 0.35 mm (high) Ca\(^{2+}\), respectively. We observed that more nucleotides could be modified by DMS after the RNA was treated in both Ca\(^{2+}\) conditions (Fig. 5D, lanes 8 and 9). Particularly, both the stem-L and stem-R regions could still be modified in high Ca\(^{2+}\) (Fig. 5D, lane 9), probably due to the relaxed structure of the UTR1. Thus, it is also unlikely for this cation to induce a terminator structure in the UTR1.

Mn\(^{2+}\)-facilitated Cleavage at the Specific Nucleotides of the UTR1—Specific divalent metal cations have been shown to induce site-specific cleavage when they bind tRNAs (41–43). Particularly, Mn\(^{2+}\) can induce hydrolysis of yeast tRNA\(^{Phe}\) and Elongator tRNA\(^{Met}\), mainly in their D-loops, and also tRNA\(^{Glu}\) in the anticodon loop (43). We incubated UTR1 RNA in a buffer supplemented with varying amounts of Mn\(^{2+}\) to examine whether Mn\(^{2+}\) could facilitate specific cleavage of UTR1 RNA when it interacted with the riboswitch domain. Specific nucleotides in which the Mn\(^{2+}\)-facilitated cleavage took place were characterized by monitoring truncated UTR1 fragments generated from wild-type full-length UTR1 RNA. We observed RNA fragments containing 47-, 54-, and 55-nt UTR1, respectively, in which the level was proportional to the Mn\(^{2+}\) concentration supplemented (Fig. 6A, lanes 2–4), indicating that Mn\(^{2+}\) induced a strong cleavage of the 3′,5′-phosphodiester bond at nucleotides A\(^{47}\), A\(^{54}\), and G\(^{55}\) in a concentration-dependent manner; these are located in stem-loop T (Fig. 5A). The effect of this ligand was validated by supplementation of EDTA, which significantly reduced cleavage at these sites (Fig. 6A, lane 5). However, all three of these sites were seldom cleaved when stem-R[mut] UTR1 was incubated with Mn\(^{2+}\) at the same levels as for wild-type UTR1. It is worth pointing out that the nucleotides at these sites keep the same nucleotides in the wild type and the substituted UTR1 (Fig. 5, A and C). This suggests

\[ \text{FIGURE 6. Mn}^{2+}\text{-facilitated cleavage of the UTR1 in vitro. A, cleavage of wild-type and stem-R[mut] UTR1 RNA in mixtures supplemented with 0, 0.1, 0.2 mm Mn}^{2+}, \text{and 0.2 mm Mn}^{2+} \text{plus 0.5 mm EDTA. Numbered nucleotides indicate their positions in the UTR1. B, cleavage of wild-type UTR1 RNA in mixtures supplemented with 0.2 mm Mn}^{2+}, \text{Mg}^{2+}\text{ and Ca}^{2+}. \text{M corresponds to a ladder prepared from a Maxam-Gilbert A}+\text{G reaction. U is untreated sample.} \]
that cleavage would take place only if nucleotides 47, 54, and 55 were placed in stem-loop $T$. We also observed minor cleavages occurring at U17 and G42 (Fig. 6A, lane 4). Interestingly, all five of these nucleotides are located in base-paired stem regions in $S_1$ (the conformation without Mn$^{2+}$/H$_{11001}$ligand), but two of them, A$^{54}$ and G$^{55}$, are switched to loop $T$ region in $S_2$. We reasoned that the loop nucleotides might be flexible for Mn$^{2+}$/H$_{11001}$binding. In stem-R[mut] UTR1, the strong cleavage sites were U17 and C27, which could be weakly or rarely cleaved in wild-type UTR1 (Fig. 6A, lanes 9 and 4). Although it remains to be investigated whether specific cleavage of the UTR1 would reflect a direct Mn$^{2+}$ interaction with these specific nucleotides, we postulated that Mn$^{2+}$ could bind to central atoms adjacent to specific cleavage sites located in stem-loop $T$ in wild-type UTR1, but located in to stem-loop $A$ in R[mut] UTR1. The products derived from cleavage at A$^{47}$, A$^{54}$, and G$^{55}$ could not be detected when wild-type UTR1 was incubated with the same amount of Mg$^{2+}$ or Ca$^{2+}$ (Fig. 6B, lane 2 versus lanes 3 and 4), which again demonstrates that the UTR1 is a riboswitch that is highly specific to Mn$^{2+}$.

Salmonella $mgtA$ Mg$^{2+}$ Riboswitch Can Sense Mn$^{2+}$—In vitro, Mn$^{2+}$ can induce similar folding of the Mg$^{2+}$ riboswitch characterized from $B$. subtilis $mgtE$ (27). However, a previous finding showed that Mn$^{2+}$/H$_{11001}$ is unable to repress a $mgtA$ transcript in Salmonella, which is controlled by its leader region (26). We reasoned that 0.025 mM Mn$^{2+}$/H$_{11001}$ supplemented in the experiment could not raise the cytoplasmic concentration of Mn$^{2+}$ over a threshold level to act on this riboswitch. Thus, we introduced a plasmid (pYS1008) carrying the $mntH$-FLAG fusion gene under the control of promoter P$_{lac}$ into the wild-type strain carrying pYS1010. Overexpression of $mntH$ to facilitate Mn$^{2+}$ uptake significantly inhibited the expression of lacZ controlled by $mgtA$-LR, as $\beta$-galactosidase activity in the wild-type strain carrying pYS1010 and pYS1008 was 25-fold lower than that carrying pYS1010 and pUHE21 (vector) when bacteria were grown in N medium with 0.01 mM Mg$^{2+}$/H$_{11001}$.

FIGURE 7. The Salmonella $mgtA$ Mg$^{2+}$ riboswitch also responds to cytoplasmic Mn$^{2+}$ in a manner similar to Mg$^{2+}$. A, $\beta$-galactosidase activity was determined in the wild-type strain harboring pYS1010 and pYS1008 (pmmnH). Bacteria were grown for 6 h in N medium (0.01 mM Mn$^{2+}$) supplemented with 0.5 mM IPTG and 0 or 0.01 mM Mn$^{2+}$. B, $\beta$-galactosidase activity was determined in mntH-FLAG corA-FLAG strain (YS10211) harboring pYS1010 and pYS1008 (pmmnH) or one of the pYS1008 derivatives (pYS1013 (D34E) or pYS1014 (E102D)) grown under the same conditions as in A. C, immunoblot analysis of MntH protein. The level of MntH-FLAG protein from the cultures in B was determined by Western blot. M2 anti-FLAG antibodies (Sigma) were used. Cellular lysate of the wild type (14028S) was used as a negative control of MntH-FLAG (shown as C). Production of CorA-FLAG is dependent on expression of MntH. The mntH-FLAG corA-FLAG strain harboring pUHE21 (vector) was used as a negative control of heterogeneous overproduction of MntH-FLAG (shown as –). D, $\beta$-galactosidase activity was determined in wild-type strain 14028S harboring pYS1008 and pYS1010 or pYS1011 grown in N medium (0.01 mM Mg$^{2+}$/H$_{11001}$) supplemented with 0.5 mM IPTG and 0 or 0.01 mM Mn$^{2+}$. E, in vitro transcription of a template harboring the $P_{lac1–6}$ promoter and the full-length wild-type $mgtA$ 5’-LR sequence conducted in buffer containing 0.35 mM Mg$^{2+}$/H$_{11001}$ without (–) or with (+) the addition (3.5 mM) of one of the tested divalent cations.
Mg\textsuperscript{2+} condition allowing transcription to pass through the 5'-LR (26), 0.01 mM Mn\textsuperscript{2+}, and IPTG (Fig. 7A, right panel, column 2 versus 1). On the other hand, lacZ expression remained similarly activated in the strains harboring pYS1010, and either pYS1008 or pUHE21, when they were grown in the medium with low Mg\textsuperscript{2+} and IPTG but without 0.01 mM Mn\textsuperscript{2+} (Fig. 7A, left panel, column 2 versus 1), indicating that only overexpression of mntH without adding Mn\textsuperscript{2+} was unable to repress this lacZ transcription. It has been shown that Asp\textsuperscript{34} and Glu\textsuperscript{102} residues are conserved in Salmonella and E. coli and are essential for MntH-dependent Mn\textsuperscript{2+} transport (45). We constructed two pYS1008 derivatives, pYS1013 and pYS1014, which directed the synthesis of two substituted MntH proteins in which Asp\textsuperscript{34} and Glu\textsuperscript{102} were changed to Glu\textsuperscript{34} and Asp\textsuperscript{102}, i.e. D34E and E102D, respectively. Expression of lacZ was similar in the wild-type strain harboring pYS1010 and pYS1008 or one of its derivatives under the low Mg\textsuperscript{2+} condition without added Mn\textsuperscript{2+} but with IPTG (Fig. 7B, columns 1–3, left panel). When 0.01 mM Mn\textsuperscript{2+} was added to the strain harboring pYS1010 and wild-type pYS1008, β-galactosidase activity was reduced ~24-fold (Fig. 7B, column 1, right panel versus column 1, left panel). However, Mn\textsuperscript{2+} supplementation could not change lacZ expression in the strain harboring pYS1010 and pYS1013 or pYS1014, as β-galactosidase activity remained similar in these strains regardless of Mn\textsuperscript{2+} (Fig. 7B, columns 2 and 3, right panel versus columns 2 and 3, left panel). Although expression of D34E or E102D MntH was unable to repress mgtA 5'-LR transcription, immunoblot results showed that the level of mutated MntH protein produced was similar to that of the wild-type protein under the same inducing condition, all of which was much higher than the level of MntH expressed from the chromosomal locus (Fig. 7C). These results indicate that mgtA Mg\textsuperscript{2+} riboswitch can sense Mn\textsuperscript{2+} when Mn\textsuperscript{2+} uptake is enhanced by MntH transporter. It has been shown that mgtA 5'-LR forms a stem-loop B at high cytoplasmic Mg\textsuperscript{2+} concentrations to mediate transcription termination (26). We sought to determine whether mgtA 5'-LR also requires this stem-loop by constructing a pYS1010 derivative, pYS1011, which carried a substituted right arm of stem-loop B. Under low Mg\textsuperscript{2+} and IPTG-containing conditions, Mn\textsuperscript{2+} supplementation reduced β-galactosidase activity in the wild-type strain harboring pYS1010 and pYS1008 by ~23-fold (Fig. 7B, column 1, right panel versus column 1, left panel), whereas β-galactosidase activity remained similar in the strain harboring pYS1011 and pYS1008 grown under the same conditions regardless of Mn\textsuperscript{2+} (Fig. 7D, column 2, right panel versus left panel). Also, we conducted an in vitro transcription assay to determine whether mgtA 5'-LR could sense Mn\textsuperscript{2+} and Ca\textsuperscript{2+} in addition to Mg\textsuperscript{2+}. When transcription of the full-length mgtA 5'-LR was initiated by P\textsubscript{lac}−1−6, consistent with a previous observation (26), the reactions with a low Mg\textsuperscript{2+} condition (0.35 mM) mainly produced a 264-nt runoff RNA (Fig. 7E, lanes 1, 3, and 5, respectively, marked −), and a high Mg\textsuperscript{2+} condition (3.5 mM) strongly facilitated transcription termination to produce a 220-nt truncated RNA (Fig. 7E, lane 2, +). Adding either 0.35 mM Mn\textsuperscript{2+} or Ca\textsuperscript{2+} to the low Mg\textsuperscript{2+} reaction induced the 220-nt truncated product to a level similar to that of Mg\textsuperscript{2+} (Fig. 7E, lanes 4 and 6 versus lane 2). Taking these findings together, we concluded that Mn\textsuperscript{2+} and Ca\textsuperscript{2+}, just like Mg\textsuperscript{2+}, are able to act on mgtA 5'-LR in a manner similar to Mg\textsuperscript{2+} and that the mgtA Mg\textsuperscript{2+} sensor should be considered a general divalent cation sensor.

**Concluding Remarks**—The discovery of the Mn\textsuperscript{2+} riboswitch from the *Salmonella mntH* gene should provide new insights into specific transcriptional regulation employing riboswitches to respond to specific divalent metal cations. It also reveals a feedback genetic control, which modulates the transcription of the bacterial Mn\textsuperscript{2+} transporter gene through control of the elongation step by sensing Mn\textsuperscript{2+}. As transcription of the *mntH* gene is regulated in response to not only Mn\textsuperscript{2+} but also other cations, the biological significance of the Mn\textsuperscript{2+} riboswitch may lie in the selective sensing of Mn\textsuperscript{2+} but not of other divalent cations that can be transported through MntH. Mn\textsuperscript{2+} also interacts with a Mg\textsuperscript{2+} riboswitch in *Salmonella*, mgtA 5'-LR, and modulates transcription of the coding region of the Mg\textsuperscript{2+} transporter. This suggests a coordinating regulation that contributes to the maintenance of divalent cation homeostasis by *Salmonella* can modulate the level of cytoplasmic Mg\textsuperscript{2+} and probably some other divalent cations by responding to Mn\textsuperscript{2+}.

In Fig. 5A we have summarized a working model that describes the regulatory action of the UTR1. In low Mn\textsuperscript{2+}, 34CAUAGC\textsuperscript{39} and the following 40AUG\textsuperscript{42} sequence play the role of anti-terminator motifs in S1, in which 40AUG\textsuperscript{42} base-pairs 46CAU\textsuperscript{48} to form stem-loop B, and 34CAUAGC\textsuperscript{39} base-pairs 56GCUAUG\textsuperscript{61}; together they prevent these two sequences from forming terminator stem-loop T. In high Mn\textsuperscript{2+}, on the other hand, the anti-terminator 20UCAU\textsuperscript{23} and the following 28UAU\textsuperscript{26} base-pair anti-terminator motifs to form stem-loop E in S2, which allows 46CAU\textsuperscript{48} as well as 49AGC\textsuperscript{51} to pair with 56GCUAUG\textsuperscript{61} and form stem-loop T.

Two independent mechanisms most likely participate in the regulation of the *mntH* expression in response to the cytoplasmic Mn\textsuperscript{2+} through transcription initiated from the +1 transcription start site. Low levels of cellular Mn\textsuperscript{2+} allow transcription to take place when the R1 sequence is not occupied by MntR. Synthesis of MntH transporter will facilitate uptake of Mn\textsuperscript{2+}, which causes an accumulation of cytoplasmic Mn\textsuperscript{2+}. The Mn\textsuperscript{2+} ion then binds to UTR1 as a nascent transcript, thus acting on its riboswitch motif to terminate *mntH* transcription before it arrives at the *mntH* coding region. We postulate that UTR1 serves as a fine-tuning device. When cytoplasmic Mn\textsuperscript{2+} reaches further, to a higher level, it will activate MntR. Then, this regulator will bind to R1 and repress transcription initiation from +1. At that time point, it remains to be investigated how MntR can differentiate the transcription initiated alternatively from +1 and +1'. Although the Fur-box is located proximally downstream of +1, it is unlikely to regulate the Mn\textsuperscript{2+}-dependent transcription of *mntH* because Fur responds to Fe\textsuperscript{3+} but not Mn\textsuperscript{2+} (18, 19).

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