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Core sequence in the RNA motif recognized by the ErmE methyltransferase revealed by relaxing the fidelity of the enzyme for its target

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ABSTRACT

Under physiological conditions, the ErmE methyltransferase specifically modifies a single adenosine within ribosomal RNA (rRNA), and thereby confers resistance to multiple antibiotics. The adenosine (A2058 in *Escherichia coli* 23S rRNA) lies within a highly conserved structure, and is methylated efficiently, and with equally high fidelity, in rRNAs from phylogenetically diverse bacteria. However, the fidelity of ErmE is reduced when magnesium is removed, and over twenty new sites of ErmE methylation appear in *E. coli* 16S and 23S rRNAs. These sites show widely different degrees of reactivity to ErmE. The canonical A2058 site is largely unaffected by magnesium depletion and remains the most reactive site in the rRNA. This suggests that methylation at the new sites results from changes in the RNA substrate rather than the methyltransferase. Chemical probing confirms that the rRNA structure opens upon magnesium depletion, exposing potential new interaction sites to the enzyme. The new ErmE sites show homology with the canonical A2058 site, and have the consensus sequence aNNNcgGAHAg (ErmE methylation occurs exclusively at adenosines (underlined); these are preceded by a guanosine, equivalent to G2057; there is a high preference for the adenosine equivalent to A2060; H is any nucleotide except G; N is any nucleotide; and there are slight preferences for the nucleotides shown in lower case). This consensus is believed to represent the core of the motif that Erm methyltransferases recognize at their canonical A2058 site. The data also reveal constraints on the higher order structure of the motif that affect methyltransferase recognition.

Keywords: 23S rRNA; magnesium-dependent RNA tertiary structure; MLS antibiotic resistance; RNA–protein interaction; rRNA modification

INTRODUCTION

The Erm methyltransferases specifically methylate adenine 2058 (A2058, *Escherichia coli* numbering) in bacterial 23S rRNA (Lai & Weisblum, 1971; Skinner et al., 1983), conferring resistance to macrolide, lincosamide, and streptogramin B (MLS) antibiotics (reviewed by Cundliffe, 1990). Comparison of the presently characterized Erm methyltransferases (Lafontaine et al., 1994; Weisblum, 1995) shows that there is sufficient homology in the regions of alignable amino acid sequences to suggest that all the Erm methyltransferases derive from a common ancestral sequence. Structural studies link some of the conserved sequence elements with catalysis or with binding of the methyl group donor, S-adenosyl methionine, whereas others sequences are suggested to be responsible for the enzyme's specificity for 23S rRNA position A2058 (Yu et al., 1997; Bussierre et al., 1998).

The conservation of the Erm methyltransferase sequences is paralleled by the rRNA conservation around A2058 (Noller, 1984; Gutell et al., 1994). This adenine is present in all bacterial sequences and is situated in a region of the 23S rRNA termed the peptidyl transferase loop. The peptidyl transferase loop is formed at the junction of five helices that are linked together by phylogenetically highly conserved single-stranded regions. This rRNA region is thus assumed to fold into a near identical structure in different organisms. In accordance with this, the ErmE methyltransferase from *Saccharopolyspora erythraea* (the actinomycete that produces the macrolide antibiotic, erythromycin) modifies rRNA from diverse Gram-positive and -negative bacteria (Skinner et al., 1983; Katz et al., 1987). Naked rRNA is the substrate for ErmE, and the rRNA cannot be methylated

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when assembled within ribosomal particles. ErmE specifically dimethylates the N6 position of A2058 (Skinner et al., 1983).

This conserved fidelity for a single site in the rRNA only holds true, however, when ErmE is used under standard, physiological conditions (i.e., in the presence of magnesium ions, with intact 23S rRNA as the substrate). This was previously noted after most of the 23S rRNA structure had been removed to leave a 625-nt domain V fragment containing the peptidyl transferase loop (Vester & Douthwaite, 1994). ErmE methyltransferase still efficiently methylates position A2058 in the 625-nt fragment, but, in addition to this, an extra site in the rRNA is also methylated. This extra site, at A2225, is apparently inaccessible to ErmE in the intact 23S rRNA under physiological conditions. Interestingly, A2225 occurs within a sequence that shows striking similarity to that at A2058.

The accessibility of a potential adenine target is important in determining whether it becomes methylated by ErmE. This has been demonstrated for A2058, the accessibility of which is lowered by nearby mutations (Vester et al., 1995) and in RNA fragments that fold in an alternative conformation (Vester et al., 1998). In all cases, a reduction in the accessibility of A2058 is concomitant with reduced Erm methylation at this site. Conversely, the methyltransferase would be expected to show higher reactivity with substrates that present an adenine target in a more exposed conformation. In this study, we show that ErmE begins to recognize and methylate numerous additional adenines in rRNAs upon depletion or removal of magnesium ions. Despite the appearance of new methylation sites, the preferred target for ErmE is still A2058. The relatively constant degree of methylation at A2058 suggests that magnesium does not directly affect the methyltransferase structure or activity, but rather that the enzyme interacts with newly exposed adenine targets that in some way resemble the canonical target at A2058. The extent to which RNA structures unfold on depletion of magnesium is monitored through the entire 16S and 23S rRNAs by chemical probing. Methylation by ErmE at the new adenine targets is correlated with increases in nucleotide accessibilities within the rRNA. Comparison of the sequences of the new sites shows that there is a consensus in the structure methylated by ErmE. This is predicted to represent the core of the RNA motif that is recognized by Erm methyltransferases under physiological conditions.

RESULTS

Sites methylated by ErmE in the absence of magnesium

The ErmE methyltransferase can be induced to dimethylate RNA nucleotides other than its canonical target at A2058 in 23S rRNA. We observe these effects when the magnesium ion concentration is dropped below 2 mM. In the study reported here, *E. coli* 16S and 23S rRNAs were incubated with the ErmE methyltransferase under three sets of conditions: in the absence of magnesium ions (buffer 1); with a depleted amount of magnesium (0.6 mM, in buffer 2); and under standard magnesium conditions for use of this enzyme (10 mM, in buffer 3). Sites of ErmE dimethylation were analyzed throughout the entire 16S and 23S rRNAs sequences using sets of primers and extending with reverse transcriptase (one primer for approximately every 200 nt). Progress of reverse transcriptase along an RNA template is terminated by dimethylation at the N6 position of adenine (Zalacain & Cundliffe, 1989). The cDNA fragments formed in this way were run on sequencing gels to locate and quantify new sites of ErmE modification. The positions of sites and the approximate degrees of ErmE dimethylation are shown for the three buffers in Table 1.

Under standard modification conditions with 10 mM magnesium, A2058 is the only position that is modified by the enzyme. On reduction of the magnesium concentration to 0.6 mM, a further 18 sites become reproducibly modified, and an additional 8 sites are modified on complete removal of magnesium. In all cases, the modified bases are adenines, and modification is dependent on the presence of the methyl-group donor, S-adenosyl methionine (SAM). Modification is by far the strongest at A2058 in all three buffers. Under the conditions used here, methylation at A2058 has reached saturation, so although the band intensities in Figure 1 seem to indicate that recognition of A2058 is largely independent of magnesium ions, kinetic studies show that modification at A2058 is slightly more efficient at magnesium concentrations between 2 and 10 mM (Vester et al., 1998). The new sites that appear at 0.6 mM magnesium are generally more strongly modified at 0 magnesium, as illustrated at A2225 (Fig. 2A) and A447 (Fig. 2C). Modification sites that appear only on complete removal of magnesium tend to be the weakest modified. There are relatively few sites in 16S rRNA, and these are only weakly modified.

Structural changes in the rRNA caused by magnesium depletion

23S rRNA was probed using the chemical reagents DMS and kethoxal to determine what structural changes occur upon depletion of magnesium ions. Ionic conditions and temperature were identical to those used in the Erm methylation experiments. In the region of A2058, the chemical modification of bases is largely unaffected by the magnesium conditions (Fig. 1). The lack of detectable magnesium-induced changes in the local RNA structure correlates with the minimal changes observed for ErmE methylation at A2058.

TABLE 1. Sites in rRNA modified by the ErmE methyltransferase at different magnesium concentrations.

Position of methylation	Buffers	Degree of methylation (%)	Sequence
23S rRNA A2058	1,2,3	>80	AAGACGG**A**AAGACCCC
23S rRNA A2225	1,2	9	GUUGCGG**A**CAGUGUCU
23S rRNA A1301	1,2	1–5	UCGCCGG**A**AGACCAAG
23S rRNA A1272	1,2	1–5	AGUAACG**A**UAAAGCGG
23S rRNA A1260	1,2	1–5	AAUGCUG**A**CAUAAGUA
23S rRNA A447	1,2	1–5	CUGACCG**A**UAGUGAAC
23S rRNA A819	1,2	1–5	CUCCCCG**A**AAGCUAUU
23S rRNA A917	1	1–5	AACUGCG**A**AUACCGGA
23S rRNA A943	1,2	0.5–1	ACGGGAG**A**CACACGGC
23S rRNA A802	1,2	0.5–1	CCGGGAG**A**UAGCUGGU
23S rRNA A2430	1,2	0.5–1	UCAACGG**A**UAAAAGGU
23S rRNA A1889	1	0.5–1	UUGAUCG**A**AGCCCCGG
23S rRNA A477	1,2	0.5–1	AAAGGCG**A**AAAGAACC
23S rRNA A125	1,2	0.5–1	AAUGGGG**A**AACCCAGU
23S rRNA A1048	1,2	0.5–1	GGCCCAG**A**CAGCCAGG
23S rRNA A1088	1,2	0.5–1	UUUAAAG**A**AAGCGUAA
16S rRNA A1179	1,2	0.5–1	CUGGAGG**A**AGGUGGGG
16S rRNA A1306	1,2	0.5–1	AGUCCGG**A**UUGGAGUC
23S rRNA A979	1,2	<0.5	AAGAGGG**A**AACAACCC
16S rRNA A1021	1,2	<0.5	AGAUGAG**A**AUGUGCCU
23S rRNA A1847	1,2	<0.5	GUGCCGG**A**AGGUUAAU
16S rRNA A238	1	<0.5	UGCCCAG**A**UGGGAUUA
16S rRNA A279	1	<0.5	CUAGGCG**A**CGAUCCCU
16S rRNA A303	1	<0.5	UGAGAGG**A**UGACCAGC
16S rRNA A994	1	<0.5	GGUCUUG**A**CAUCCACG
23S rRNA A761	1	<0.5	AAACCGG**A**UGACUUGU
23S rRNA A1126	1	<0.5	UGCGCGG**A**AGAUGUAA
Consensus sequence			aNNNcgG**A**HAgNNNNN

The entire sequences of *E. coli* 16S and 23S rRNAs were screened by reverse transcriptase primer extension after ErmE methylation in three different buffers. All the reverse transcriptase stops are dependent on the presence of both the methyltransferase and the SAM cofactor. Buffer 1 contains no magnesium; buffer 2 contains 0.6 mM magnesium; and buffer 3 contains 10 mM magnesium. Methylation was observed only in the buffers indicated (i.e., methylation at 23S rRNA A2058 occurs in all three buffers, but there was no methylation in buffer 3 at any other site). The degrees of methylation were quantified as described in Materials and Methods from at least three independent measurements. Sites are listed in descending degrees of reactivity; bands of less than 0.2% of the total radioactivity in a lane are not recorded. When the methylation degree is different in buffers 1 and 2, the more reactive result is used in the ranking. In the consensus, methylation occurs exclusively at an adenosine (in bold, equivalent to A2058 at the canonical site). There is also an absolute requirement for guanosine (at the G2057 equivalent). Adenosine occurs at the site equivalent to A2060 in 55% of the total sequences and in over 70% of the more strongly methylated sites (thus the sites closest to the top of the list generally conform best with the consensus). The position equivalent to A2059 (designated by H) shows a slight preference for adenosine (50%), but, more interestingly, guanosine is excluded from this position. Nucleotides in lower case occur in between 40 and 50% of the sequences. N indicates that preference for any one nucleotide is less than 40% at these positions. In addition to adherence to the consensus sequence, a favorable conformation of the RNA structure is also required for effective methylation to take place (as discussed in the text).

In other regions of 23S rRNA, clear opening of the structure is evident on magnesium depletion. This is shown in Figure 2B for the RNA region in which three adenines (at A1260, A1272, and A1301) become methylated by ErmE when magnesium is reduced. Several nearby residues (Figs. 2B, 3B) are inaccessible, or only weakly accessible, to the probes at 10 mM magnesium. These bases become more readily modified as magnesium is removed, indicating an opening of the local RNA structure. Similar increases in base accessibilities were observed in other rRNA regions neighboring new ErmE methylation sites, as illustrated for structures adjacent to A447 (Fig. 2C) and A2225 (Fig. 2A). It is noteworthy, however, that there is no magnesium-induced change in DMS modification directly at any of the new adenosine sites. This is illustrated in Figure 2, where the new ErmE targets are weakly to moderately accessible to DMS at 10 mM magnesium, and this accessibility is not significantly changed on removal of magnesium.

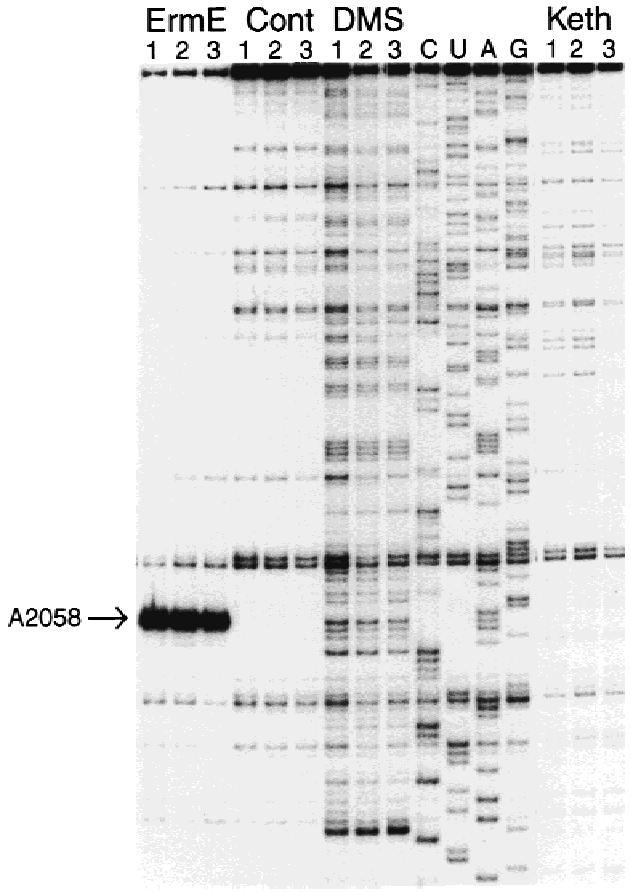

FIGURE 1. Autoradiogram of cDNAs of 23S RNA templates extended with the 2141–2157 primer. RNAs were methylated with methyltransferase (ErmE), or modified with DMS, or modified with kethoxal (Keth) in buffers 1, 2, or 3 as indicated above the gel lanes. The control samples (Cont) were untreated. Dideoxy-sequencing lanes are shown (C, U, A, and G). The canonical site of ErmE methylation at A2058 is indicated.

Consensus in the sequences at the new ErmE methylation sites

Each of the new sites at which ErmE methylates is an adenosine. No reproducible reverse transcriptase stops, which are dependent on the methyltransferase and SAM, were observed at other nucleotides in the RNA sequences. Each of the methylated adenosines is preceded by a guanosine, which corresponds to G2057 at the canonical site. The adenosine at the position equivalent to A2060 occurs with significantly high frequency, although it is not absolutely required for methylation. This nucleotide is prevalent in the more strongly methylated sites, but is more rare in the weaker sites towards the bottom of Table 1. At the position after the methylated adenosine (corresponding to A2059) there is a slight preference for an adenosine, although this position can be occupied by any nucleotide except a guanosine. Slight preferences are also noted for the nucleotides corresponding to A2051, C2055, G2056, and G2061 in the canonical sequence. There does not appear to be selection for specific bases at other positions.

Sequences that conform well to this consensus but are not methylated also give information on the requirements for recognition by ErmE. Accessibility is important. For instance, A1502 (bold) occurs in the sequence ANNNCGG**A**AA, which conforms perfectly with the consensus, but there is no methylation here. The lack of DMS modification (not shown) indicates that the N1 position of A1502 is inaccessible, and probably sheltered in a stable Watson–Crick base-paired helix (Noller, 1984; Gutell et al., 1994). This would also hide the adenosine N6 position from ErmE. Additionally, 16S rRNA A1080 and 23S rRNA A631 are situated in sequences that conform well to the consensus, but these are structured in highly stable tetra-loops (Gutell & Woese, 1990; Gutell et al., 1994) where they are inaccessible and remain unmethylated. Another important factor is exclusion of G immediately after the potential target adenosine. Adenosines that are accessible to the DMS probe and occur in sequences that, with the exception of the G2059 equivalent, match the consensus (e.g., at 23S rRNA A800 and A941) are not methylated by ErmE.

DISCUSSION

Under normal physiological conditions, the ErmE methyltransferase modifies with absolute specificity a single adenine in naked 23S rRNA (Skinner et al., 1983; Skinner & Cundliffe, 1982). However, this fidelity can be interfered with: an additional site of methylation appears on truncation of the 23S rRNA (Vester & Douthwaite, 1994), and here we report the occurrence of multiple methylation sites on depletion of magnesium ions. The reduced specificity of ErmE results mainly from unfolding of the rRNA substrate structure, rather than a change within the enzyme itself. This is demonstrated by the fairly constant methylation at the A2058 canonical site, despite the appearance of new methylation sites. Also, the A2225 site is accessible to ErmE methylation in the presence of magnesium after opening the rRNA structure by truncation (Vester & Douthwaite, 1994). Last, the chemical probes show that the rRNA structure opens upon magnesium depletion.

Each of the new sites of artefactual methylation displays some aspects of the A2058 canonical site, and the most strongly methylated sites show the greatest homology with A2058. This information has been pieced together to elucidate the motif with which ErmE interacts. ErmE recognizes the core sequence G**A**HA, methylating at the nucleotide in bold (H is any nucleotide except guanosine). There is an absolute requirement for a guanosine (equivalent to G2057 at the canonical site) immediately 5′ to the methylated adenosine. In addition, there are minor preferences for other nucleotides indicated in lower case letters, giving the

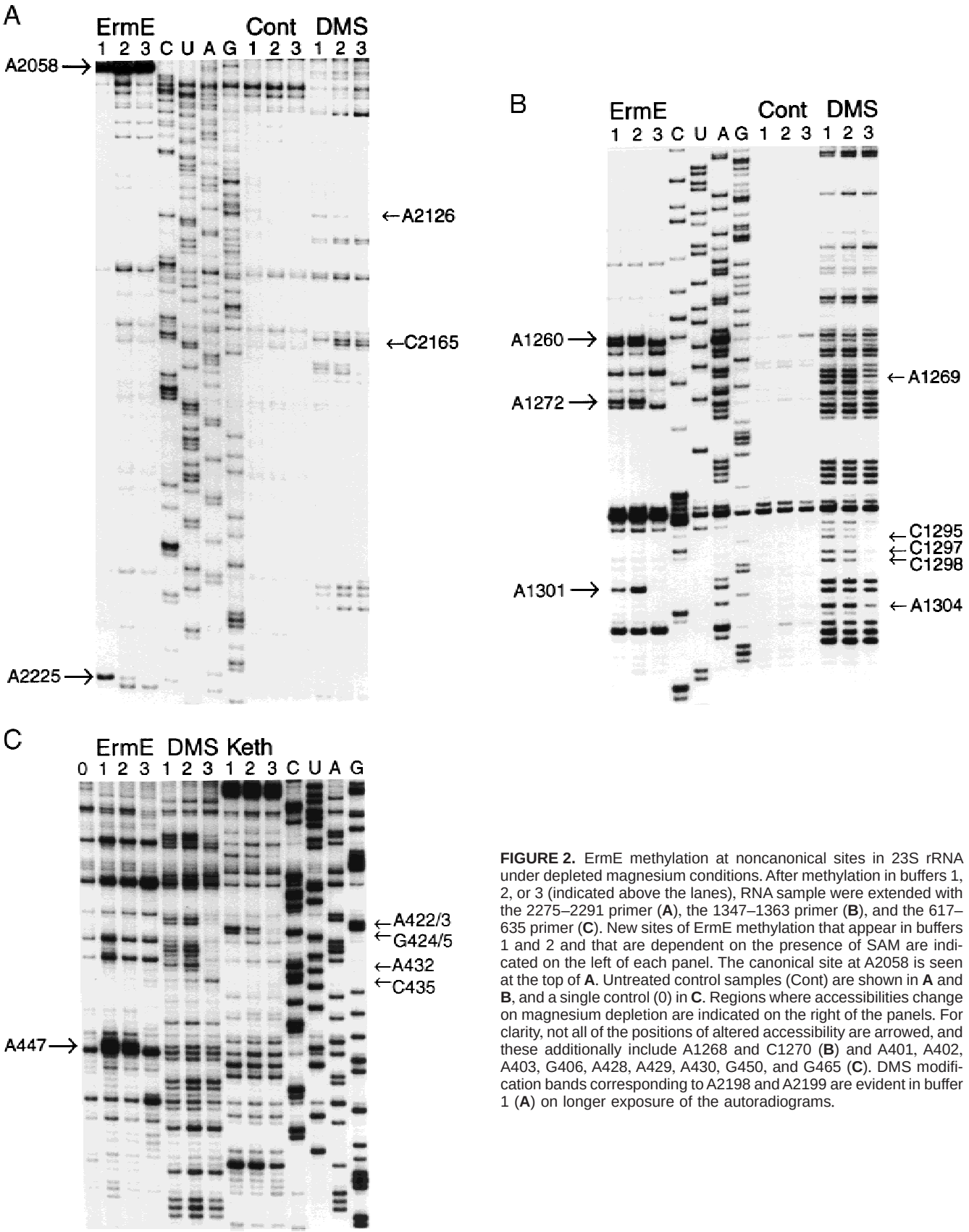

FIGURE 2. ErmE methylation at noncanonical sites in 23S rRNA under depleted magnesium conditions. After methylation in buffers 1, 2, or 3 (indicated above the lanes), RNA sample were extended with the 2275–2291 primer (**A**), the 1347–1363 primer (**B**), and the 617–635 primer (**C**). New sites of ErmE methylation that appear in buffers 1 and 2 and that are dependent on the presence of SAM are indicated on the left of each panel. The canonical site at A2058 is seen at the top of **A**. Untreated control samples (Cont) are shown in **A** and **B**, and a single control (0) in **C**. Regions where accessibilities change on magnesium depletion are indicated on the right of the panels. For clarity, not all of the positions of altered accessibility are arrowed, and these additionally include A1268 and C1270 (**B**) and A401, A402, A403, G406, A428, A429, A430, G450, and G465 (**C**). DMS modification bands corresponding to A2198 and A2199 are evident in buffer 1 (**A**) on longer exposure of the autoradiograms.

extended consensus sequence aNNNcgG**A**HAg (N is any nucleotide).

In addition to the consensus sequence, the secondary and tertiary structures play an important role in determining whether, and to what extent, an RNA substrate becomes methylated by ErmE. At the canonical A2058 site, the irregular base-paired structure of stem 73 (Fig. 3) is necessary for methylation. Destabilization of stem 73 by point mutations (Vester et al., 1995; I. Villsen, unpubl.) reduces methylation, and removal of the lower (3′) strand of this stem abolishes methylation (Vester et al., 1998). This suggests that Erm either directly interacts with stem 73, and/or that the stem supports a conformation of A2058 that is favorable for methylation. An overly tight structure in the RNA can also prevent methylation by ErmE, and this has been observed upon truncation of the rRNA by deletion of distal portions of the peptidyl transferase loop. In truncated RNA substrates, magnesium ions support a tightly folded conformation with reduced accessibility at A2058 (Vester et al., 1998). As observed in the present study, secondary structure in the form of a stable base-paired stem or tetra-loop can obscure potential adenosine targets from methylation by the enzyme.

It is well established that magnesium ions often maintain the tertiary structures of RNAs (e.g., Peattie & Gilbert, 1980; Laing et al., 1994). In intact 23S rRNA, removal of magnesium increases the accessibility of numerous nucleotides including those around A429 (Fig. 2C), which is involved in a tertiary interaction with U234, and A1269/C1270 (Fig. 2B), which interact with G2010/U2011 (Haselman et al., 1989; Gutell et al., 1994). This would indicate that 23S rRNA tertiary structures maintained by magnesium probably obscure potential sites of ErmE methylation. However, it should be noted that although removal of magnesium makes many new adenines accessible to ErmE, the reactivities of these adenines towards the DMS probe is not detectably altered. This to a large extent probably reflects the difference in sizes of the bulky ErmE protein (43,600 Da) and the smaller DMS probe (126 Da). An additional important factor could be that unfolding of the 23S rRNA increases its surface area available for nonspecific electrostatic interactions with the methyltransferase. Such

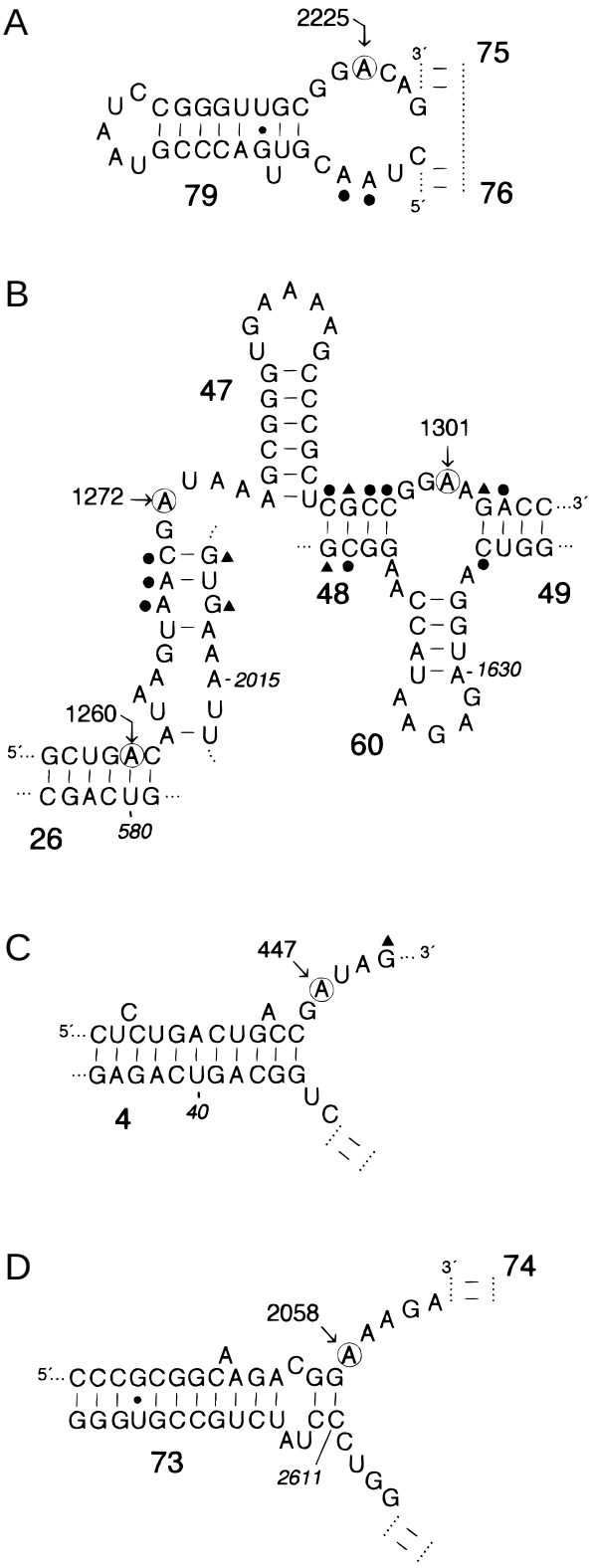

FIGURE 3. Putative rRNA secondary structures of the ErmE methylation sites (encircled) at A2225 (**A**); A1260, A1272, and A1301 (**B**); and A447 (**C**) that appear on reduction of the magnesium ion concentration. The canonical site at A2058 is shown in **D**. Helices are labeled according to the standard numbering system for 23S rRNA (e.g., Brimacombe et al., 1990; Egebjerg et al., 1990). The secondary structures are derived from phylogenetic sequence comparisons (Noller, 1984; Egebjerg et al., 1990; Gutell et al., 1994) and more closely reflect the structures in functional ribosomal particles rather than in naked rRNA (e.g., Moazed et al., 1986), and should therefore be viewed with mild caution in this context. The filled triangles and circles indicate sites of increased reactivity to kethoxal and DMS, respectively, on removal of magnesium. Sequences in **B** possibly form a structural core in the 23S rRNA (Haselman et al., 1989) and show extensive unfolding on removal of magnesium. Minor effects with the chemical probes were evident in the region of A2225 (**A**) and A447 (**C**), although there are more extensive changes neighboring the structures shown here (see Fig. 2C). No structural changes were detected close to A2058 (**D**).

contacts have been shown to be important for the kinetics of methylation at the canonical A2058 site (Zhong et al., 1995; Vester et al., 1998) and probably play a role in methylation at the new sites.

There are parallels between the induced loss of ErmE fidelity for its canonical site and the aberrant reactivities of other enzymes that normally interact with specific sites in nucleic acids. The cytotoxin alpha-sarcin and group II restriction endonucleases can be induced under nonoptimal assay conditions to interact at noncanonical sites. Under physiological conditions with eucaryotic 80S ribosomes as the substrate, alpha-sarcin specifically cleaves a single phosphodiester bond in a tetra-loop motif within a highly conserved rRNA sequence (Wool, 1984). With naked RNA as the substrate in 2 mM magnesium, the cytotoxin presumably gains unimpeded access to a multitude of new sites. Here the loss of fidelity is extreme, and alpha-sarcin begins to cleave randomly on the 3′ side of purines, irrespective of the primary or secondary structure of the RNA (Huber & Wool, 1984). The case of star cleavage activity at noncanonical sites exhibited by restriction endonucleases is somewhat different, because loss of fidelity here is probably a result of changes in the enzyme itself (or the manner in which it interacts with the nucleic acid) rather than a change in the substrate. Star activity in endonucleases such as *Taq*I, *Eco*RI and *Hin*dIII is promoted by organic solvents (Nasri & Thomas, 1986; Barany, 1988), raising the pH (Polisky et al., 1975) or by lowering the ionic strength or magnesium concentration in the buffer (Polisky et al., 1975; Hsu & Berg, 1978). The star sites show high homology with the canonical sites, but are degenerate at the ends of the recognition sequence. The canonical site core sequence is present in all the star sites. As with ErmE, the endonucleases interact best at their canonical nucleic acid sequences, despite the altered buffer conditions.

The importance of the sequence around A2058 for recognition by various Erm methyltransferases has been investigated by mutagenesis studies on RNA substrates. A transition mutation at G2057 (or its equivalent) markedly lowered the methylation by ErmE (Vester et al., 1995) and by ErmC′, whereas an A-to-U change at 2059 has a more minor effect on methylation (Zhong et al., 1995). These findings are consistent with the nucleotide frequencies in the consensus sequence (Table 1). A mutagenesis study of a 23S domain V substrate (625 nt) for ErmE is also in good agreement with the consensus (I. Villsen, B. Vester, S. Douthwaite, unpubl.). Smaller RNAs have been used as substrates for Erm methyltransferases, and have identified the location of the motif that is recognized by ErmSF (Kovalic et al., 1995) and by ErmE (Douthwaite et al., 1995) as lying adjacent to A2058. However, the predictions in the ErmSF study about the importance of the individual nucleotides within this motif do not fit with the consensus reported here. Present evidence would suggest that this discrepancy is unlikely to be due to the different Erm methyltransferases studied, but rather the structures of the substrates employed. Although the RNA motif for Erm can be presented in small RNA substrates (50 nt and less) the structural constraints that are placed upon the conformation of A2058 play an important role in whether, and to what extent, it is methylated by the enzyme (Vester et al., 1998).

In conclusion, we present a consensus sequence for the RNA motif that is recognized and methylated by the ErmE methyltransferase, after the specificity of the enzyme has been relaxed. The consensus presumably defines the essential nucleotides that target the A2058 canonical site for ErmE methylation under physiological conditions. In addition, it is shown that RNA secondary and tertiary structure elements around the consensus sequence can either promote or interfere with the methylation reaction. The secondary structure of stem 73 aids ErmE recognition at the canonical site, but the participation of potential target adenosines in stable structures can prevent them from being methylated by the enzyme. As there is high phylogenetic conservation of both the RNA binding domain within the Erm methyltransferases and the methyltransferase target site on the rRNA, the consensus is proposed to represent the motif that is recognized by all the members of this group of enzymes.

MATERIALS AND METHODS

Preparation of rRNA

E. coli strain DH1 was grown to an A_{450} of 0.4. Cells were harvested by centrifugation, and were washed and resuspended in TMN buffer (50 mM Tris-HCl, pH 7.8; 10 mM $MgCl_2$; 100 mM NH_4Cl). The cells were lysed by sonication, and cell debris was removed by centrifugation. Ribosomes were isolated and fractionated by centrifugation as previously described (Douthwaite et al., 1989). RNA was prepared essentially as described by Stern et al. (1988) by extracting twice with phenol, once with chloroform and precipitating with ethanol prior to redissolving in TE buffer (10 mM Tris-HCl, pH 7.8; 1 mM EDTA).

E. coli plasmids encoding *ermE*

The *erm*E methyltransferase gene has been brought under control of the *lac* promoter in the R1-derivative plasmid pREK47, and expressed in *E. coli* (Vester & Douthwaite, 1994). This type of plasmid is present in the cell as a single copy per chromosome when the incubation temperature is kept below 37 °C, but it exhibits runaway replication at 42 °C, generating up to 1,000 copies/cell (Larsen et al., 1984). Plasmid pREK48 gives a slightly improved yield of ErmE methyltransferase, and was formed by deleting a 250-base-pair *BglII* fragment from pREK47 (Vester et al., 1995). This inactivates the *copB* gene, giving a constitutive copy number of 8–10 plasmids per chromosome at temperatures below 37 °C, and the plasmid

still exhibits runaway replication at higher temperatures (Larsen et al., 1984). The pREK plasmids additionally encode *aph*, conferring kanamycin resistance.

Preparation of ErmE methyltransferase

DH1 cells harboring pREK47 or pREK48 plasmids were grown at 35 °C with aeration in LB medium (twice concentrated; Sambrook et al., 1989) with 0.4% glucose and kanamycin at 25 mg/L. At an A_{450} of 0.7, the temperature was raised to 42 °C for 1.5 h, and cultures were then transferred back to 35 °C for 3 h. Cells were harvested by centrifugation, and washed and resuspended in 20 mM Tris-HCl, pH 7.8; 10 mM $Mg(OAc)_2$; 100 mM NH_4Cl; 6 mM β-mercaptoethanol; and 10% glycerol. Cell were lysed by sonication, and ErmE methyltransferase was considerably purified by exploiting its association with ribosomes (Skinner & Cundliffe, 1982). Briefly, ribosomes were pelleted as described above and the methyltransferase was washed from the ribosomes with high-salt buffer. Ribosomes were repelleted, and the methyltransferase present in the supernatant was dialyzed and concentrated as described by Vester & Douthwaite (1994).

Methylation conditions

rRNA (6 μg) was dissolved in 150 μL of either buffer 1 (20 mM HEPES, pH 7.8; 100 mM NH_4Cl; 5 mM EDTA; 1 mM dithiothreitol; and 10% glycerol), buffer 2 (as buffer 1, with 0.6 mM $MgCl_2$ and without EDTA), or buffer 3 (as buffer 1, with 10 mM $MgCl_2$ and without EDTA). Samples were heated at 50 °C for 5 min followed by 10 min at 30 °C. Four hundred fifty nanograms purified methyltransferase and 1.5 mM S-adenosylmethionine were preincubated in 150 μL of the respective buffers at 30 °C for 10 min prior to addition to the rRNA. Methylation reactions were carried out at 30 °C for 40 min. Reactions were stopped by extracting with phenol followed by chloroform extraction, and the rRNA was recovered by precipitation with ethanol. Enough rRNA is methylated in such a reaction for reverse transcriptase analyses from several primers.

Structural probing

Eight-microgram samples of rRNA were renatured by warming for 5 min at 50 °C in 200 μL of buffer 1, 2, or 3. Samples were preincubated for 10 min at 30 °C, before modification (for 30 min at 30 °C) with either dimethyl sulfate (DMS; 2 μL of a 1:6 (v/v) dilution in ethanol) or kethoxal (3 μL of a 40 mg/mL solution in 30% (v/v) ethanol). Reactions were stopped and the rRNA was recovered as described by Moazed et al. (1986).

Analysis of rRNA by primer extension

Bases in rRNA that are accessible to modification by DMS or kethoxal or to ErmE dimethylation were analyzed by primer extension. Primers complementary to the *E. coli* 16S rRNA sequences 334–351, 682–699, 858–875, 1054–1070, 1195–1213, 1390–1407, and 1502–1520, and 23S rRNA sequences 228–245, 417–433, 617–635, 888–906, 1009–1026, 1169–1187, 1347–1363, 1510–1528, 1687–1703, 1906–1922, 2061–2078, 2141–2157, 2227–2254, 2275–2291, 2500–2515, 2677–2694, 2795–2812, and 2888–2904 were extended with 1 mM dATP, dCTP, dGTP, and dTTP (Stern et al., 1988). Samples were run on polyacrylamide/urea sequencing gels alongside extensions of untreated control RNA and sequencing lanes to detect sites of modification in the RNA. Gels were autoradiographed and scanned on a Molecular Dynamics Storm 840 phosphorimager. The degree of methylation at A2058 was determined by adapting the method described by Sigmund et al. (1988). The 2061–2078 primer, complementary to the sequence immediately 3′ to A2058, was hybridized to the rRNA and extended with reverse transcriptase together with dTTP and ddCTP for 20 min at 41 °C. The extension products were run on a 13% polyacrylamide denaturing gel, and the intensities of the bands corresponding to methylated or unmethylated at A2058 (Vester & Douthwaite, 1994) were quantified by scanning. Methylation at the A2225 site was quantified in a similar manner by extension with dGTP, dTTP, and ddCTP from the 2227–2254 primer (S. Helm-Petersen, unpubl.). Methylation at the other methylated adenosines was estimated by comparison with the amount of read-through by reverse transcriptase. Because there are longer stretches of sequence between priming sites and these adenosines, their quantification is necessarily less precise than at A2058 and A2225 (see Table 1).

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