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Biochemistry

A functional peptide encoded in the *Escherichia coli* 23S rRNA

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ABSTRACT **A pentapeptide open reading frame equipped with a canonical ribosome-binding site is present in the *Escherichia coli* 23S rRNA. Overexpression of 23S rRNA fragments containing the mini-gene renders cells resistant to the ribosome-inhibiting antibiotic erythromycin. Mutations that change either the initiator or stop codons of the peptide mini-gene result in the loss of erythromycin resistance. Nonsense mutations in the mini-gene also abolish erythromycin resistance, which can be restored in the presence of the suppressor tRNA, thus proving that expression of the rRNA-encoded peptide is essential for the resistance phenotype. The ribosome appears to be the likely target of action of the rRNA-encoded pentapeptide, because *in vitro* translation of the peptide mini-gene decreases the inhibitory action of erythromycin on cell-free protein synthesis. Thus, the new mechanism of drug resistance reveals that in addition to the structural and functional role of rRNA in the ribosome, it may also have a peptide-coding function.**

Ribosomal RNA plays a fundamental role as a structural and functional component of the ribosome (1, 2). Because of its size and sequence complexity, rRNA also has considerable protein-coding capacity; indeed, relatively long open reading frames (ORFs) can be found in the rRNA of *Escherichia coli* and other organisms (3, 4). However, attempts to identify rRNA-encoded proteins in the cell have not been successful so far (5). Until now, the possible functional significance of the smaller ORFs in rRNA has been essentially ignored, even though biological activity of short peptides has been well documented (6–8). Furthermore, short peptides can modulate ribosomal activity (9–11), and peptide-like antibiotics are potent inhibitors of translation (12–14). In this paper we present experimental evidence that a pentapeptide mini-gene is present in *E. coli* rRNA; this mini-gene codes for a biologically active peptide capable of affecting ribosomal function.

MATERIALS AND METHODS

Strains, Enzymes, and Chemicals. *E. coli* strain JM109 was used in most experiments. *E. coli* strains 3000YA14 and CA161 were obtained from the *E. coli* Genetic Stock Center Yale University. All restriction enzymes, *Taq* DNA polymerase, and T7 RNA polymerase were from Promega or New England Biolabs. Radioisotopes were from Amersham. MS2 RNA, pyruvate, pyruvate-kinase, and total *E. coli* tRNA were from Boehringer Mannheim; folinic acid was from Sigma; and glass fiber filters were from Fisher Scientific. The Met-Arg-Met-Leu-Thr (MRMLT) and *N*-formyl-Met-Arg-Met-Leu-Thr (fMRMLT) peptides were synthesized by TANA Bio-Systems (Houston) and Chiron (San Diego), respectively, and purified by HPLC.

RNA Expression Vectors pPOT1 and pPOT72. The RNA expression vectors pPOT1 and pPOT72 were constructed on the base of the pGEX-2T plasmid (Pharmacia). The *Tth*III–*Bsp*M1 segment of the pGEX-2T plasmid was replaced with the *Kpn*1 linker to generate pGEX-K plasmid. A DNA fragment containing sequences of the *tac* promoter (15), *lac* operator, *Nhe*1 cloning site, and *trp* terminator was introduced into the *Kpn*1 site of pGEX-K to produce pPOT1 vector (see Fig. 1*A*). The transcripts originated at P*tac* contain sequences of the *lac* operator and *trp* terminator at the 5′- and 3′-termini, respectively; these extra sequences form internal hairpins (see Fig. 1*B*) and consequently, should not interfere with folding of the inserted rRNA fragments and may even increase the stability of the transcript (16, 17). Replacement of the pPOT1 *tac* promoter with T7 RNA polymerase promoter and elimination of the upstream *Kpn*1 restriction site resulted in the generation of pPOT72 vector. When cut at the unique *Kpn*1 site, pPOT72 can be used for *in vitro* run-off transcription of cloned rDNA fragments. The RNA transcripts generated *in vitro* from pPOT72 are almost identical to *in vivo*-generated transcripts from pPOT1 (Fig. 1*B*), except for the presence of several extra uridines at the 3′-end and three guanosines at the 5′-ends of the *in vitro* transcripts.

Construction of the rRNA Random Fragment Library and Selection of Erythromycin-Resistant Clones. The complete *E. coli rrn*B operon was excised from the pKK3535 plasmid (3) as a 7.5-kb *Bam*H1 fragment, circularized by overnight incubation with DNA ligase, and randomly fragmented by partial cleavage with DNAse I in the presence of Mn^{2+} (18). The resulting fragments, which ranged in size from ≈30 bp to several hundred base pairs, were blunt-ended, ligated to the *Spe*1 linkers, and cloned into the *Nhe*1 site of the pPOT1 vector (neither *Nhe*1 nor *Spe*1 sites are present in the *rrn*B operon). About 10^4 clones were obtained after introducing the plasmid library into the *E. coli* JM109 cells.

To identify rRNA fragments which can render cells resistant to erythromycin, 5×10^4 library clones were plated onto agar plates containing 100 μg of ampicillin per ml, 1 mM isopropyl β-D-thiogalactopyranoside (IPTG), and 150 μg of erythromycin per ml. Ery^r colonies that appeared on the plate after 24 hr of incubation were grown in liquid cultures; plasmids were isolated and used to transform fresh cells. Phenotypes of the secondary transformants were tested by replica plating onto ampicillin (100 μg/ml)/erythromycin (150 μg/ml) and ampicillin (100 μg/ml)/erythromycin (150 μg/ml)/IPTG (1 mM) plates. Clones that showed IPTG-dependent Ery^r phenotype ("E-RNA" clones) were used for further analysis.

Deletion Analysis of E-RNA. One of the initially selected E-RNA clones, expressing a fragment of the 23S rRNA gene spanning nucleotides 1233–1348, was subjected to deletion analysis. Deletions in rDNA were generated by PCR amplifying segments of the rDNA insert and reintroducing them into the pPOT1 vector. Phenotypes of the cells transformed with deleted versions of the original clone were tested by replica plating on antibiotic-containing plates as described in the previous section.

Introducing Mutations into E-RNA. To introduce random mutations into E-RNA, an oligodeoxyribonucleotide corresponding to the 23S rRNA sequence 1234–1276, flanked by *Spe*1 restriction sites, was synthesized so that all possible nucleotide substitutions were introduced at each position of

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Abbreviations: IPTG, isopropyl-β-D-thiogalactopyranoside.

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the 23S rRNA sequence with a frequency of 1% (97% of the wild-type nucleotide). After synthesis of the second DNA strand and cleavage of the product with *Spe*1, the double-stranded DNA fragment was ligated into the *Nhe*1 site of the pPOT1 vector. The recombinant plasmids were introduced into JM109 cells and clones exhibiting Ery^s or Ery^r phenotypes were selected by replica plating.

Fusion of the E-RNA Ribosome-Binding Site to the *cat* Reporter Gene. The *cat* gene was PCR-amplified from the pACYC 184 plasmid. The upstream PCR primer, CACTAGT-GGAGGTATCAGAAGTGGAGAAAAAAATCAC, contained the sequence of the ribosome binding site of the E-RNA mini-gene (underlined) and 15 nucleotides from the 5′-end of the *cat* gene starting from the second codon. The downstream PCR primer corresponded to the 3′-end of the *cat* gene. The PCR product was cut at the *Spe*1 sites present within the PCR primers and cloned into the *Nhe*1 site of the pPOT1 vector. The resulting plasmid was introduced into JM109 cells, and the *in vitro* CAT assay was performed as described (19).

Introduction of an Opal Suppressor tRNA Gene into E-RNA Clones. The gene of an opal suppressor $tRNA^{Arg}$ with its own promoter was PCR-amplified from the pGFIBI plasmid (20) and introduced into the unique *Eco*0109 restriction site (Fig. 1*A*) of recombinant pPOT1 plasmids expressing wild-type or mutant E-RNAs corresponding to the 23S rRNA segment 1234–1268. The plasmids were introduced into JM109 cells, and individual colonies were isolated and grown in liquid cultures containing 100 μg of ampicillin per ml. Cultures were diluted, and 20-μl aliquots containing ≈20 and ≈100 cells were spotted onto agar plates containing ampicillin (100 μg/ml)/IPTG (1 mM) and ampicillin (100 μg/ml)/IPTG (1 mM)/erythromycin (150 μg/ml) (21). Plates were incubated at 37°C 16 hr for ampicillin/IPTG plates and 40 hr for ampicillin/IPTG/erythromycin plates.

***In Vitro* Translation.** The rDNA segment (positions 1234–1268 in the 23S rRNA) was PCR-amplified from the recombinant pPOT1 plasmid and inserted into the pPOT72 plasmid vector under the control of the T7 RNA polymerase promoter. The resulting plasmid was linearized by cutting with *Kpn*1 and transcribed *in vitro* as recommended by the manufacturer of the T7 RNA polymerase (Promega). The RNA transcript was gel-purified and used in the cell-free translation system.

Ribosomes and S100 enzyme fraction used in the cell-free translation system were prepared from the *E. coli* strain MRE600 as described previously (22). The preincubation step of the cell-free translation was carried out in a 50-μl reaction mixture containing 8 mM $MgCl_2$, 80 mM KCl, 80 mM NH_4Cl, 5 mM 2-mercaptoethanol, 20 mM Tris·HCl (pH 7.6), 1.5 mM ATP, 0.5 mM GTP, 5 mM pyruvate, 1.2 μg of pyruvate kinase per ml, 12 μg of folinic acid per ml, 1.2 mg of total *E. coli* tRNA per ml, 6 pmol of ribosomes (activated 5 min at 42°C), 90 μM each of 19 amino acids (excluding phenylalanine), and 4 μl of the S100 fraction. The mixture was incubated with 10 pmol of phage MS2 RNA or with 10 pmol of *in vitro*-transcribed E-RNA34 at 37° for 20 min. Eight microliters of erythromycin solution in 8 mM $MgCl_2$/80 mM KCl/80 mM NH_4Cl/1.5 mM 2-mercaptoethanol/20 mM Tris·HCl, pH 7.6 was added to the reaction mixture to bring erythromycin concentration to 0.4 or 0.8 μg/ml, and the mixture was incubated 5 min at 37°C. This was followed by adding 42 μl of a solution containing 8 mM $MgCl_2$, 80 mM KCl, 80 mM NH_4Cl, 1.5 mM 2-mercaptoethanol, 20 mM Tris·HCl (pH 7.6), 1.8 mM ATP, 0.6 mM GTP, 6 mM pyruvate, 1.4 μg of pyruvate kinase per ml, 14 μg of folinic acid per ml, 1.4 mg of total *E. coli* tRNA per ml, 107 μM each of 19 amino acids (excluding phenylalanine), 60 μM [^{3}H]phenylalanine [7.5 Ci/mmol (1 Ci = 37 GBq)], 10 pmol of phage MS2 RNA, and 4 μl of S100 fraction. The reaction mixture was incubated 20 min at 37°C. RNA was hydrolyzed by adding 200 μl of 1M NaOH and incubating 10 min at 37°C. The synthesized polypeptides were precipitated by adding 1 ml of 25% trichloroacetic acid/2% casamino acids and incubating 30 min on ice. Precipitates were collected in glass fiber filters; filters were dried and counted in a scintillation counter.

RESULTS

For studies of rRNA functions, an expression library of random rRNA fragments was prepared. Random fragments of the *E. coli rrn*B operon were inserted downstream of the strong *tac* promoter in the plasmid vector pPOT1 (Fig. 1), and the resulting plasmid library was introduced into *E. coli* cells. About ten thousand clones were obtained which, upon induction of transcription from the *tac* promoter, generated rRNA fragments (or their complements) ranging in size from ≈30 to 500 nucleotides. The representation of rRNA sequences in the generated library appeared to be random; PCR analysis of the total library and sequencing of 20 randomly picked clones did not show predominance of any specific rRNA fragment.

The library was screened for the presence of clones where expression of rRNA fragments increased cell resistance to ribosome-targeted antibiotics. A number of colonies appeared when cells were plated on agar medium containing erythromycin. Erythromycin resistance was shown to be dependent on the presence of rDNA-containing plasmids rather than chromosomal mutations, as Ery^r phenotype of the selected clones cotransferred with the plasmids. Furthermore, transcription of a plasmid-borne rDNA segment was essential for erythromycin resistance, because transformed cells exhibited Ery^r phenotype only in the presence of IPTG, an inducer of the *tac* promoter. The rRNA fragments produced in these clones were designated E-RNA for erythromycin resistance RNA. All the analyzed rDNA inserts from >20 different Ery^r clones over-

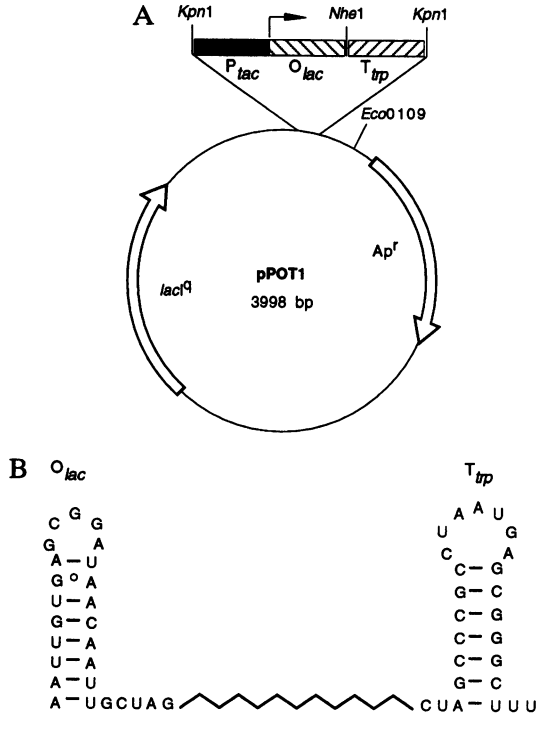

FIG. 1. Random rRNA fragment library. (*A*) The schematic map of the pPOT1 plasmid vector. Relative location and orientation of the $lacI^q$ and β-lactamase (Ap^r) genes are shown by open arrows. An inducible *tac* promoter (P_{tac}) is shown by a black bar; *lac* operator (O_{lac}) and tryptophan terminator (T_{trp}) are shown by hatched bars; and transcription start site is shown by an arrow. The position of the *Eco*0109 site used for introducing the suppressor tRNA gene is indicated. (*B*) The general secondary structure of the rRNA transcripts expressed from the pPOT1 library plasmids. rRNA segment of the transcript is represented by a zigzag line flanked by hairpins formed by the *lac* operator (O_{lac}) and *trp* terminator (T_{trp}) sequences.

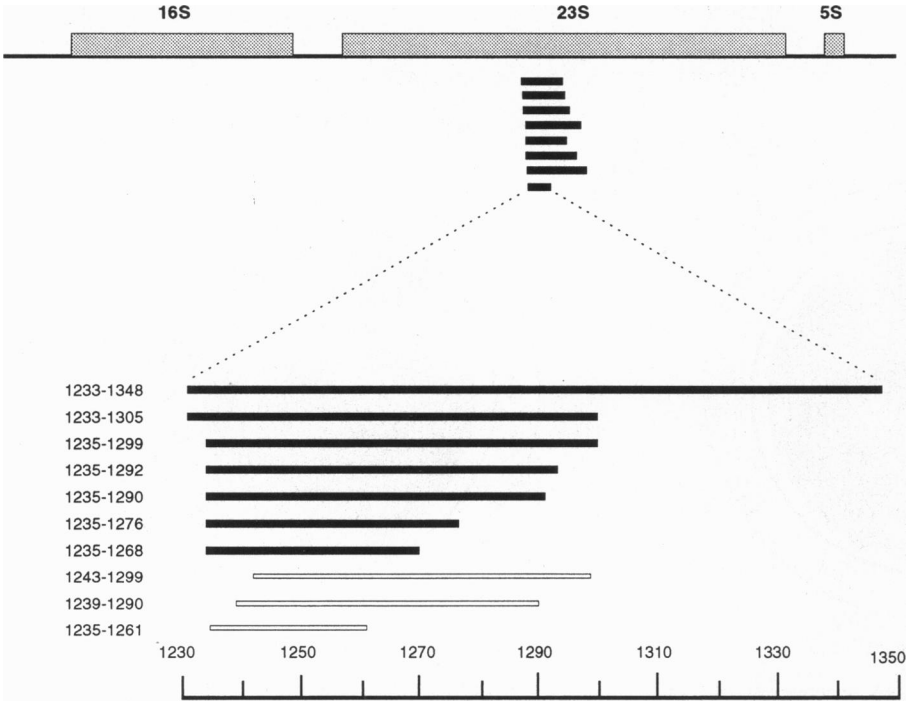

FIG. 2. rRNA fragments expressed in the selected and constructed Ery^r clones. The black bars shown under the schematic map of the rRNA operon correspond to E-RNAs expressed in the originally selected Ery^r clones. The bottom portion shows deletion analysis of the clone that expressed E-RNA corresponding to the 23S rRNA at positions 1233–1348. Filled bars correspond to the rRNA fragments that rendered cells resistant to erythromycin. Open bars represent fragments that did not confer erythromycin resistance. The scale at the bottom corresponds to the 23S rRNA numeration (23).

lapped in the region corresponding to positions 1233–1348 of the 23S rRNA (Fig. 2). In all the clones, the inserts were present in the direct orientation so that their transcription should result in production of sense 23S rRNA fragments. The smallest of the E-RNAs in the selected Ery^r clones corresponded to a 116-nt long segment of the 23S rRNA; subsequent deletion analysis showed that expression of an rRNA fragment only 34 nt long (E-RNA34), corresponding to the *E.*

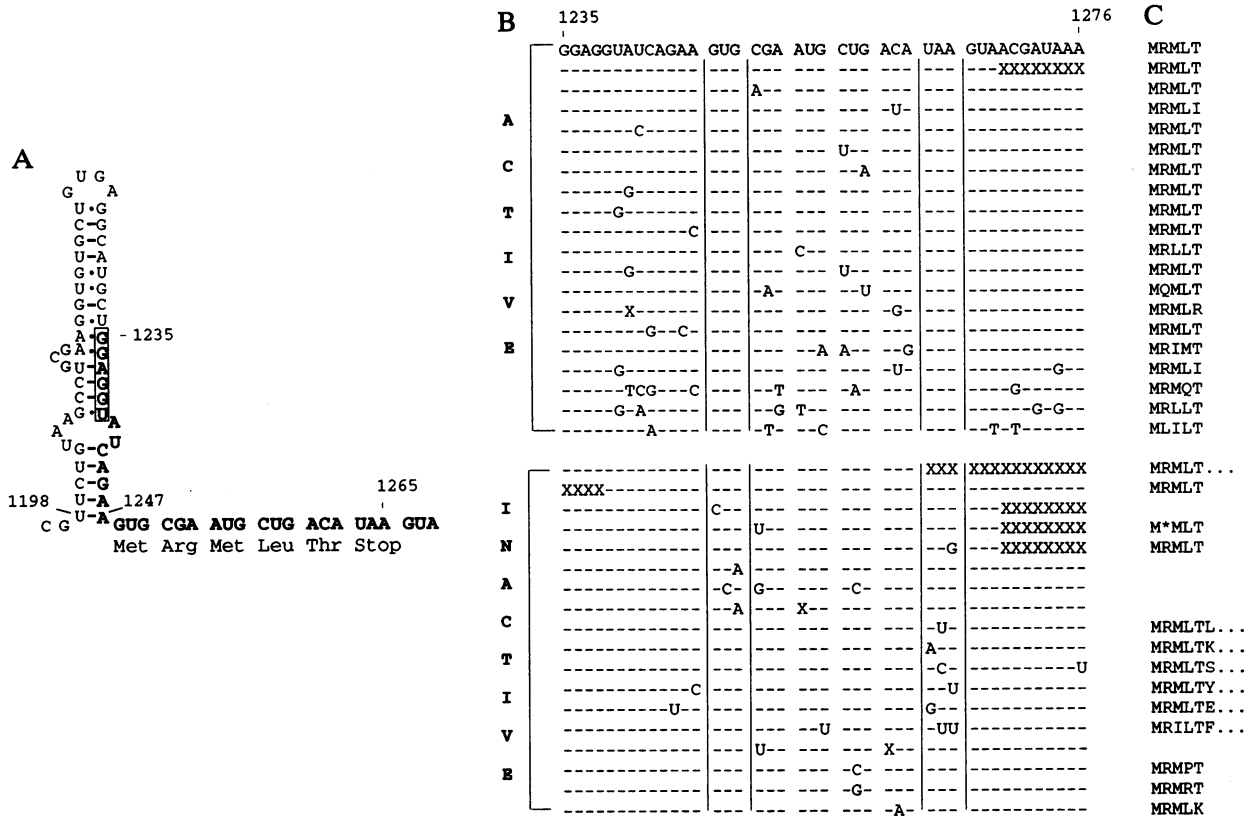

FIG. 3. E-RNA mutants. (*A*) The nucleotide sequence and secondary structure of the *E. coli* wild-type 23S rRNA segment encoding the pentapeptide mini-gene. The minimal rRNA fragment capable of conferring erythromycin resistance (E-RNA34) is shown in boldface. Shine–Dalgarno region (24) of the pentapeptide gene is boxed, and the sequence of the encoded peptide is indicated. (*B*) E-RNA mutants that retained ("active") or lost ("inactive") their ability to confer erythromycin resistance. Nucleotides identical with the wild-type rRNA are indicated by dashes; deletions are marked by "X." Positions corresponding to the initiation codon and stop codon of the pentapeptide gene are outlined by vertical lines. (*C*) Amino acid sequences of the peptides that can be expressed from the wild-type and mutant E-RNA.

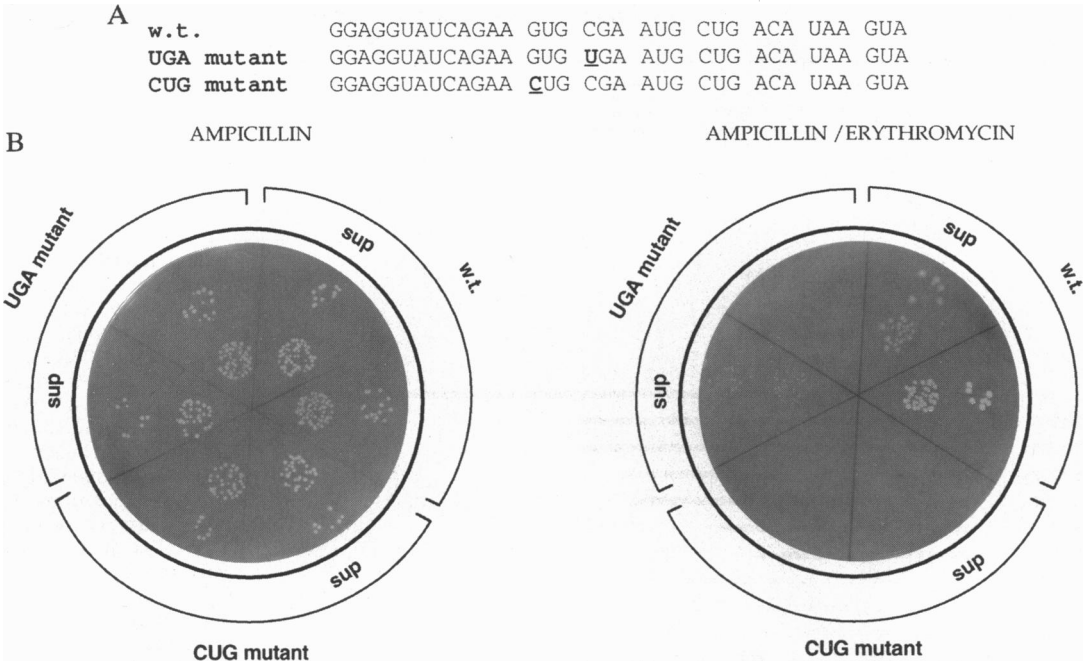

FIG. 4. Effect of the *opal* suppressor tRNA on activity of wild-type and mutant E-RNA34. (*A*) Sequences of the wild-type E-RNA34 and CUG and UGA mutants. (*B*) Effect of erythromycin on growth of cells expressing wild-type and mutant E-RNA34 in the absence or in the presence of the *opal* suppressor $tRNA^{Arg}$. Strains that were transformed with the wild-type or mutant E-RNA plasmids containing the suppressor $tRNA^{Arg}$ gene are marked "sup." Diluted cell cultures were spotted on plates containing 100 μg of ampicillin per ml and 1 mM IPTG (left) or 100 μg of ampicillin per ml, 150 μg of erythromycin per ml, and 1 mM IPTG (right). The level of suppression caused by the suppressor $tRNA^{Arg}$ is ≈20% (19), which accounts for the smaller size of the colonies produced on erythromycin plates by cells expressing the UGA mutant of E-RNA34.

coli 23S rRNA sequence between positions 1235 and 1268, could render cells resistant to erythromycin (Fig. 2).

To get insights into the mechanism of E-RNA action, mutations were introduced randomly into the structure of the E-RNA (Fig. 3*B*). Distribution of mutations that abolished E-RNA activity revealed two critical regions: GUG at positions 1248–1250 and UAA at positions 1263–1265. Remarkably, these two segments corresponded to initiator and terminator codons of a pentapeptide ORF present in E-RNA (Fig. 3*A*). Moreover, a canonical Shine–Dalgarno sequence, GGAGGU (positions 1235–1240), which is essential for initiation of translation in prokaryotes (24), is present seven nucleotides upstream from the initiator codon, at the 5′-end of E-RNA, and is important for its function, because deletion of GGAG (positions 1235–1238) renders E-RNA inactive (Fig. 2). These results raised the possibility that expression of a 23S rRNA-encoded pentapeptide Met-Arg-Met-Leu-Thr (E-peptide) may be required for the observed erythromycin resistance in recombinant clones.

To verify that the putative ribosome binding site of the E-peptide mini-gene could direct initiation of translation, the first 16 nucleotides of E-RNA34, comprising the putative Shine–Dalgarno sequence and the initiator GUG codon, were fused to the second codon of the chloramphenicol acetyltransferase reporter gene. Cells, transformed with the resulting chimeric construct, became resistant to chloramphenicol and significant chloramphenicol acetyltransferase activity was detected in the cell extracts (data not shown). This result confirmed that the putative ribosome binding site of the pentapeptide mini-gene present in the *E. coli* 23S rRNA can be efficiently used for initiation of translation. To directly test whether translation of the E-peptide mini-gene was required for erythromycin resistance, we investigated whether function of an E-RNA with a nonsense mutation in the peptide ORF can be restored in the presence of a suppressor tRNA. We used E-RNA34 mutant, in which E-RNA function was abolished by a nonsense mutation in the second codon of the mini-gene, which converted the arginine codon CGA into the *opal* stop codon UGA (see Fig. 4). As a negative control, we used another inactive E-RNA mutant with a mutation in the initiator codon (CUG mutant, Fig. 4). A gene of the opal suppressor $tRNA^{Arg}$ (20), expressed from its own promoter, was introduced into the same plasmid, and phenotypes of the transformed cells were assessed by their plating on antibiotic-containing agar plates (Fig. 4). Presence of the suppressor $tRNA^{Arg}$ restored ability of the UGA mutant, but not the CUG mutant, to confer resistance to erythromycin, thus proving that synthesis of the rRNA-encoded E-peptide was necessary for drug resistance.

Effects of the E-peptide and E-RNA on translation apparatus was studied in the cell-free translation system. The synthetic E-peptides, MRMLT and f-MRMLT, did not influence erythromycin sensitivity of *in vitro* protein synthesis up to 1 mM of the peptide concentration (data not shown). Yet, when E-RNA was added to the cell-free system, instead of the E-peptide, a reproducible protection against erythromycin was observed (Fig. 5). Without the E-RNA, translation of the phage MS2 RNA was reduced ≈3-fold in the presence of 0.4–0.8 μg of erythromycin per ml. If, however, ribosomes were allowed to translate E-RNA before addition of the phage RNA, then residual protein synthesis in the presence of erythromycin was ≈2-fold higher. Because E-RNA reduces the effects of erythromycin not only *in vivo* but also *in vitro*, the translation apparatus appears to be a likely target of action of the rRNA-encoded peptide.

DISCUSSION

In this paper we demonstrated that production of specific rRNA fragments can render cells resistant to erythromycin. The rRNA sequences that conferred resistance were identified using an expression library of random rRNA fragments. This approach has considerable potential for isolating rRNA fragments with specific functions, because phenotypic selection makes it possible to screen thousands of clones expressing different rRNA fragments in a quest for sequences that can confer selectable phenotypes.

In our selection, we used the ribosome-targeted antibiotic erythromycin as a selective agent. Erythromycin has a single binding site located on the large ribosomal subunit. Bound erythromycin protects positions A2058, A2059, and G2505 in

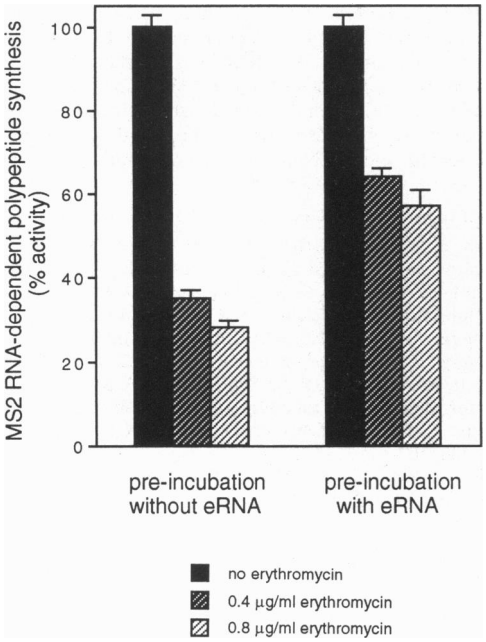

FIG. 5. Effect of the E-RNA translation on erythromycin sensitivity of the cell-free translation system. Ribosomes were preincubated with E-RNA or MS2 RNA and subsequent MS2 RNA-dependent polypeptide synthesis was assessed both in the absence and in the presence of erythromycin. Polypeptide synthesis in the absence of the drug was taken as 100%.

the central loop of domain V from chemical modification (25, 26). Mutations in the same region result in Ery^r phenotype (27–29). Adenine, corresponding to A2058 of the *E. coli* 23S rRNA, is methylated in the erythromycin producer, *Streptomyces erythreus*, rendering the ribosomes of the producer resistant to the drug; methylation of the similar position in the rRNA of *E. coli* and other bacteria prevents binding of erythromycin (30). Thus, there is ample experimental evidence suggesting that the primary binding site of erythromycin involves domain V of the 23S rRNA. Much to our surprise, the clones isolated from our library expressed E-RNAs derived from a different region of the 23S rRNA, the junction of domains II and III. The mode of E-RNA action began to emerge when mutations that affected initiator or terminator codons of the pentapeptide mini-gene present within E-RNA abolished resistance; this suggested that E-RNA action may be mediated by the expression of the rRNA-encoded E-peptide. The idea was corroborated by the observation that the ribosome binding site of the E-peptide mini-gene, fused to a reporter gene, could be efficiently used for initiation of translation. The final confirmation of this hypothesis was received from an experiment in which activity of an E-RNA with a nonsense mutation in the E-peptide gene was restored in the presence of a suppressor tRNA (Fig. 4). This clearly demonstrated that translation of E-RNA was required for erythromycin resistance of the E-RNA-expressing clones.

Translation of E-RNA solved the problem of its relatively low abundance. At the initial stages of our experiments, we noticed that the amount of E-RNA accumulated in Ery^r clones was relatively low, <1 mol % of the amount of 5S rRNA (data not shown), and was not enough to directly affect a significant portion of the ribosome population or to sequester a substantial amount of intracellular erythromycin. Translation of E-RNA resolves this contradiction, because multiple E-peptide molecules can be translated from one molecule of E-RNA.

The mutational analysis revealed some functionally important structural features of the E-peptide (Fig. 3*B*). Conversion of the mini-gene's stop codon into a sense codon abolished the peptide-mediated erythromycin resistance suggesting that the presence of extra sequences at the C-terminus of the E-peptide is unfavorable for its activity. The importance of the translation termination site was also evident from the fact that the E-RNA mutant, in which the stop codon of the E-peptide mini-gene was changed from *ochre* UAA to *amber* UAG, remained active in the *E. coli* strain 3000YA14 but not in the isogenic strain CA161 (31) that carried an *amber* suppressor mutation (data not shown). The necessity for efficient termination of E-peptide translation suggests that the size of the E-peptide is essential for its activity. In contrast, the primary structure of the E-peptide appears to be less strictly constrained; whereas certain amino acid changes were deleterious for the E-peptide function (see "inactive mutants" in Fig. 3*B*), the majority of missense mutations did not eliminate activity of E-RNA. Analysis of a bigger collection of E-peptide sequence variations should help to elucidate E-peptide sequence constraints (Tenson and Mankin, unpublished data).

How can expression of a short peptide make cells resistant to an antibiotic? In general, E-peptide can sequester erythromycin into an inactive complex, decrease uptake of the drug, or prevent its binding to the ribosome. To examine these possibilities, we studied effects of the E-peptide and E-RNA on cell-free translation. *In vitro* translation of the MS2 phage RNA is inhibited by micromolar concentrations of erythromycin. Addition of up to 1 mM of the synthetic E-peptide (Met-Arg-Met-Leu-Thr or *N*-formyl-Met-Arg-Met-Leu-Thr) to the cell-free translation system did not reduce erythromycin inhibition. This ruled out direct sequestering of the drug by the E-peptide, because the peptide concentration was three orders of magnitude higher than that of the drug. However, when the *in vitro*-transcribed E-RNA was present instead of E-peptide, the erythromycin effect was notably diminished. This effect was most prominent when ribosomes could translate E-RNA before the addition of a reporter cistron (phage MS2 RNA). In experiment, shown in Fig. 5, the ribosomes were preincubated with either E-RNA or, in the control, MS2 RNA, in the presence of 19 amino acids (excluding phenylalanine). Under these conditions, ≈3 pmol of E-peptide is translated from E-RNA (data not shown), corresponding to one-half of the molar amount of the ribosomes present. After preincubation, an excess of MS2 RNA was added to the reaction mixture accompanied by ^{3}H phenylalanine; because E-peptide does not contain phenylalanine, the label is incorporated only into the MS2 RNA-coded proteins. Fig. 5 shows that the ribosomes that were allowed to translate E-RNA became less sensitive to erythromycin, whereas erythromycin sensitivity of the ribosomes preincubated with MS2 RNA or without mRNA did not change (Fig. 5). It should be noted that a seemingly small effect of E-RNA on the erythromycin sensitivity of the cell-free protein synthesis can easily translate into a 3- to 4-fold increase of the drug's minimal inhibitory concentration observed when E-RNA is expressed *in vivo* (27). Because direct sequestering of erythromycin was ruled out in experiments with the synthetic peptide and drug transport does not affect a cell-free system, the results of *in vitro* translation experiments suggest that the ribosome is a likely target of action of the E-RNA-encoded E-peptide. One possible model is that the E-peptide remains tightly associated with the nascent peptide channel of the ribosome and hinders binding of erythromycin; this model is compatible with the known mode of erythromycin action (12, 13, 32, 33). An interesting consequence of the model is that E-peptide should be placed into its target site cotranslationally and function in cis, affecting erythromycin sensitivity of the ribosome on which it has been translated. This suggestion is consistent with the observation that exogenously added synthetic E-peptide was not effective in the cell-free system at physiological concentrations; the site of cotranslational placement of functionally active peptide may be inaccessible for binding a peptide from the solution. Similar conclusions can be

drawn from the results of Lovett and coworkers (9, 10), who have shown that surprisingly high concentration of synthetic peptides were required to reproduce *in vitro* the effects of cis-acting peptides involved in translation attenuation.

In our experiments, E-peptide was translated from a rRNA fragment expressed from a strong plasmid promoter. It is unclear yet whether E-peptide is expressed naturally. In the intact ribosome, the peptide mini-gene apparently remains cryptic because its ribosome-binding site is sequestered in the rRNA secondary structure (Fig. 3*A*). As is evident from our results, the E-peptide expression can be activated by a specific RNA fragmentation. Cutting of the rRNA in the apex stem-loop of the helix at positions 1198–1247 of the 23S rRNA, which may happen, for example, under conditions of physiological stress, can generate translatable rRNA fragments leading to production of the E-peptide. Mutations are yet another way to activate expression of the rRNA-encoded E-peptide. A spontaneous deletion of 12 nucleotides (positions 1219–1230) from the 23S rRNA gene has been described as causing resistance to erythromycin (34). The effect of this deletion in domain II was difficult to reconcile with the known location of the erythromycin-binding site in domain V of the 23S rRNA. Our results offer a new explanation for the erythromycin resistance caused by deletion in domain II. The 12-nt deletion destabilized the hairpin at positions 1198–1247, thus making the ribosome-binding site of the E-peptide mini-gene more accessible and activating E-peptide expression. Analysis of effects of other deletions and nucleotide substitutions in the hairpin at positions 1198–1247 on erythromycin resistance strongly supports our explanation (35).

In the absence of direct evidence for expression of E-peptide in normal cells, we can only speculate about its biological significance. It is not clear whether the presence of a functional peptide gene in rRNA is a suspicious coincidence or a result of evolutionary selection. At the very least, the fact that the rRNA-encoded peptide can affect translation raises a possibility that such arrangement can be used by the cell. Erythromycin resistance is probably not a primary activity but a side effect of interaction of the rRNA-encoded peptide with the ribosome. Similar to other peptides that can affect the function of the ribosome in cis, the E-peptide may be involved in regulation of translation (36–38); in this case it is expected to be expressed only under certain conditions that are compatible with the cryptic nature of the E-peptide gene in the intact 23S rRNA.

It is worth noting that E-peptide effects were not restricted to a particular *E. coli* strain. Thus, strains DH5α, JM109, and HB101, which significantly differ in their erythromycin sensitivity, all exhibited E-peptide-dependent erythromycin resistance. All the tested strains, upon transformation with the E-RNA-expressing plasmid, could tolerate erythromycin concentration 2 to 3 times higher than the respective minimal inhibiting concentrations. Because translation apparatus is extraordinarily conserved, it is expected that ribosomes of other bacteria may also exhibit erythromycin resistance upon interaction with the E-peptide or its analogs. Therefore, it is possible that some cases of erythromycin resistance observed in clinical isolates can be due to mutations in the rRNA genes activating expression of the E-peptide. Such a mutation, even in one of multiple rRNA gene copies, is expected to be dominant; ribosomes expressed from the wild-type rRNA genes would become resistant due to interaction with E-peptide translated from the mutant rRNA. Many, but not all, prokaryotic 23S rRNA sequences contain a pentapeptide mini-gene at the junction of domains II and III. However, it might well be that functional peptides are encoded in other short ORFs which are found in rRNA of practically all organisms. It would be also interesting to analyze whether expression of other short peptides, not necessarily encoded in rRNA, could contribute to appearance of bacterial strains resistant to low erythromycin concentrations.

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1. Noller, H. F., Moazed, D., Stern, S., Powers, T., Allen, P. N., Robertson, J. M., Weiser, B. & Triman, K. (1990) in *The Ribosome: Structure, Function and Evolution*, eds. Hill, W. E., Dahlberg, A., Garrett, R. A., Moore, P. B., Schlessinger, D. & Warner, J. R. (Am. Soc. for Microbiol., Washington, DC), pp. 73–92.
2. Egebjerg, J., Larsen, N. & Garrett, R. A. (1990) in *The Ribosome: Structure, Function and Evolution*, eds. Hill, W. E., Dahlberg, A., Garrett, R. A., Moore, P. B., Schlessinger, D. & Warner, J. R. (Am. Soc. for Microbiol., Washington, DC), pp. 168–179.
3. Brosius, J., Dull, T. J., Sleeter, D. D. & Noller, H. F. (1981) *J. Mol. Biol.* **148,** 107–127.
4. Peng, G., Taylor, J. D. & Tchen, T. T. (1992) *Biochem. Biophy. Res. Commun.* **189,** 445–449.
5. Berg, K. L., Squires, C. L. & Squires, C. (1987) *J. Bacteriol.* **169,** 1691–1701.
6. Hardie, D. G. (1991) *Biochemical Messengers: Hormons, Neurotransmitters and Growth Factors* (Chapman & Hall, London).
7. McCann, S. M. & Dhindsa, D. S., eds. (1983) *Role of Peptides and Proteins in Control of Reproduction* (Elsevier, New York).
8. Frankel, A. D. (1992) *Protein Sci.* **1,** 1539–1542.
9. Gu, Z., Harrod, R., Rogers, E. J. & Lovett, P. S. (1994) *J. Bacteriol.* **176,** 6238–6244.
10. Gu, Z., Harrod, R., Rogers, E. J. & Lovett, P. S. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 5612–5616.
11. Mayford, M. & Weisblum, B. (1990) *J. Bacteriol.* **172,** 3772–3779.
12. Vazquez, D. (1979) *Inhibitors of Protein Biosynthesis*, Kleinzeller, A., Springer, G. F. & Wittmann, H. G., eds. (Springer, Berlin).
13. Cundliffe, E. (1981) in *The Molecular Basis of Antibiotic Action* (Wiley, London), pp. 402–547.
14. Kolter, R. & Moreno, F. (1992) *Annu. Rev. Microbiol.* **46,** 141–163.
15. de Boer, H. A., Comstock, L. J. & Vasser, M (1983) *Proc. Natl. Acad. Sci. USA* **80,** 21–25.
16. Emory, S. A., Bouvet, P. & Belasco J. G. (1992) *Genes Dev.* **6,** 135–148.
17. McLaren, R. S., Newbury, S. F., Dance, G. S. C., Causton, H. C. & Higgins, C. F. (1991) *J. Mol. Biol.* **221,** 81–95.
18. Holzmayer, T. A., Pestov, D. G. & Roninson, I. B. (1992) *Nucleic Acids Res.* **20,** 711–717.
19. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY), 2nd Ed., pp. 16.60–16.62.
20. McClain, W. H., Foss, K., Jenkins, R. A. & Schneider, J. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 9260–9264.
21. Powers, T. & Noller, H. F. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 1042–1046.
22. Saarma, U. & Remme, J. (1992) *Nucleic Acids Res.* **20,** 3147–3152.
23. Noller, H. F., Kop, J., Wheaton, V., Brosius, J., Gutell, R. R., Kopylov, A. M., Dohme, F. & Herr, W. (1981) *Nucleic Acids Res.* **9,** 6167–6189.
24. Shine, J. & Dalgarno, L. (1974) *Proc. Natl. Acad. Sci. USA* **71,** 1342–1346.
25. Moazed, D. & Noller, H. F. (1987) *Biochimie* **69,** 879–884.
26. Douthwaite, S. & Aagaard, C. (1993) *J. Mol. Biol.* **232,** 725–731.
27. Sigmund, C. D. & Morgan, E. A. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 5602–5606.
28. Sigmund, C. D., Ettayebi, M., Borden, A. & Morgan, E. A. (1988) *Methods Enzymol.* **164,** 673–690.
29. Sigmund, C. D., Ettayebi, M., & Morgan, E. A. (1984) *Nucleic Acids Res.* **12,** 4653–4663.
30. Skinner, R. E., Cundliffe, E. & Schmidt, F. J. (1983) *J. Biol. Chem.* **258,** 12702–12706.
31. Signer, E., Beckwith, J. R. & Brenner, S. (1965) *J. Mol. Biol.* **14,** 153–166.
32. Vester, B. & Garrett, R. A. (1987) *Biochimie* **69,** 891–900.
33. Andersson, S. & Kurland, C. G. (1987) *Biochimie* **69,** 901–904.
34. Douthwaite, S., Prince, J. B. & Noller, H. F. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 8330–8334.
35. Dam, M., Douthwaite, S., Tenson, T. & Mankin, A. S. (1996) *J. Mol. Biol.* **259,** 1–6.
36. Geballe, A. P. & Morris, D. R. (1994) *Trends Biochem. Sci.* **19,** 159–164.
37. Lovett, P. S. (1994) *J. Bacteriol.* **176,** 6415–6417.
38. Tenson, T. & Mankin, A. S. (1995) *Biochem. Cell Biol.* **73,** 1061–1070.