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Negative in vitro selection identifies the rRNA recognition motif for ErmE methyltransferase

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ABSTRACT

Erm methyltransferases modify bacterial 23S ribosomal RNA at adenosine 2058 (A2058, *Escherichia coli* numbering) conferring resistance to macrolide, lincosamide, and streptogramin B (MLS) antibiotics. The motif that is recognized by Erm methyltransferases is contained within helix 73 of 23S rRNA and the adjacent single-stranded region around A2058. An RNA transcript of 72 nt that displays this motif functions as an efficient substrate for the ErmE methyltransferase. Pools of degenerate RNAs were formed by doping 34-nt positions that extend over and beyond the putative Erm recognition motif within the 72-mer RNA. The RNAs were passed through a series of rounds of methylation with ErmE. After each round, RNAs were selected that had partially or completely lost their ability to be methylated. After several rounds of methylation/selection, 187 subclones were analyzed. Forty-three of the subclones contained substitutions at single sites, and these are confined to 12 nucleotide positions. These nucleotides, corresponding to A2051–A2060, C2611, and A2614 in 23S rRNA, presumably comprise the RNA recognition motif for ErmE methyltransferase. The structure formed by these nucleotides is highly conserved throughout bacterial rRNAs, and is proposed to constitute the motif that is recognized by all the Erm methyltransferases.

Keywords: MLS resistance; negative in vitro selection; protein–RNA interaction; rRNA modification

INTRODUCTION

Antibiotic resistance is a widespread and ever increasing problem in the treatment of bacteria-related diseases. Resistance to clinically important macrolide, lincosamide, and streptogramin B type (MLS) antibiotics is conferred by mono- or dimethylation of bacterial 23S rRNA by Erm methyltransferases (Weisblum, 1995). Erm methyltransferases are found in a wide variety of bacteria ranging from antibiotic-producing actinomycetes to clinical pathogens. Despite the diverse occurrence, there is sufficient structural homology in the methyltransferases to indicate that they have a common ancestral origin and have retained the same function (Lafontaine et al., 1994). The ErmE methyltransferase originates from *Saccharopolyspora erythraea*, an actinomycete that produces the macrolide antibiotic, erythromycin (McGuire et al., 1952; Labeda, 1987). As in the case of the other Erm methyltransferases, the rRNA target for modification is adenosine 2058 (A2058, *Escherichia coli* 23S rRNA numbering), which becomes dimethylated by ErmE at the N6 position (Skinner et al., 1983). The structure around A2058 is distinctive, and is conserved within bacterial rRNAs (Noller, 1984; Gutell et al., 1994), suggesting that A2058 is displayed in a unique structural motif that is recognized by the Erm methyltransferases (Vester et al., 1998). The existence of such a motif would explain the high specificity of Erm methyltransferases for A2058, and also their ability to modify this nucleotide and confer drug resistance in phylogenetically diverse bacteria (Skinner et al., 1983). A possible means of combating this form of drug resistance would be to create molecular analogs of the RNA motif that could occupy the active site of the methyltransferase. Identifying the structure of the RNA motif would be a first step towards this goal.

Here, we employed an in vitro selection approach to define the features of the motif around A2058 that are essential for its recognition by the ErmE methyltransferase. In vitro selection and evolution procedures have been used with considerable success to enrich and isolate RNA aptamers with improved binding or catalytic properties (see, e.g., Dai et al., 1995; Ringquist

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Abbreviations: RT-PCR, reverse transcriptase-polymerase chain reaction; SAM, S-adenosyl methionine.

et al., 1995; Wilson & Szostak, 1995; Burke et al., 1997; Welch et al., 1997; Zhang & Cech, 1997). The strategy taken in this study differed from previous ones in that we selected for the *loss* of a desired characteristic. The starting point for this study was a good RNA substrate for Erm methylation. From this RNA, pools of RNA molecules were constructed with degenerate sequences that extended over and beyond the putative Erm recognition motif. After methylation of the RNA molecules by Erm, the pool was subjected to reverse transcriptase-PCR (RT-PCR) amplification, followed by T7 RNA polymerase transcription to form a new pool for the next selection round. Molecules that are the best substrates for Erm are most readily dimethylated at the adenosine N6, and this modification blocks reverse transcription. Good substrate molecules were thus preferentially removed from the pool. At the same time, there was strong selection for RNAs with alterations at nucleotide positions that are important for Erm recognition and methylation. These substrates escaped methylation and were therefore subsequently amplified by RT-PCR. After several rounds of methylation and selection, RNAs remaining in the pool were analyzed by cloning and sequencing. Comparison of the selected RNA subclones with the original pool revealed structures in the RNA motif that are essential for recognition by Erm methyltransferase.

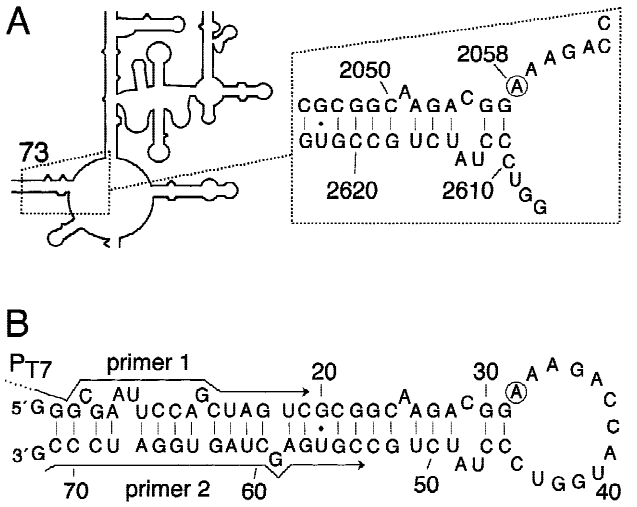

FIGURE 1. A: Schematic secondary structure of a portion of domain V of 23S RNA (Gutell et al., 1994; Noller, 1984). The structure of helix 73 and the adjacent target for Erm methyltransferases at A2058 is shown in detail (*E. coli* sequence and nucleotide numbering). **B**: The 72-nt reference RNA, which is analogous to 23S rRNA helix 73 and the adjacent single-stranded region containing A2058. The hairpin secondary structure of this sequence has been verified using chemical and ribonuclease probes (Vester et al., 1998). Under the ionic conditions used here, the reference RNA is efficiently and specifically methylated at A32 (encircled) by ErmE methyltransferase. Degenerate pools of RNA were derived by doping the reference RNA sequence between positions 20 and 53; sequences 1–19 and 54–72 (indicated by the arrow shafts) remained invariant. The invariant sequences serve as annealing sites for RT-PCR selection and amplification of RNA–DNA using oligodeoxynucleotide primers 1 and 2. Primer 1 has the sense RNA sequence and additionally encodes a T7 RNA polymerase promoter (P_{T7}); primer 2 is complementary to the RNA. The invariant sequences additionally encoded *Eco*RI and *Bam*HI restriction endonuclease sites to facilitate subcloning.

RESULTS

Experimental strategy

A negative selection procedure was developed to identify RNAs that have lost their function as effective substrates for the ErmE methyltransferase. Comparison of poorly methylatable RNAs with the RNAs in the initial pool revealed sequences that are essential for the methyltransferase reaction. The starting material for the study was a 72-nt "reference RNA" corresponding to 23S rRNA helix 73 plus the single-stranded region containing the adenosine target for Erm methyltransferases (Fig. 1). RNAs with this structure are good substrates for specific methylation by ErmE (Vester et al., 1998).

Two degenerate pools of RNAs, based on the reference RNA structure, were transcribed from doped oligodeoxynucleotides. The first of these, pool A, was a pilot study designed to test the feasibility of the approach and to define the experimental parameters for a more comprehensive study (with pool B). After methylation, RNAs in each pool were amplified by RT-PCR using the priming sites in the invariant regions that flank the doped sequences (Figs. 1 and 2); these invariant regions additionally encode restriction sites for subcloning of individual sequences. Only unmethylated RNAs are extended to form full-length cDNAs, as N6-dimethylation of adenosine blocks extension by reverse transcriptase (Zalacain & Cundliffe, 1989).

In the pool A starting material, ten positions were doped (Fig. 3A), including the Erm modification target at A32, plus other positions that have previously been implicated in the Erm interaction (Vester et al., 1998; Hansen et al., 1999). These mutated RNAs were expected to function as poor methylation substrates, and therefore there would be strong selection for them. In addition, this pool was enriched with a high proportion of reference RNA. The reference RNA was predicted to be preferentially methylated, and therefore would be quickly removed from the pool. In pool B, the starting material was more heterogeneous with 33 degenerate positions covering the putative ErmE recognition motif (Fig. 3D). The importance of the methylation target A32 was established in pool A. This position was not doped in pool B, so that RNAs with mutations here would not overrun the selected pools.

At the beginning of each round of selection, the RNA pools were incubated with ErmE methyltransferase under conditions that would methylate approximately 60–70% of the reference RNA molecules. This degree of methylation over four or five rounds of selection was

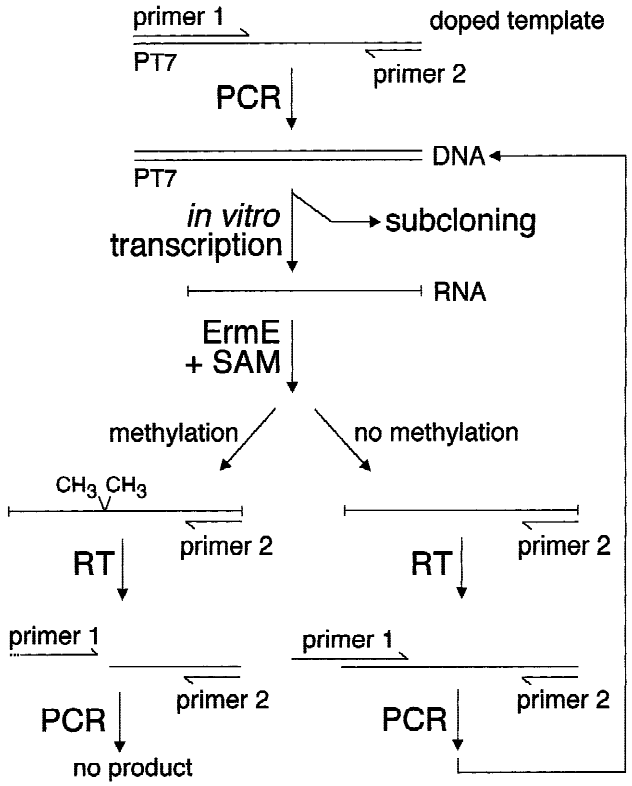

FIGURE 2. Flow scheme of the negative selection process for RNAs with reduced capacity to be methylated by ErmE.

estimated to capture phenotypes ranging from moderately poor substrates to unmethylatable RNAs. At the same time, these conditions were predicted to remove from the pool most of the reference RNA molecules and RNAs with neutral mutations.

RNA methylation and selection

The initial distribution of doped bases in the RNA pools before selection was checked by cloning and sequencing of 43 clones. The base distribution in pool A was additionally screened by sequencing the doped oligodeoxynucleotide. The substitutions in the initial pools were distributed at the designated positions (Fig. 3), and the frequencies of the substitutions (tested by chi-squared analysis) were consistent with the theoretical expectation given in Materials and Methods (data not shown).

Pool A initially showed a high methylation capacity because of the large content (55%) of reference RNA. The proportion of methylatable RNAs fell steeply during the first three rounds of selection, and the fall was less obvious in subsequent rounds (Fig. 4). RNAs were subcloned after the fourth round, and sequence analysis showed that the occurrence of reference RNA had dropped to less than 4% (2 of 55 subclones). The remaining subclones displayed a range of single, double, and multiple substitutions, and in a few cases nucleotide deletions had occurred. The overall distribution and frequencies of the substitutions and single-nucleotide deletions are depicted in Figure 3B. Mutations at A32, which is equivalent to A2058 in 23S rRNA and essential for Erm methylation (Vester et al., 1995; Zhong et al., 1995), were particularly prevalent. The depletion of the reference RNA, together with the amplification of deleterious mutations, showed that the negative selection had been successful.

RNA from pool B was subjected in the same manner to several rounds of methylation and selection, and the methylation capacity of the pool was assayed after each round. After the fifth round, the RNAs remaining in the pool were subcloned, and 132 individual subclones were analyzed. The prevalence of reference RNA fell from 37% in the initial pool to less that 1% (one subclone out of 132) after round five, again indicating that good substrates were being excluded from the pool. The remaining subclones all contained mutations that were predominantly distributed in the upper helical strand from position 25 past the target adenosine to position 34, and on the lower helical strand at position 45, with additional hot spots at positions 20, 36, 39, and 41 (Fig. 3E).

Thirty-one of the subclones had single-site substitutions, and these resolved which positions are of primary importance for methylation (Fig. 3F). The single-site substitutions are limited to positions 25–34, 45, and 48. In addition, 11 subclones with single-nucleotide deletions (presumably caused by replication errors) at C29 (four subclones) and at G30 or G31 (seven subclones) were isolated. The other mutational hotspots (Fig. 3E) were only observed in subclones with multiple mutations. Single-site changes were markedly absent from the remainder of the loop region from position 35 to 44, and from most of the lower strand of the helix, indicating that the identities of these nucleotides are not important for the methylation reaction.

Individual RNA subclones

RNA was transcribed from the isolated subclones to evaluate the effects of the single-site mutations on the methylation reaction (Fig. 5A). The identities of the individual nucleotide substitutions are shown in Figure 5B, together with the degree to which they affect methylation by Erm. Substitutions at positions A25, G27–A34, and C45 had marked effects, reducing the rate of substrate methylation to less than 15% of the reference RNA molecule. At A28 and G30, the effects of transition and transversion mutations are indistinguishable, suggesting that either type of mutation has a similar effect in disrupting the RNA structure or its interaction with the methyltransferase. At other positions, however, differences were observed in the effects of different types of substitution. This is most notable at the bulged nu-

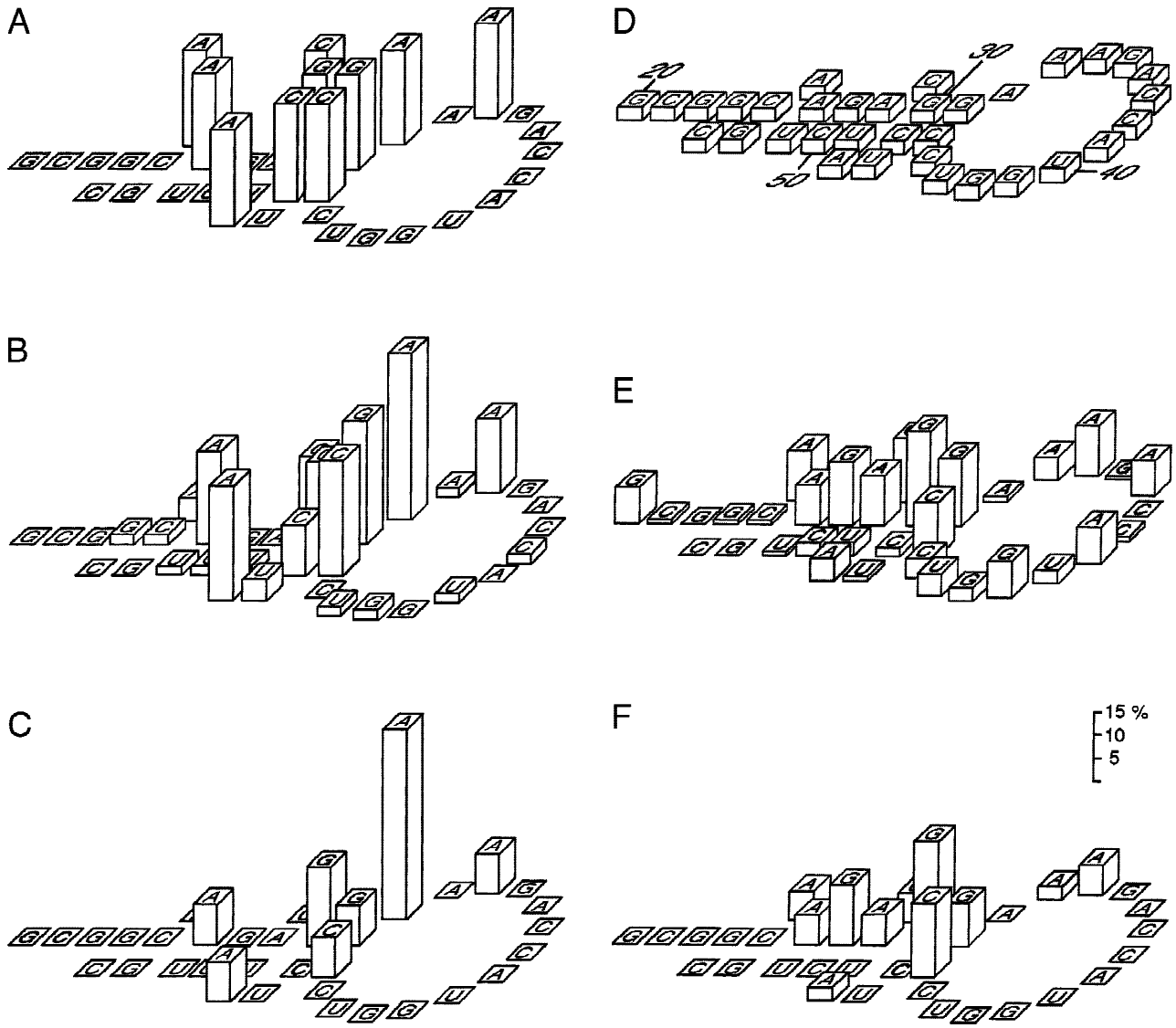

FIGURE 3. Negative in vitro selection from pools of doped RNA sequences. The course of experiments with pool A (**A**–**C**) and pool B RNA (**D**–**F**) are illustrated in the doped sequence between positions 29 and 53; the invariant sequences (Fig. 1) were present in all RNAs, but are omitted here for clarity. The starting material for pool A (**A**) and pool B (**D**) was doped at ten and thirty-three positions, respectively, with 7% and 1% of each of the other 3 nt (indicated by the heights of the columns at these positions); reference (i.e., undoped) RNA was added to pool A to constitute 55% of the starting material. Before selection, the distributions and approximate frequencies of substitutions in the initial pools were confirmed by sequencing. After four rounds of selection within pool A, 55 subclones were isolated and sequenced. These consisted of 2 reference sequences, 12 subclones with single-site substitutions, 15 with double mutations, and 26 with multiple changes. The mutation frequency for each nucleotide within the total set of pool A subclones is shown in **B**. In pool B, 132 subclones were isolated after five rounds of selection: one subclone had the reference sequence; 31 subclones had single-site substitutions; 11 had single-nucleotide deletions; 37 had double substitutions; and 52 had multiple changes (21 of the last group of subclones additionally had single-nucleotide insertions and deletions). The overall mutation frequencies for the pool B subclones is depicted in **E**. The subclones with single-site mutations in pool A and pool B are shown in **C** and **F**, respectively. These single-site mutations resolve which nucleotide positions are important for the Erm methyltransferase reaction.

cleotide A25, where a guanosine functions almost as well as the adenosine; however, substituting cytidine in this position produces a virtually nonfunctional substrate.

An inverse correlation could be expected between the efficiency of methylation of a substrate and the frequency of selection of that substrate. Although a much larger set of subclones would have to be analyzed to test this hypothesis conclusively, an inverse correlation was observed for the extreme cases of very good or very bad substrates. For instance, the position 32 substitutions (from pool A) and the G30/G31 single-nucleotide deletions (pool B) are nonfunctional substrates for methylation, and these mutant RNAs were predominant in their respective pools. Conversely, RNAs without mutations were almost totally excluded from both pools, and no neutral mutations were isolated in

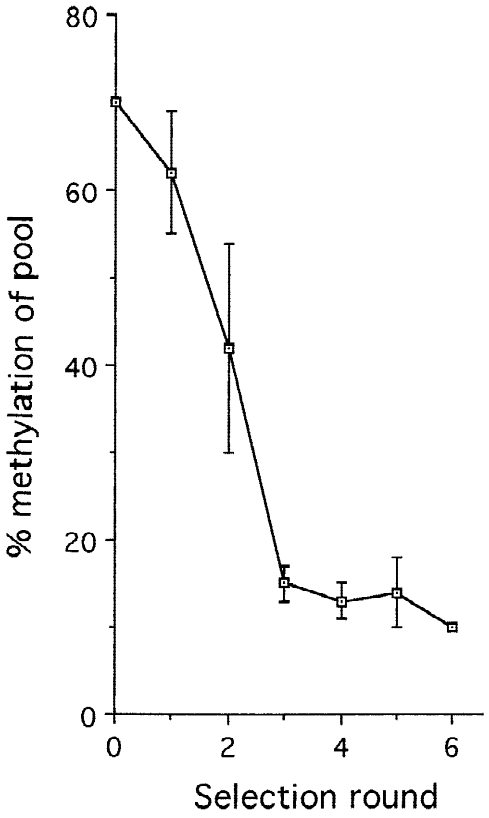

FIGURE 4. Methylation of the pool A RNAs followed over six rounds of in vitro selection. The methylation capacity of the RNA pools was assayed after each round by reverse transcriptase extension from primer 2, followed by gel electrophoresis and phosphorimager scanning of the cDNA products. The spread of results in two independent experiments is shown.

the single-site subclones. The selection conditions were adjusted so that a few substitutions such as A25G and A26U, which give an only mild reduction in methylation efficiency, would make it through the selection procedure. These RNAs necessarily represent only a small fraction of the selected subclones, and probably other substitutions conferring similarly mild phenotypes existed in the initial pool, but went undetected here.

Although the frequency of selection was often a good indication of the functional importance of a mutation, direct analysis of methylation of an RNA (Fig. 5A) is a more consistently reliable means of evaluating its interaction with Erm. Some mutations, or combinations of mutations (for example at positions 20, 36, 39, and 41 in Fig. 3E), could cause peculiarities in an RNA structure that influence its ability to be amplified. In such cases, the frequency of selection of a mutant RNA would not indicate a causal link with the importance of the mutated nucleotide for the methylation reaction.

DISCUSSION

The structure of the natural target for the Erm methyltransferases at A2058 within domain V of 23S rRNA is depicted in Figure 1. Transcripts of domain V are methylated as efficiently as intact 23S rRNA by ErmE and by the homologous ErmSF and ErmC′ methyltransferases (Kovalic et al., 1994; Vester & Douthwaite, 1994; Zhong et al., 1995). The substrate size has been further truncated (Kovalic et al., 1995), and RNAs with structures essentially no bigger than the region from nucleotides 20 to 53, which is degenerate in the pool B RNA (Fig. 3D), have been shown to contain the motif required for specific recognition by ErmE (Vester et al., 1998).

The unique RNA motif recognized by the methyltransferase appears to be formed by a specific sequence of bases that is displayed in a particular secondary structure. In 23S rRNA, nucleotides between A2051 and A2060 have been implicated in recognition by ErmE (Hansen et al., 1999), which is consistent with the selection of single-site mutations at positions A25 to A34 (Fig. 3F). Nucleotide A32 (A2058) is the key nucleotide, as its exocyclic amino group is the methyl acceptor, and thus any substitution here abolishes methylation of the reference RNA (and of 23S rRNA). Of the remaining nucleotides, some presumably play a primary role in the Erm interaction, whereas others could function in a more subsidiary capacity to maintain the secondary structure of the motif. Nucleotides A33 and A34 are probably not involved in maintaining the secondary structure of the RNA. The base identity at A34 appears to be of direct importance for the Erm interaction. At position 33, however, exclusion of a guanine base seems to be more important than the presence of an adenine (Hansen et al., 1999). This idea cannot be reliably tested with the present approach without the screening of a much larger set of selected subclones (although the selection of one A34G subclone is consistent with the idea).

The roles of nucleotides A25–G31 can be characterized to varying degrees. The irregular base-paired structure of helix 73 is essential for methylation, and the RNA is no longer methylated after removal of the 3′ side of the helix (Vester et al., 1998). Changes in the G2057-C2611 base pair (Vester et al., 1995) reduce methylation, and the same observation is made here for the corresponding G31-C45 base pair. The other individual base pairs in the helix seem to be less important, however, as single-site mutations were only selected at C45 (C2611) and A48 (A2614) on the 3′ side of the helix. Probably the identities of the other bases on the 3′ side of helix 73 are unimportant, as long as mutations do not disrupt the overall helical structure. This is consistent with a study on 23S rRNA domain V (Villsen et al., 1999), in which compensatory base pair substitutions in helix 73 show that A2051 to C2055 serve to maintain the irregular secondary structure, whereas the identities of nucleotides G2056 to A2060 are of primary importance for Erm recognition. The requirement for nucleotide C29 (C2055) to be un-

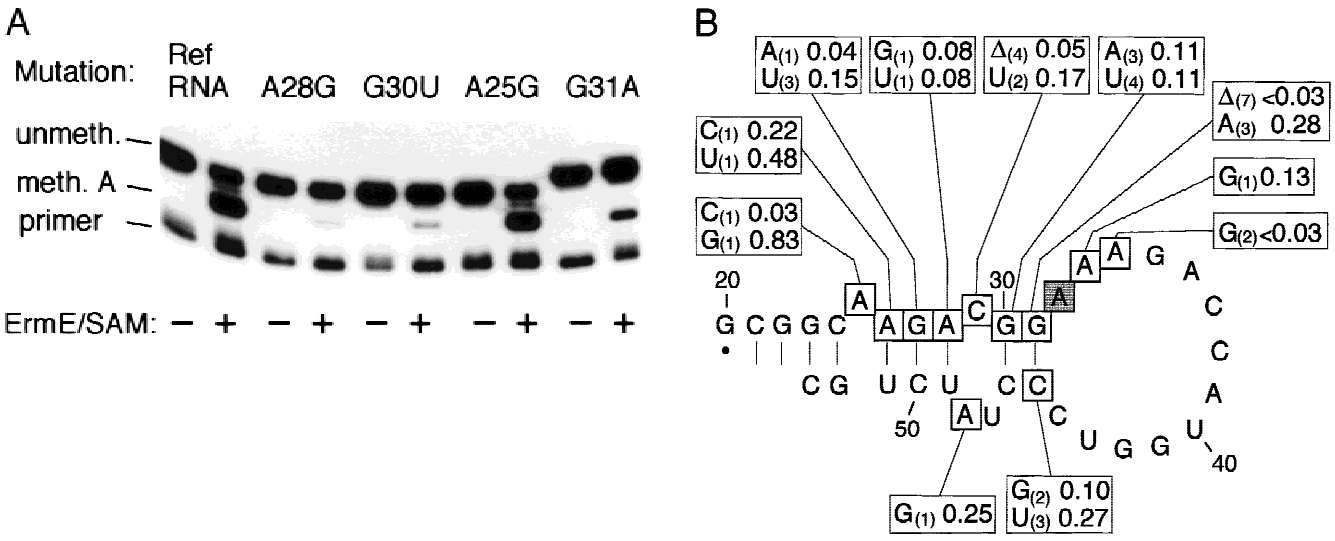

FIGURE 5. A: Methylation assay of individual RNA subclones containing either no mutation (Ref RNA), or single A28G, G30U, A25G, or G31A mutations. Methylation kinetics were compared as previously described (Vester et al., 1998), and differences in methylation are illustrated here with a single time point after 45 min. After incubation with ErmE and SAM, methylated (+) and untreated control RNAs (−) were hybridized to a primer complementary to positions 35–52, which was then extended with reverse transcriptase, and dTTP and ddCTP (Vester & Douthwaite, 1994). After gel analysis and autoradiography, the bands corresponding to the primer and cDNAs from the methylated (meth. A) and unmethylated RNAs (unmeth.) are visible. **B**: Effects of single-site mutations in the RNA on methylation by ErmE methyltransferase. The positions and identities of the single-site mutations are indicated. The methylation rates (averages of at least two experiments) of these RNAs relative to the reference RNA (set to 1.00) are shown. Values of less than 0.03 cannot be significantly distinguished from gel background levels. The values in parentheses indicate the number of times a particular single-site mutation was independently isolated within the 132 subclones from pool B. Five single-site mutations at position A32 (three U, one C, and one G substitution), all of which prevent methylation by ErmE, were isolated within the 55 subclones from pool A.

paired is emphasized by the single-site mutations that alter this structure (Fig. 5B). Methylation is greatly reduced by deletion of C29; and the C29U and A48G substitutions also effectively remove the bulge at position 29 by potentially creating a base pair between positions 29 and 48. No other single-site mutations were selected at these positions. Taken together with site-directed mutagenesis data (Villsen et al., 1999), this suggests that the irregularity in the helix is more important than the identities of the nucleotides that form it.

In conclusion, selection and analysis of 187 RNA subclones, of which 43 subclones have single-site substitutions, reveals which nucleotides are essential for interaction with ErmE methyltransferase. The single-site substitutions are limited to 12 nt at positions A25–A34, C45, and A48, which correspond to A2051–A2060, C2611, and A2614 in 23S rRNA. As indicated in Figure 5B, substitutions at some of these positions drastically reduce methylation, and thus are presumably of primary importance for the interaction. Substitutions at other positions give only a mild reduction in methylation, suggesting that these nucleotides play a less direct role, such as supporting the secondary structure of the motif. The high phylogenetic conservation of both the 23S rRNA motif (Gutell et al., 1993) and of the putative RNA binding region of Erm methyltransferases (Yu et al., 1997; Bussiere et al., 1998) suggests that the data presented here for ErmE are valid for all the Erm methyltransferases.

MATERIALS AND METHODS

The RNA substrate

The structure of the 72-nt-long reference RNA, which contains the recognition motif for Erm methyltransferase, is shown in Figure 1. The RNA was formed by PCR amplification of an 89-deoxynucleotide template, 5′-CGGGATCCAC TAGCTCA CGG CAGATAGGGA CCATGGTCTT TCCGTCTTGC CGC GACTAGC TGGAATTCGC CCTATAGTGA GTCGTATTA, with oligodeoxynucleotide primer 1 (5′-TAATACGACT CACTATA GGG CGAATTCCAG CTAGTC) and primer 2 (5′-CGGGAT CCAC TAGCTCACG), followed by transcription from the encoded T7 RNA polymerase promoter (Fig. 2). PCR was performed with *Taq* DNA polymerase under conditions recommended by the enzyme supplier (Promega) in 50 μL 10 mM Tris-Cl, pH 9.0, 50 mM KCl, 3 mM $MgCl_2$, 0.1 mM Triton X-100, and 0.4 mM of each dNTP with 25 cycles denaturing at 94 °C, annealing at 60 °C, and elongation at 72 °C. The resulting double-stranded PCR product was purified on a PCR column (Qiagen). Transcription from the PCR product was performed as recommended by the supplier of T7 RNA polymerase (Promega).

After transcription, the DNA template was eliminated with 2 U of DNAse (Boehringer Mannheim). RNA transcripts were

extracted with phenol and chloroform. Intact RNAs of the correct length were isolated on 13% denaturing polyacrylamide gels, and extracted from excised gel bands in 10 mM Tris-HCl, pH 7.5, and 1 mM ethylene diamine tetraacetic acid (EDTA). Phenol/chloroform extraction was repeated, the RNA was recovered by precipitation with ethanol, and was resuspended in H_2O.

RNA substrates pools for in vitro selection

Pool A

The 89-mer template was doped at 10 nucleotide positions with 7% of each of the other 3 nt (Fig. 3A). The doped nucleotides include the position coding for the methyltransferase target (A32, Fig. 1). Generation of double-stranded DNA templates, in vitro transcription, and RNA purification were performed as described above. After transcription, reference RNA was added to this pool so that it constituted 55% of the RNA.

Pool B

The 89-mer template was doped at 33 positions, each with 1% of each of the other 3 nt (Fig. 3D). The methyltransferase target at A32 was not doped, and no extra reference RNA was added to the pool. The DNA template was amplified and transcribed as above.

The degrees of doping in the oligonucleotides for both pools were calculated to obtain a desired level of single and double mutations while keeping multiple mutations to a minimum. The pool A oligonucleotide encoded 9% reference RNA (before addition of extra reference RNA to the pool), 25% with a single change, 30% with double changes, 36% with multiple changes. The pool B oligonucleotide was predicted to encode 37% reference RNA, 37% with single-base substitutions, 17% with double substitutions and 9% with multiple changes. The initial distribution of doped bases in the RNA pools before selection was checked by cloning and sequencing. Base distribution in the pool A oligodeoxynucleotide was additionally screened by sequencing directly from the primer 5′-TAATACGACT CACTATAG with T7 DNA polymerase and ^{33}P-labeled ddNTP's (Amersham Pharmacia).

Selection of poor methylation substrates

One hundred nanograms ($\sim 2.5 \times 10^{15}$ molecules) of RNA in 100 μL 20 mM HEPES, pH 7.6, 6 mM β-mercaptoethanol, 10% glycerol, 0.05% Triton X-100, 100 mM $NHCl_4$ and 5 mM EDTA was renatured by warming at 50 °C for 5 min, followed by 5 min at 30 °C. The RNA pool was incubated with 0.4 μg ErmE methyltransferase (Vester et al., 1998) and 2.8 mM S-adenosyl methionine (SAM) for 45 min at 30 °C. The methylation reaction was stopped by phenol/chloroform extraction. Carrier tRNA (0.3 μg) was added to facilitate precipitation with ethanol, and the RNA was redissolved in 2.5 μL H_2O.

One picomole of primer 2 (complementary to the 3′ end of the RNA) was hybridized to the RNAs and extended in 10 μL of 50 mM Tris-Cl, pH 8.3, 60 mM KCl, 7.5 mM $MgCl_2$ and 7.5 mM dithiothreitol (DTT), with 0.3 mM dNTP and 1 U AMV reverse transcriptase (Life Sciences) for 20 min at 40 °C. cDNA was recovered by precipitation with ethanol and redissolved in 15 μL H_2O. One microliter of the cDNA was amplified by PCR, and the double-stranded template was used for T7 RNA polymerase transcription as described above. Rounds of methylation and selection were continued until there was no further appreciable drop in the degree of methylation of the RNA pool.

Measurements of dimethylation

The degree of methylation of the RNA pools was estimated after each round of selection. 5′-^{32}P end-labeled primer 2 was hybridized to 1 μg RNA and extended with reverse transcriptase and dNTP (Stern et al., 1988). The extension products were concentrated by ethanol precipitation and run on a 13% denaturing polyacrylamide gel. The intensity of the bands representing methylated (stopping at A32) and unmethylated (read-through) fractions of the RNA were quantified by phosphorimager scanning (Storm 840, Molecular Dynamics).

For selected subclones, methylation was measured more precisely by using a primer (5′-CAGATAGGGA CCATGGTC) that hybridizes immediately 3′ to A32 in the RNA. These RNAs were extended for 20 min at 40 °C with reverse transcriptase using one or more deoxynucleoside triphosphates and a single dideoxynucleoside triphosphate (Sigmund et al., 1988; Vester & Douthwaite, 1994) and then analyzed as above on gels.

Cloning and sequencing of selected RNAs

After the fourth round of selection from pool A and the fifth round of selection from pool B, PCR-amplified DNA was digested with the restriction enzymes *Eco*RI and *Bam*HI and ligated into the same sites in pAlter phagemid (Promega), a vector that confers tetracycline resistance. *E. coli* strain TG1 (Sambrook et al., 1989) was transformed with recombinant phagemids. Individual clones were picked from agar plates containing 10 μg/mL of tetracycline, and the phagemid sequences were determined by a standard dideoxy procedure (Sanger et al., 1977).

Purified ssDNA from individual clones was PCR amplified with primers 1 and 2. RNA was transcribed from the PCR product, and its function as a methylation substrate was assayed as described above.

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