DISSERTATIONES BIOLOGICAE UNIVERSITATIS TARTUENSIS
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RIBOSOMES, PEPTIDES AND ANTIBIOTIC RESISTANCE

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TARTU UNIVERSITY
PRESS

Submitted to comply with the Ph.D. requirements of the Institute of Molecular and Cell Biology, Tartu University May, 07th 1997

Dissertation is accepted for the commencement of the Doctor of Philosophy (in Molecular Biology) on August 28th, 1997 by the Council of the Institute of Molecular and Cell Biology, University of Tartu

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Commencement: October 24th, 1997

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CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original papers which will be referred to by their Roman numerals:

I. **Tenson, T.,** and Mankin, A. (1995) Comparison of functional peptide encoded in the *Escherichia coli* 23S rRNA with other peptides involved in cis-regulation of translation. Biochem. Cell Biol., 73, 1061–1070.

II. **Tenson, T.,** DeBlasio, A. and Mankin, A. (1996) A functional peptide encoded in the *Escherichia coli* 23S rRNA. Proc. Natl. Acad. Sci. USA, 93, 5641–5646.

III. Dam, M., Douthwaite, S., **Tenson, T.,** and Mankin, A. S. (1996) Mutations in domain II of 23 S rRNA facilitate translation of a 23 S rRNA-encoded pentapeptide conferring erythromycin resistance. J. Mol. Biol., 259, 1–6.

IV. **Tenson, T.,** Xiong, L., Kloss, P., and Mankin, A. S. (1997) Erythromycin resistance peptides selected from random peptide libraries. J. Biol. Chem., 272: 17425–17430.

LIST OF ABBREVATIONS

CPA1	carbamoyl-phosphate synthase A
E-peptide	erythromycin resistance peptide
E-RNA	erythromycin resistance RNA
Ery^r	erythromycin resistant
gp	glycoprotein
Ile	isoleucine
IPTG	isopropyl-ß-galactoside
Leu	leucine
mRNA	messenger RNA
ORF	open reading frame
rDNA	DNA coding for ribosomal RNA
rRNA	ribosomal rRNA
tRNA	transport RNA
uORF	upstream open reading frame
Val	valine

1. INTRODUCTION

Ribosome is a big nucleoprotein complex (molecular weight 2.5 MD), *Escherichia coli* ribosome contains three different RNAs and 54 ribosomal proteins. Its function is to synthesize proteins according to mRNA program. During this process ribosome interacts with many ligands. mRNA determines the sequence of the synthesized polypeptide. It interacts mainly with the small ribosomal subunit. tRNA brings activated amino acids to the ribosome. According to the classical model there are three tRNA binding sites in the ribosome: one for aminoacyl-tRNA (A site), one for peptidyl-tRNA (P site) and one for nonaminoacylated tRNA (E site) (Fig. 1). In all three sites tRNA has contact with rRNA (Noller, 1991) and ribosomal proteins (Wower *et al.*, 1995). Two elongation factors interact with the ribosome: EF-Tu brings aminoacyl-tRNA to the ribosomal A site and EF-G catalyzes translocation of tRNAs from A site to P site and from P site to E site after peptide bond formation. Several regions of rRNA and ribosomal proteins are involved in the interactions with the elongation factors (Möller and Maassen, 1986; Noller, 1991).

In addition to the well known interactions between ribosome and its ligands mentioned above, there is one more interaction that is often dismissed. It is the interaction between ribosome and nascent peptide. Formation of the peptide bond is catalyzed by the ribosomal peptidyl transferase center. Nascent peptide exits the ribosome through a tunnel (Yonath *et al.*, 1987) or channel (Ryabova *et al.*, 1988). Recently it has been discovered that there is bigger communication between the ribosome and the nascent peptide than thought before. It has been shown that protein folding can start already on the ribosome (Kolb *et al.*, 1994; Hardesty *et al.*, 1995; Komar *et al.*, 1997). And nascent peptide can regulate the work of the ribosome (Chapter 2.2.).

Ribosomal ligands described above are all ubiquitous parts of the normal translational machinery. In addition to the usual ligands there are also ribosomal ligands of big practical importance that do not help to synthesize proteins but inhibit the process instead. These molecules are called antibiotics. One of the ribosome targeted antibiotics is erythromycin which is widely used in medical practice. Erythromycin binds to the nascent peptide binding site and blocks growth of the nascent peptide chain (Chapter 2.1.). Antibiotic resistance is a growing problem in the treatment of infections. And the same problem is present with erythromycin — resistance has been described in many medical isolates. In some cases the resistance mechanism is known but there are also cases where resistance mechanism is not clear.

In the present work a new erythromycin resistance mechanism mediated by small ribosome-targeted peptides is described. First, relevant literature about erythromycin and ribosome-targeted peptides is reviewed. In the second part experimental results are discussed.

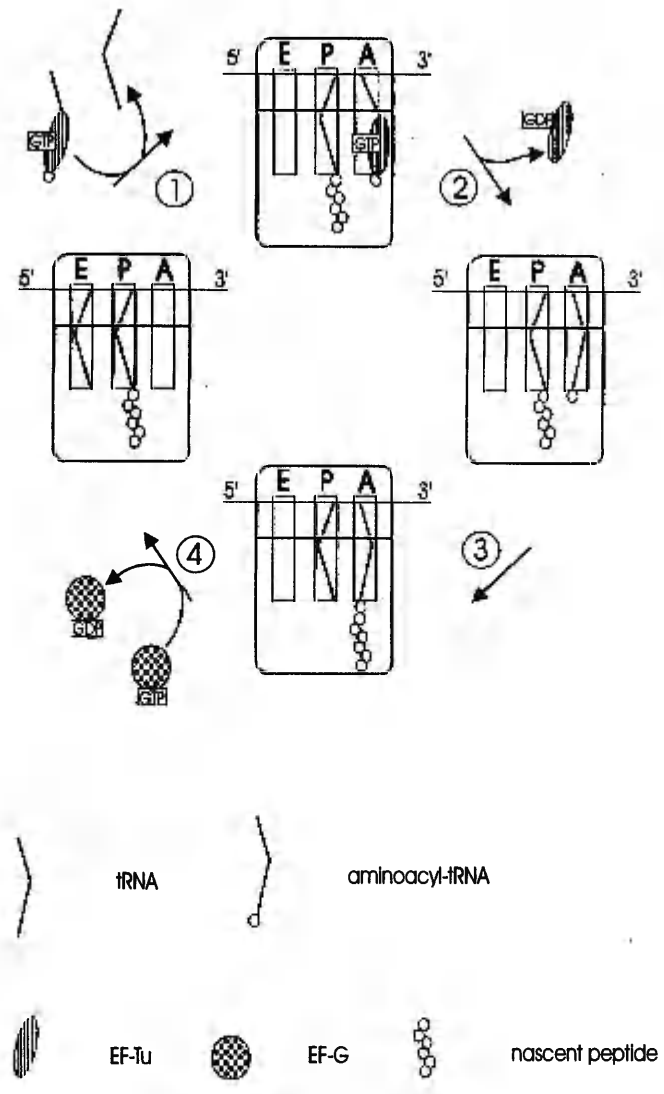

Figure 1. The three site model for the elongation cycle. E site tRNA is released and [aminoacyl-tRNA: EFTu: GTP] complex binds to the A site (1). GTP is hydrolyzed and [EF-Tu: GDP] complex leaves the ribosome; aminoacyl-tRNA in the A site is ready to act as acceptor in the peptidyl transferase reaction (2). Peptidyl transferase reaction occurs (3). EF-G catalyzes translocation of the peptidyl-tRNA from A site to P site and nonaminoacylated tRNA from P site to E site; during this process one GTP molecule is hydrolyzed (4).

2. REVIEW OF LITERATURE

2.1. Erythromycin

Macrolides are a group of commonly used antibiotics. They all contain a large lactone ring of 12–22 atoms which contains few or no double bonds and no nitrogen atoms; in addition they have one or more sugars attached to the lactone ring (Pestka, 1977). Erythromycin is the most widely used macrolide with 14 atom lactone ring and two attached sugars (Fig. 2). Erythromycin inhibits protein synthesis in procaryotes but not in eukaryotic cytoplasm or mitochondria (Pestka, 1977).

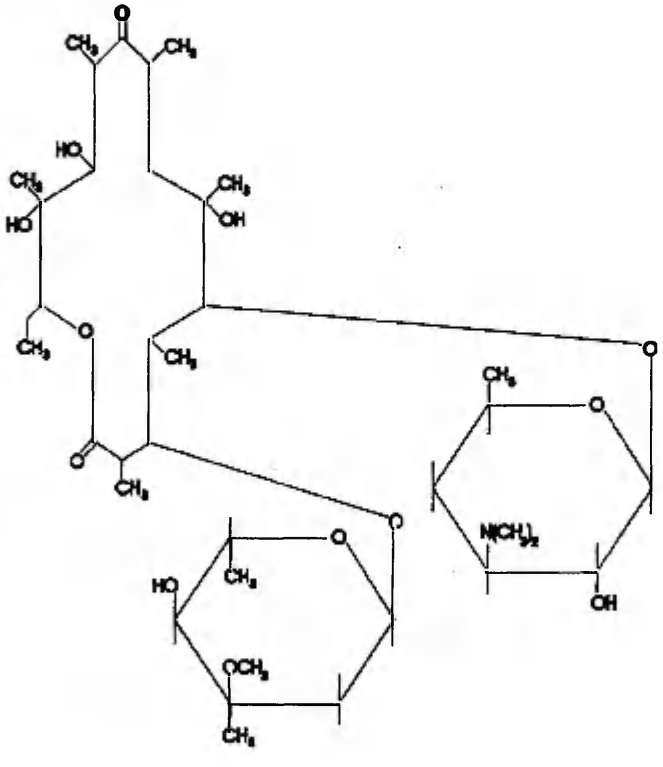

Figure 2. Chemical structure of erythromycin (Pestka, 1977).

The single binding site of erythromycin is located on the large ribosomal subunit in the vicinity of the peptidyl transferase center (Vazquez, 1979). 23S RNA has direct role in erythromycin binding because: (i) postranscriptional methylation of rRNA can confer resistance, (ii) mutations in rRNA can confer resistance, and (iii) erythromycin protects nucleotides in the 23S RNA from chemical modification.

The drug binds to vacant ribosomes or large ribosomal subunits and inhibits protein synthesis (Vazquez, 1979). Erythromycin causes the breakdown of polysomes and ribosomes enter a cycle of repetitive abortive initiations (Tai *et al.*, 1974). Andersson and Kurland (1987) studied the effect of erythromycin on ß-galactosidase synthesis *in vivo* and concluded that an initial stage of translation is susceptible to erythromycin while the elongating ribosome is insensitive to the drug action. In the model peptidyltransferase reaction erythromycin does not inhibit formation of the first peptide bond. However it inhibits peptidyltransferase when the donor substrate has peptide chain from two to five amino acids in length (Mao and Robishaw, 1972). Erythromycin does not inhibit translation when the nascent peptide chain is longer than 5 amino acids (Vester and Garrett, 1987). Peptidyl-tRNAs are released from the ribosome in response to erythromycin treatment, suggesting that erythromycin and peptidyl-tRNA have overlapping binding sites (Otaka and Kaji, 1975; Menninger and Otto, 1982).

From these observations it has been suggested that erythromycin inhibits nascent peptide growth only in a narrow window of the peptide length of 2 to 5 amino acids: during the synthesis of the first peptide bond the nascent chain does not reach the erythromycin binding site and after the nascent chain is longer than 5 amino acids the binding site is covered and erythromycin cannot bind (Vester and Garrett, 1987; Weisblum, 1995b).

It has been reported recently that erythromycin inhibits also assembly of the 50S subunit (Chittum and Champney, 1995). For inhibition of cell growth the effects of the drug on nascent peptide growth and 50S subunit assembly seem to contribute equally (Champney and Burdine, 1996). The mechanism how erythromycin inhibits 50S subunit assembly is currently not known.

The known mechanisms of erythromycin resistance include active efflux of the drug from the cell, modification of erythromycin and modification of ribosomal RNA (methylation or mutation of 23S rRNA bases) and ribosomal protein mutations. Active efflux mediated by a membrane transporter is a common cause for high level resistance (Weisblum, 1995a). Structural modification of erythromycin includes phosphorylation (Ohara *et al.*, 1989), glycosylation (Jenkins and Cundliffe, 1991), and lactone ring cleavage by erythromycin esterase (Ounissi and Courvalin, 1985; Arthur *et al.*, 1987).

Methylation of the 23S RNA resulting in erythromycin resistance occurs at nucleotide A2058 and was originally observed in clinical isolates of *Staphylococcus aureus* (Lai and Weisblum, 1971; Lai *et al.*, 1973; Skinner *et al.*, 1983). The gene responsible for methylation was designated as *ermA*. Later additional *erm* genes from other organisms have been found. The Erm family comprises a group of homologous methylases that use S-adenosylmethionine (SAM) as the methyl donor to modify a single adenine residue in the 23S rRNA to form either N^6-mono- or dimethyladenine (Weisblum, 1995a). There appear to be at least two functionally different classes of Erm methylases. The first class in-

cludes those that only monomethylate adenine, e.g., Lrm from *Streptomyces lividans* (Jenkins and Cundliffe, 1991), Clr from *Streptomyces caelestis* (Calcutt and Cundliffe, 1990), and TlrD from *Streptomyces fradiae* (Zalacain and Cundliffe, 1991). The second class includes enzymes that predominantly dimethylate adenine, e.g. ErmC from *S. aureus* (Denoya and Dubnau, 1989), ErmE from *Saccharopolyspora erythrea* (Calcutt and Cundliffe, 1990) and TlrA from *S. fradiae* (Zalacain and Cundliffe, 1989).

Mutations at three nucleotides in the 23S RNA are known to cause erythromycin resistance. All the three positions are located close to each other in the secondary structure model (Fig. 3) (Brimacombe *et al.*, 1990). Mutations C2611U (Vannuffel *et al.*, 1992; Harris *et al.*, 1989) and C2611G (Harris *et al.*, 1989) affect 5' half of helix 73, mutations G2057A (Ettayebi *et al.*, 1985), A2058G (Vester and Garrett, 1987) and A2058U (Sigmund *et al.*, 1988) affect 3' half of the same helix. Location of erythromycin binding site at the base of helix 73 is also confirmed by footprinting studies (Moazed and Noller, 1987). The drug protects bases A2058 and A2059 from dimethylsulfate modification; in addition, erythromycin also strongly protects G2505 (Fig. 3). Erythromycin resistance mutations are also described in domain II of 23S RNA (Douthwaite *et al.*, 1985, 1989). Their mechanism of action will be discussed in chapter 3.4.

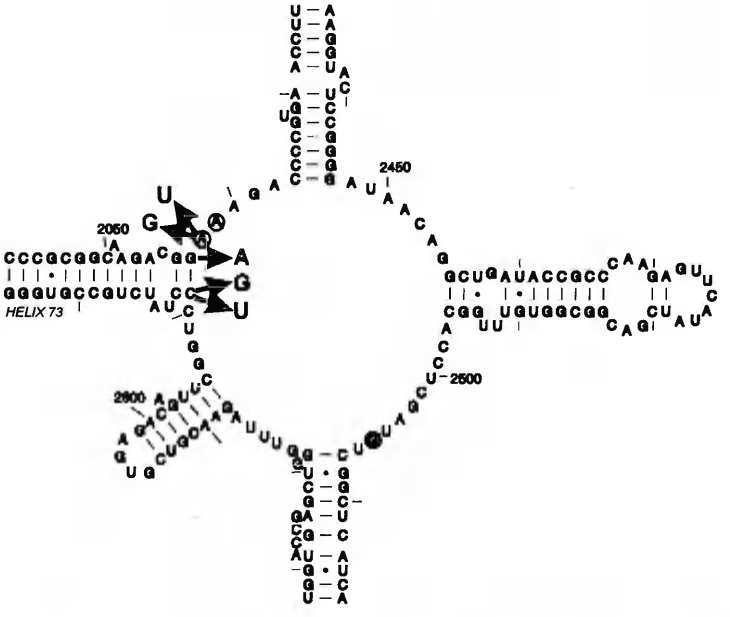

Figure 3. Secondary structure model of the central part of 23S rRNA domain V. Nucleotides protected from chemical attack by erythromycin are circled; mutations leading to erythromycin resistance are indicated by arrows.

Though rRNA is important for erythromycin binding, ribosomal proteins also contribute to the formation of erythromycin binding site. Mutations in ribo-

somal proteins L4 and L22 are known to cause erythromycin resistance in laboratory isolates (Wittmann *et al.*, 1973; Pardo and Rosset, 1977; Arévalo *et al.*, 1988). Erythromycin can be chemically crosslinked to L22 (Arévalo *et al.*, 1988). L4 and L22 are associated with domain I of the 23S RNA (Liiv *et al.*, 1996). These data are not easily compatible with the known erythromycin binding site in domain V. At least L4 can influence erythromycin binding indirectly, through RNA conformation/50S subunit assembly, similarly to the streptomycin resistance conferred by mutations in S12 (Noller *et al.*, 1990). Erythromycin resistance mutations in ribosomal proteins have never been observed in clinical isolates, probably because of their temperature-sensitive phenotype and severe defects in ribosome assembly (Pardo *et al.*, 1979). Ribosomal protein L15 has been shown to bind erythromycin in solution (Teraoka and Nierhaus, 1978), but the binding affinity is three orders of magnitude lower than the binding between ribosomes and erythromycin (affinity constant 10^{-8} M) (Pestka *et al.*, 1976).

2.2. Peptides and ribosomes (Reference I)

It is frequently assumed that the ribosome is indifferent to the sequence of the newly synthesized polypeptides. This concept, which stems from the fact that various cellular proteins are efficiently assembled by the ribosome, is however, oversimplified and there are a number of examples when amino acid sequence of the nascent peptide affects ribosome functions (Lovett and Rogers, 1996).

Translational attenuation is used in bacteria to regulate expression of chloramphenicol-resistance (*cat* and *cmlA*) and erythromycin-resistance (*erm*) genes in response to low concentrations of the corresponding drugs (Lovett, 1990; Weisblum, 1995b). The ribosome binding sites of these antibiotic resistance genes are sequestered in the RNA secondary structure; ribosome stalling at a specific codon of a short ORF preceding the resistance cistron changes the secondary structure of mRNA and permits translation of the downstream main ORF. Such stalling occurs at codon 6 of the cat leader ORF and at codon 9 of the leader ORFs of cmlA and erm genes. Curiously, ribosome stalling depends on the amino acid sequence of the translated short peptide; missense mutations in the leader ORF changing the nature of the encoded amino acids reduced efficiency of induction of the main cistron translation (Mayford and Weisblum, 1989, 1990; Gu *et al.*, 1994a). In the stalled ribosome, the nascent peptide is present in the form of peptidyl-tRNA and, thus, is positioned close to the peptidyl transferase center. Synthetic peptides corresponding to the first 5 codons of cat or first 8 codons of cmlA leader ORFs were able to inhibit *in vitro* peptidyl transferase reaction (Gu *et al.*, 1993, 1994b). Therefore, Lovett and co-workers have suggested that the nascent peptide causes site-specific pausing of translation by inhibiting activity of the peptidyl transferase (Gu *et al.*, 1993); it has

been proposed further that the pausing ribosome is converted into the stalled state by the inducer, chloramphenicol. However, since little similarity can be found between *cat*, *cmlA*, and *erm* leader peptides, it remains unclear what properties of the peptide are important for its regulatory effect. Similar to the cases of translational attenuation, sequence of the peptide translated from the leader ORF has been shown to be important for transcriptional attenuation of the *E. coli* tryptophanase operon (Gish and Yanofsky, 1995).

Regulation of translation, mediated by ribosome stalling on short upstream open reading frames (uORFs), was described also for several eukaryotic genes (Geballe and Morris, 1994). In some of these cases stalling depends on the sequence of the translated peptide rather than on the sequence of mRNA. For example, expression of the glutaminase subunit of carbamoyl-phosphate synthase A (CPA1) in yeast is controlled posttranscriptionally by the concentration of arginine (Messenguy *et al.*, 1983). The arginine-dependent repression of CPA1 translation requires the presence of a 25 codon uORF in the 250 nucleotide long leader region of the CPA1 mRNA (Werner *et al.*, 1987). It seems plausible that the nascent uORF-encoded peptide remains associated with the translating ribosome, causing it to stall and preventing it from proceeding to the initiation codon of the CPA1 ORF. Interestingly, fusion of the first 23 codons of the uORF in frame with an ORF of a reporter protein made expression of this protein repressible by arginine, indicating that precise termination of CPA1 uORF translation is not required for the regulatory function of the encoded peptide (Delbecq *et al.*, 1994). Functional analogue of CPA1 in *Neurospora crassa*, arg-2, is regulated in a similar way to CPA1.

There are also several other examples were translation of the uORF is inhibiting translation of the main ORF. For mammalian S-adenosyl methionine decarboxylase (Hill and Morris, 1993), $ß_2$ adrenergic receptor (Parola and Kobilka, 1994), Lc transcriptional activator of maize (Damiani and Wessler, 1993) and cytomegalovirus glycoprotein gene gp48 (Degnin *et al.*, 1993) it has been shown that the peptide coded by the uORF is important for inhibition of translation. In these cases it has been shown that proper placing of the termination codon is important; when the uORF is fused to a longer ORF then the inhibitory effect is no longer present. For gp48 it has been shown that ribosome stalls at the stop codon of the uORF and therefore creates a block for scanning ribosomes (Cao and Geballe, 1996a). It seems that the nascent peptide inhibits termination because the peptide stays in the ribosome in the form of peptidyl-tRNA (Cao and Geballe 1996b).

An exciting example of the influence of nascent peptide on ribosome function has been described by Weiss *et al.* (1990). Bacteriophage T4 gene *60* mRNA contains an internal 50 nucleotide long untranslated coding cap, separating the first 46 codons of the gene from the last 114 codons. This cap is not excised from the RNA as an intron but rather is bypassed by elongating ribosomes. Nascent peptide amino acids 17 to 32 are implicated in activating

such ribosome hopping, though the exact requirements for peptide sequence remains obscure. However, in another described example of translational bypass, the nascent peptide sequence is apparently not important (Benhar and Engelberg-Kulka, 1993).

In all the cases described above the regulatory (in most cases inhibitory) properties of the nascent peptide are probably caused from specific interaction of the peptide with the ribosome. What is known about interaction of different peptides with the ribosome? First, in the classic "fragment reaction" experiments, the efficiency of peptidyl transfer correlates with the chemical structure of the donor amino acid residue, which structurally and functionally corresponds to the C-terminal amino acid of the nascent peptide (Krayevsky and Kukhanova, 1979). Second, the termination of translation may be influenced by the protein's last two amino acids. Lysine is found more frequently at the C-termini of proteins in which ORF is terminated at UAA, while a similar position is occupied more often by phenylalanine, or serine in case of the UGA stop codon (Arkov *et al.*, 1993). Issakson and co-workers demonstrated that the frequency of read-through of UGA and UAG termination codons depends on the penultimate amino acid (Mottagui-Tabar *et al.*, 1994). Termination is inhibited by several (or most?) regulatory peptides. In the case of the *cat* leader peptide it has been shown that synthetic peptide inhibits *in vitro* termination reaction (Moffat *et al.*, 1994). For gp48, the fact that ribosome stops on termination codon in complex with peptidyl-tRNA indicates that uORF coded peptide inhibits termination (Cao and Geballe, 1996b).

In many (and maybe all) of the cases discussed above, newly synthesized peptide may exert its effect on translation while still located in the ribosome. However, interaction of the nascent peptides with external factors "outside" of the ribosome is also known to play a role in regulation of translation. The best characterized case is a transient arrest of translation during synthesis of secreted proteins when the signal recognition particle binds to the signal sequence of a secreted protein emerging from the ribosome and halts translation until the complex binds to a docking protein in the membrane (Walter and Johnson, 1994). Another example of cotranslational interaction of the nascent peptide with external factors outside of the ribosome comes from the studies of regulation of ß-tubulin synthesis. Increased concentration of free ß-tubulin subunits in the cell triggers the degradation of ß-tubulin mRNA in polysomes (Cleveland, 1989). The four N-terminal amino acids of ß-tubulin nascent peptide are essential for such autoregulation (Yen *et al.*, 1988; Bachurski *et al.*, 1994). When this segment of the nascent peptide is blocked by antibodies, the tubulin-dependent autoregulation is prevented, suggesting that binding of additional factors (possibly, free ß-tubulin subunits) to the nascent peptide N-terminus is necessary for mRNA degradation (Theodorakis and Cleveland, 1992).

For all of the examples described above it is common that the peptides can act only *in cis*, on the same ribosome where they have been synthesized. It is

likely that many of these peptides affect the ribosome while they are still bound in the nascent peptide channel. This is evident for the nascent regulatory peptides of the bacterial antibiotic resistance operons. Translation of these peptides is not yet complete when they cause ribosome pausing; therefore, the peptide must still be in the form of peptidyl-tRNA.

The sequences of the regulatory peptides discussed above are very different from each other, making it impossible to find structurally similar groups (Fig. 4). Moreover, there is no case where the sequence requirements are systematically studied.

We have described a new example of nascent peptide regulation of translation and also characterized the peptide sequence requirements.

ermC	**MGIFSIFVISTVHYQPNKK**
cat-86	**MVKTDKISS**
cmlA	**MSTSKNADK**
AdoMetDC	**MAGDIS**
gp48	**MQPLVLSAKKLSSLLTCKYIPP**
CPA1	**MFSLSNSQYTCQDYISDHIWKTSSH**
ß2 adrenergic receptor	**MKLPGVRPRPAAPRRRCTR**
gene *60*	**MKFVKIDSSSVDMKKYKLQNNVRRSIKSSSMNYANVAIMTDADHDG**
E-peptide in the 23S RNA	**MRMLT**
E-peptide from peptide library	**MSLKV**

Figure 4. Examples of short peptides involved in *cis*-regulation of translation. Sequences correspond to the peptides involved in regulation of translation of cistrons coding for bacterial chloramphenicol (*cat* and *cmlA*) and erythromycin (*ermC*) resistance proteins, mammalian S-adenosyl methionine decarboxylase (*AdoMetDC*), glutaminase subunit of yeast carbamoyl phosphate synthase A (*CPA1*), cytomegalovirus glycoprotein gene (*gp48*), and murine ß$_2$ adrenergic receptor. Also shown are 46 N-terminal amino acids of bacteriophage T4 gene *60* protein, E-peptide encoded in the *Escherichia coli* 23S RNA and E-peptide from random peptide library giving highest erythromycin resistance. Amino acids in which substitution affects functions of corresponding peptides are underlined.

3. RESULTS AND DISCUSSION

Ribosome is a big nucleoprotein complex and therefore very hard to study. Finding of functional subribosomal complexes is essential for understanding the ribosome at molecular level. In the current work a small functional part of ribosomal RNA was isolated and characterized.

3.1. Experimental approach: libraries

Libraries of biological macromolecules have been efficiently used to solve many biological problems. Genomic and cDNA libraries are routinely used for gene cloning and sequencing. Moreover, libraries can be used for functional mapping of a single gene. Using this strategy, random pieces of the gene are generated and expressed *in vivo*. If some pieces of the gene are coding for functional domains of the protein or RNA then they should interfere with the normal function of the gene. This approach has been used to search for regions in the genome of phage λ responsible for resistance to hyperinfection (Holzmayer *et al.*, 1992), to find genes responsible for anticancer drug resistance in mammalian cells (Gudkov *et al.*, 1994), for functional mapping of the gene for oncoprotein p53 (Ossovskaya *et al.*, 1996) and to find new tumor suppressor genes (Garkavtsev *et al.*, 1996).

We used a similar approach to study ribosomal RNA. *Escherichia coli* rRNA operon was cut randomly into small fragments with DNaseI (Fig. 5). The size of the fragments was from 100 to 1000 basepairs, coding potentially for RNA fragments ranging in size from small hairpins to secondary structure domains. DNA fragments were cloned into two RNA expression vectors. In pPOT1, transcription starts from *tac* promoter and terminates on *trp* terminator. In pMAG transcription starts from rRNA promoter P1 and terminates on rRNA terminators T1 and T2. Both plasmids have *lac* operator after the promoter that makes RNA expression inducible with IPTG. *lac* operator and the terminators code for RNA hairpins. RNA hairpins have been shown in many systems to stabilize RNA in the cell (McLaren *et al.*, 1991; Emory *et al.*, 1992). The expression levels of RNA fragments from library plasmids were estimated on Northern blots using reporter constructs. It was found that both vectors express RNA in the cell at levels one or two orders of magnitude smaller than the amount of ribosomal RNA. The copy number of the vectors is around 60 compared to the 7 ribosomal RNA operons in the chromosome and the promoters should be as strong as chromosomal rRNA promoters. Therefore the small amount of the transcript is probably caused by instability of the small RNA fragments in the cell. For our studies it was desirable to have rRNA fragments in the cell at the level comparable to the amount of ribosomal RNA. To increase the RNA level we tried to use T7 promoter directed expression system.

Unfortunately, there are two drawbacks of the T7 system. First, 23S RNA transcribed by T7 polymerase has been shown to form inactive 50S subunits; the reason for the inactivity of the T7 transcript is not known (Lewicki *et al.*, 1993). Second, transcription with T7 polymerase significantly changes cell physiology which makes many *in vivo* screening schemes impossible or very difficult. Therefore we decided to continue our work using rRNA fragment library in expression vector pPOT1 which expresses 1.5–2 times more RNA in the cell than the vector pMAG.

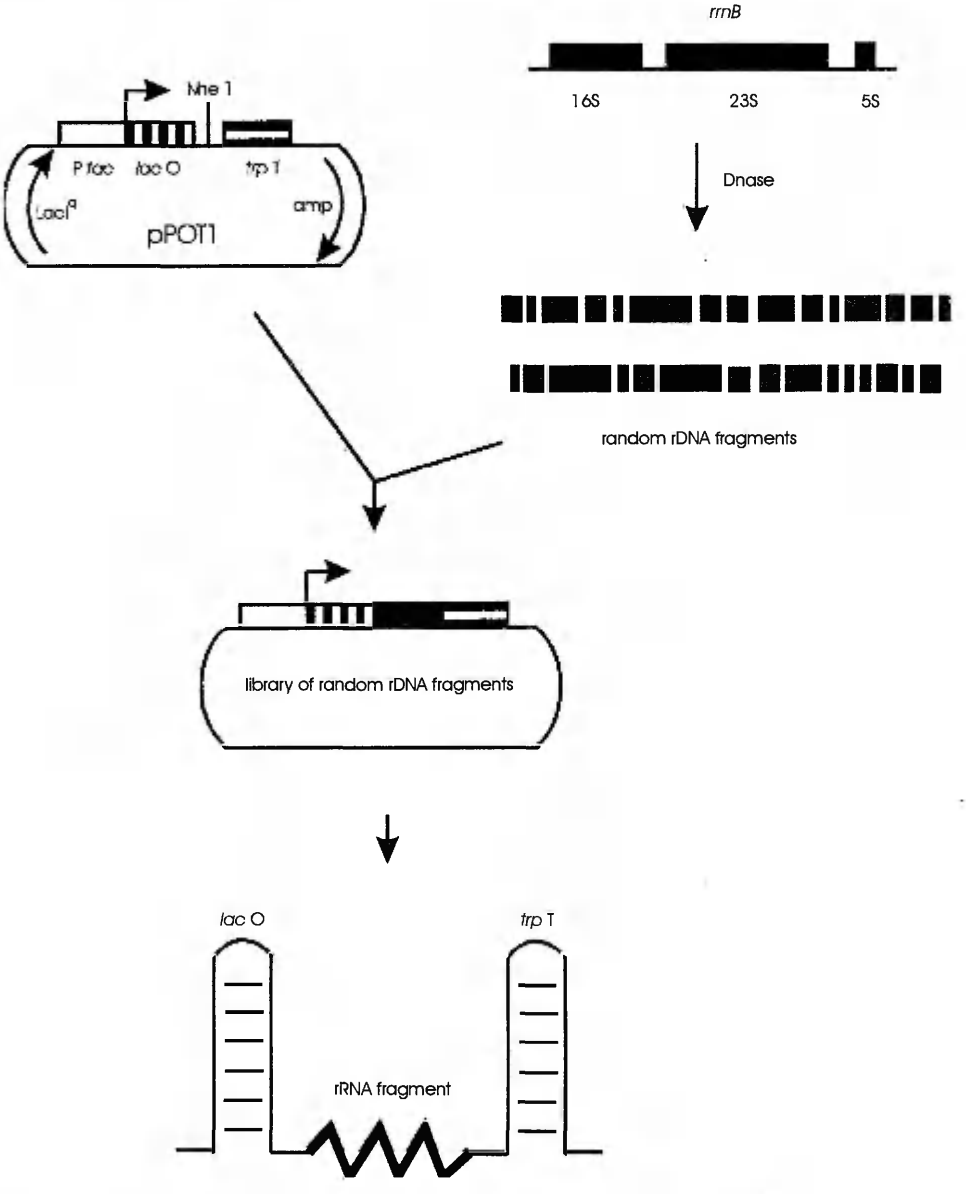

Figure 5. Construction of the random rRNA fragment library.

3.2. E-peptide (Reference II)

The first selection scheme used to look for functional rRNA fragments was selection for rRNA fragments that render cells resistant to ribosome targeted antibiotics. When cells carrying library plasmids were plated on plates containing erythromycin and IPTG several colonies appeared. Erythromycin resistance was shown to be dependent on the presence of rDNA-containing plasmids rather than chromosomal mutations, since 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 overlapped in the region corresponding to positions 1233–1348 of the 23S RNA (Fig. 6). 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 nucleotide long segment of the 23S rRNA; subsequent deletion analysis showed that expression of an rRNA fragment only 34 nucleotides long (E-RNA34), corresponding to the 23S rRNA sequence between positions 1235 and 1268, could render cells resistant to erythromycin (Fig. 7).

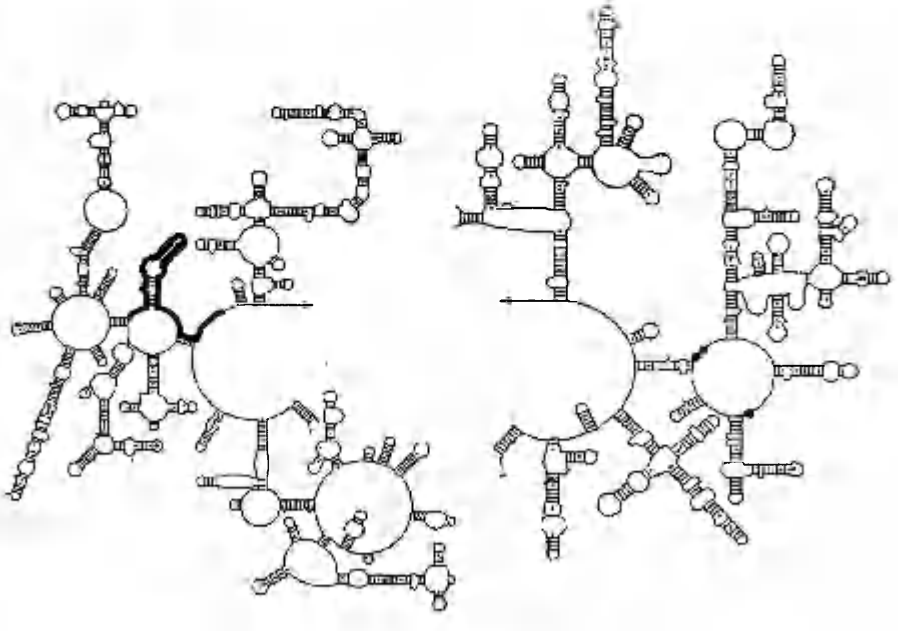

Figure 6. Positions of the eRNA (bold line) and erythromycin footprints (dots) in the secondary structure model of the 23S rRNA.

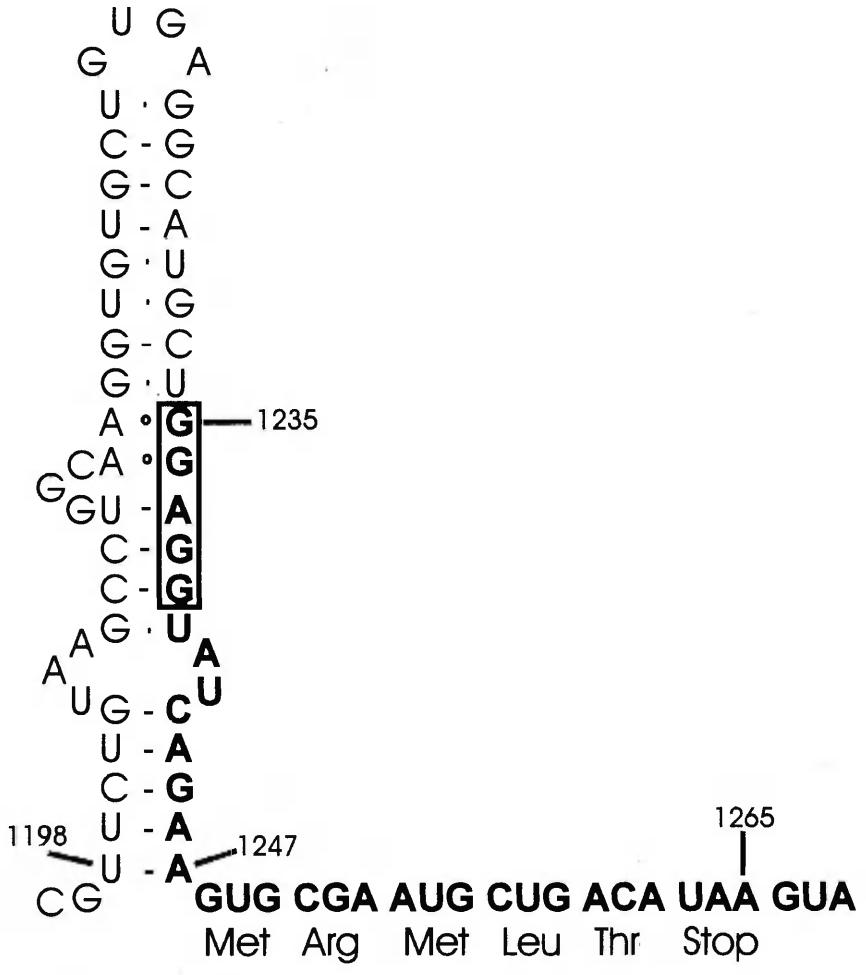

Figure 7. The nucleotide sequence and secondary structure of the *E. coli* wild-type 23S rRNA segment encoding the pentapeptide mini-gene. The minimal rRNA fragment capable of conferring erythromycin resistance (E-RNA34) is shown in boldface. Shine-Dalgarno region of the pentapeptide gene is boxed, and the sequence of the encoded peptide is indicated.

To get insights into the mechanism of E-RNA action, mutations were introduced randomly into the E-RNA. 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 correspond to initiator and terminator codons of a pentapeptide ORF present in E-RNA. Moreover, a canonical Shine-Dalgarno sequence, GGAGGU (positions 1235–1240), which is essential for initiation of translation in prokaryotes (Shine and Dalgarno, 1974), is present seven nucleotides upstream from the initiator codon, at the 5'-end of E-RNA, and is important for its function, because its

deletion renders E-RNA inactive. 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.

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. This result confirmed that the putative ribosome binding site of the pentapeptide mini-gene present in the *E.coli* 23S RNA 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. As a negative control, we used another inactive E-RNA mutant with a mutation in the initiator codon. A gene of the opal suppressor $tRNA^{Arg}$ (McClain *et al.*, 1990), expressed from its own promoter, was introduced into the same plasmid, and phenotypes of the transformed cells were assessed by plating on antibiotic-containing agar plates. 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 were studied in the cell-free translation system. The synthetic E-peptides, MRMLT and N-formyl-MRMLT, did not influence erythromycin sensitivity of *in vitro* protein synthesis up to 1 mM of the peptide concentration. Yet, when E-RNA was added to the cell-free system, instead of the E-peptide, a reproducible protection against erythromycin was observed. 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. These data also suggest that the peptide can act only on the same ribosome where it was synthesized (*cis*-mode of action). This can explain the inactivity of the synthetic peptide.

The E-peptide is reminiscent of the *cat*, *cmlA*, and *erm* leader peptides (Chapter 2.2.) in its size and *cis*-mode of action. Therefore, it is likely that all

of these peptides may interact with the same site in the vicinity of the peptidyl transferase center, probably overlapping with the erythromycin binding site.

3.3. Libraries again (Reference IV)

To elucidate sequence requirements for the E-peptide we decided to create a big collection of active peptides. To achieve this, we used random mini-gene libraries. The libraries, where each bacterial cell expresses a specific mini-gene coding for an oligopeptide, were subjected to phenotypic selection based on IPTG-inducible erythromycin resistance. Two libraries were used: one of them has 5 (4 of them randomized) and the other 21 (20 of them randomized) codons in the ORF. Comparing sequences of peptides encoded in the mini-genes in erythromycin resistant cells isolated from 21-codon and 5-codon random libraries allowed us to draw first conclusions about the sequence and size requirements for the peptide activity. Screening of the 21-codon library revealed the preferred size of erythromycin resistance peptides. There are 3 termination codons in the genetic code. Accordingly, the probability that out of 20 random codons none will be a terminator codon is $(61/64)20 = 0.38$, and thus, about 2/3 of the clones in the 21-codon library are expected to have in-frame stop codons. Therefore, this library provides a very good tool for investigating the size requirement for functional peptides. As expected, a broad distribution of sizes of the encoded peptides were found in unselected, randomly picked clones. In contrast, the majority of peptides expressed in Ery^r clones fell within an amazingly narrow size range; 11 out of 12 peptides were four, five or six amino acids long. Of course, there is a possibility that a more extensive screening could reveal some functional peptides larger than hexapeptides; nevertheless, this experiment showed a clear tendency of erythromycin resistance peptides to be four to six amino acids long. In agreement with this conclusion, the originally described rRNA-encoded E-peptide was five amino acids long.

Previously it had been demonstrated that any mutation eliminating the stop codon of the rRNA-encoded E-peptide abolished erythromycin resistance. This showed that a mere presence of the E-peptide sequence at the N-terminus of a longer polypeptide could not render ribosomes resistant to erythromycin. The results of screening a 21-codon library not only confirmed this conclusion, but also indicated that proximity of the "active" sequence to the peptide C-terminus was also not sufficient for erythromycin resistance (otherwise we could isolate clones coding for long peptides where critical sequence would be located close to the C-terminus). Thus, we can conclude an erythromycin resistance peptide cannot be part of a longer protein and the size of the peptide is essential for its activity. The strict size requirement for peptide activity may mean that the peptide binding site is not very big and cannot accommodate a longer polypeptide.

If analysis of clones isolated from the 21-codon library revealed peptide size preference, then screening the 5-codon library provided clues to the sequence features that are important for E-peptide activity. Comparison of sequences of 52 pentapeptides found in Ery^r clones showed a strong tendency for having Leu or Ile in the third position and a hydrophobic amino acid in the C-terminal position. Not only did these sequence signatures appear in the majority of isolated E-peptides, but there was also a definite correlation of peptide activity with the presence of Leu or Ile in the third position and a hydrophobic amino acid at the C-terminus.

Interestingly, if E-peptides act in the immediate vicinity of peptidyl transferase center so that the C-terminal peptide residue is positioned in the P-site, then the third residue from the C-terminus would be located very close to the erythromycin binding site since the drug starts to inhibit protein synthesis at a step when the third amino acid is added to the growing nascent peptide (Mao and Robishaw, 1972). Also noteworthy is the fact that 5 out of 12 peptides expressed in Ery^r clones isolated from the 21-codon library have either Leu or Ile in position –3 relative to the C-terminus. The bulky hydrophobic side-chain of leucine or isoleucine may interfere with interaction of erythromycin with its binding site in the vicinity of the peptidyl transferase center.

Expression of E-peptide rendered cells resistant to other macrolide antibiotics, oleandomycin, which similar to erythromycin, has a 14-atom lactone ring and to spiramycin, a macrolide with a 16-atom ring. At the same time, E-peptide did not affect cell sensitivity to structurally different chloramphenicol and clindamycin. All tested drugs compete with erythromycin for binding to the ribosome (Chang *et al.*, 1969), however, the binding sites of chloramphenicol and clindamycin do not precisely coincide with the binding site of macrolides as demonstrated by RNA footprinting and the difference in the mode of action of these drugs (Moazed and Noller, 1987; Vazquez, 1979). Thus, the site of E-peptide action probably overlaps specifically with binding site of macrolides, but not with that of other antibiotics interacting with the ribosome in the vicinity of the peptidyl transferase center.

A model of how E-peptide may interact with the ribosome and cause erythromycin resistance is shown in Fig. 8. The binding site of E-peptide is located most probably in the large ribosomal subunit, in or immediately near the nascent peptide channel, and overlaps with the erythromycin binding site (which is shown in gray in Fig. 8). The peptide has to be placed in this site co-translationally from the peptidyl transferase side which would explain a *cis*-mode of E-peptide action. Three positions in E-peptide appear to be important for peptide activity. Besides Leu or Ile in the third position and a hydrophobic residue (more frequently Val) at the C-terminus, the N-terminal formylmethionine may be also essential.

Though importance of fMet is difficult to assess since by default it is present in all library-coded E-peptides, the fact that E-peptide cannot be part of a longer

protein indicates that the position or formylation of the N-terminal methionine is critical for peptide activity. Amino acids located in the critical positions of the peptide may form specific contacts with the ribosome (shown by thin lines in the figure). One or several E-peptide amino acids may overlap with the erythromycin binding site thus conferring resistance to the drug. Lack of resistance to peptidyl transferase inhibitors chloramphenicol and clindamycin suggests that E-peptide does not overlap with the binding site of these drugs on the ribosome. The simplest explanation of how E-peptide can protect the ribosome from antibiotic action is that the E-peptide remains associated with the ribosome after completion of its translation and prevents binding of the drug to the ribosome. The presence of E-peptide on the vacant ribosome should make it immune to erythromycin. When translation of a new protein is initiated, the nascent peptide may possibly go "around" the bound E-peptide, or alternatively, displace it.

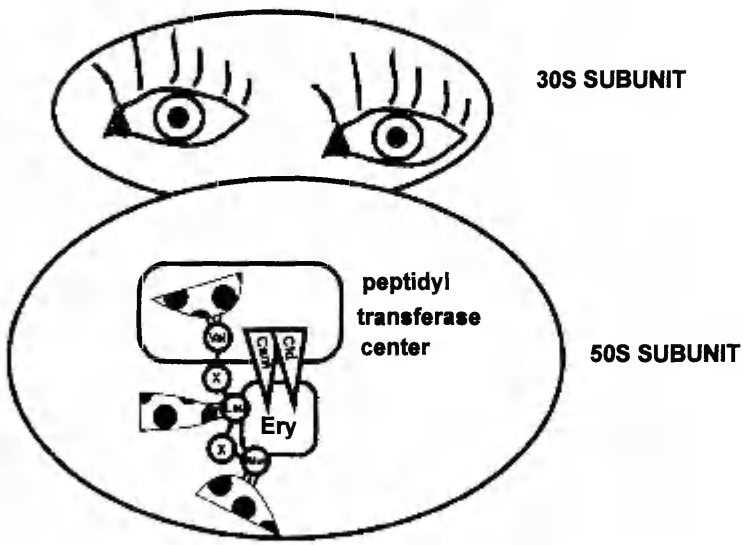

Figure 8. A model of E-peptide action. Erythromycin binding site (Ery) is shown gray and binding sites of chloramphenicol (Cam) and clindamycin (Cld) are shown as open triangles. The third position of the peptide commonly represented by Leu (as shown in the figure) or Ile is assumed to overlap with the erythromycin binding site. The conserved peptide positions, N-terminal formyl methionine, the third Leu (or Ile) and C-terminal hydrophobic amino acid (commonly represented by Val, as shown in the figure) may form specific contacts with rRNA or ribosomal proteins.

Our random peptide library approach has a number of advantages compared to the other combinatorial methods exploiting libraries of synthetic peptides (Houghten, 1993) or phage display libraries (Scott and Smith, 1990). Firstly, it is much easier to synthesize a random DNA sequence of the peptide gene than a random amino acid sequence of the peptide itself, leading to better representa-

tion of a random peptide sequence space in a mini-gene library compared to synthetic peptide libraries. Secondly, phenotypic selection permits not only screening of hundreds of thousands of peptide sequences in a single experiment, but also to amplify the "signal" (the selected sequence) by allowing cells that passed the selection to form colonies; signal amplification is usually impossible with the use of synthetic peptide libraries. Thirdly, in contrast to phage display libraries where a random amino acid sequence is expressed as a segment of a large protein, the mini-gene library peptides are expressed in their free form which can be critical for assessing functionality of the random sequence. For example, inasmuch as E-peptides cannot act as a part of a longer protein, isolation of E-peptides from a phage display library would be impossible. In our experiments, we used random mini-gene libraries for isolation of a particular class of functional peptides. Similar approach can be used for isolation of other functional peptides which may serve as enzyme co-factors, inhibitors, etc.

3.4. Translation of ribosomal RNA (Reference III)

Originally, ribosomal RNA was perceived as a messenger RNA that encodes proteins (Crick, 1958). This view later underwent a transition: for a time, rRNA was considered to be a scaffold for the ribosomal proteins; nowadays, rRNA is more commonly viewed as a functional, maybe even catalytic, constituent of the ribosome (Noller *et al.*, 1990). Still, the idea that the most abundant RNA may also carry protein-coding information was not abandoned completely. Attempts to find protein genes in the rRNA have been undertaken; a few relatively long suspicious open reading frames have been identified (Brosius *et al.*, 1978; Peng *et al.*, 1992) and that translation of one of such ORFs is possible has been demonstrated (Berg *et al.*, 1987). Nevertheless, the translation products of these ORFs were never detected and functions of the putative proteins were never recognized.

Finding of the E-peptide raises the question about translation of ribosomal RNA again. 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. As is evident, 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 (Fig. 7), which may happen, for example, under conditions of physiological stress, can generate translatable rRNA fragments leading to production of the E-peptide. Specific fragmentation of ribosomal RNA has been shown to occur in apoptotic mammalian cells (Houge *et al.*, 1995).

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 (Douthwaite *et al.*, 1985, 1989). 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 deletions in domain II. The 12-nucleotide 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.

3.5. Concluding remarks

We have found that a small fragment of 23S RNA can be translated to a functional pentapeptide. This ORF is present in large subunit rRNA of most eubacteria and many eukaryotes. The fact that E-peptide ORF is not universally conserved can explained in two ways: first, the E-peptide can be a regulatory molecule used for fine turning of translation, it is not absolutely essential and is lost in many organisms; second, the presence of the E-peptide ORF in the rRNA can be a coincidence. If the presence of the E-peptide ORF has some function, then, obviously, erythromycin resistance is not its primary function. What can be its function remains unclear. Unclear is also in what physiological conditions could E-peptide be expressed.

From the other hand, we have described a new mechanism of antibiotic resistance which can account for Ery^r phenotypes of some clinical bacterial isolates. Mutations not only in rRNA but also in mRNA can create short ORFs which can code for functional peptides. E-peptide is also a new addition to the growing number of *cis*-acting regulatory peptides.

The amount of E-RNA accumulated in Ery^r clones was relatively low, about 1 mol % of the amount of 5S RNA, 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 low amount of expressed RNA is hindering the use of our rRNA fragment libraries. Therefore it is surprising that Prescott's group, using similar approach found a fragment of 16S RNA which gives resistance to the 30S subunit targeted antibiotic spectinomycin (Howard *et al.*, 1995). The fragment corresponds to the known binding site of spectinomycin to the 16S RNA and therefore it was proposed that the RNA fragment works by sequestering the drug. Unfortunately, the amount of the spectinomycin resistance RNA in the cell was not estimated and the resistance mechanism has not been published.

The peptide library can be used to find other functionally active peptides. Indeed, using negative selection with cycloserine we found several peptide mini-genes with cytostatic activity. According to the puls-labelling experiments, at least some of the cytostatic peptides are inhibiting protein synthesis. Studying of such inhibitory peptides may yield further insight in interactions between the ribosomes and nascent peptides.

4. CONCLUSIONS

1. A new mechanism of antibiotic resistance, mediated by interaction of small peptides with the ribosome has been described.
2. Possibility of translation of a small open reading in the 23S RNA was discovered.
3. The mechanism of erythromycin resistance caused by mutations in domain II of 23S rRNA was explained; it is mediated by activation of translation of the functional peptide encoded in 23S rRNA.
4. A method to study functions of small peptides in the cell and find new pharmaceuticals based on *in vivo* expression of the random peptide libraries has been developed.
5. Sequence requirements for the erythromycin resistance peptides were characterized and the mechanism of interaction of short peptides with the ribosome has been proposed.

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RIBOSOOMID, PEPTIIDID JA ANTIBIOOTIKUMI RESISTENTSUS

Kokkuvõte

Ribosoomid seonduvad translatsioonitsükli vältel hulga molekulidega: tRNAde ja translatsioonifaktoritega. Peale selle seonduvad ribosoomid mitme antibiootikumiga. Ühelgi juhul ei ole teada täpset ribosoomi piirkonda, mis seondumise eest vastutab.

Käesoleva töö eesmärk oligi funktsionaalsete subribosomaalsete komplekside isoleerimine. Eeldati, et ekspresseerides rakus funktsionaalseid ribosoomi-RNA (rRNA) fragmente, võiksid need mõjutada translatsiooniprotsessi. Valmistati juhuslike rRNA-fragmentide ekspressiooni kogu ja selekteeriti erütromütsiiniresistentseid kloone. Selgus, et erütromütsiiniresistentsust põhjustab 23S RNA teise domeeni fragmentide ekspressioon rakus.

Deletsioonanalüüs võimaldas isoleerida minimaalse funktsionaalse fragmendi. See on 34 nukleotiidi pikk, vastates 23S RNA positsioonidele 1235 kuni 1268. Mutatsioonanalüüs näitas, et erütromütsiiniresistentsuseks võib olla oluline selles fragmendis asuv avatud lugemisraam, mille ees on translatsiooni initsiatsiooniks vajalik Shine'i-Dalgarno järjestus. Kui fragmendi 5'osa, kuni lugemisraami teise koodonini, klooniti kloramfenikool-atsetüülitransferaasi geeni translatsiooni initsiatsiooni ala asemele, siis täheldati kloramfenikool-atsetüülitransferaasi sünteesi. Seega võimaldab 23S RNA fragmendi initsiaatorkoodon ja sellele eelnev ala translatsiooni initsiatsiooni. Seega võis oletada, et erütromütsiiniresistentsuse põhjustab sünteesitav pentapeptiid MRMLT. Selle hüpoteesi kontrollimiseks kasutasime mutanti, mille teine koodon oli muteeritud arginiini kodeerivast CGA-koodonist UGA-terminaatorkoodoniks. Selline mutant põhjustas erütromütsiiniresistentsust ainult supressor-tRNA juuresolekul, mis kodeerib UGA-koodonit arginiiniga. Sellest võib järeldada, et erütromütsiiniresistentsuseks on tõepoolest vajalik pentapeptiidi translatsioon. *In vitro* translatsiooni süsteemis keemiliselt sünteesitud pentapeptiidi lisamine resistentsust ei põhjustanud. Seevastu tekkis resistentsus, kui ribosoomidele lisati rRNA fragment ja lasti transleerida peptiid. Sellest võib oletada, et peptiid mõjub *in cis*: ainult sellele ribosoomile, kus ta sünteesiti.

Selgitamaks, millised peptiidi omadused on vajalikud erütromütsiiniresistentsuse põhjustamiseks, konstrueeriti kaks raamatukogu. Esimene ekspresseerib rakus RNAsid, milles on olemas translatsiooni initsiatsiooniks vajalikud järjestused ja initsiaatorkoodonile järgneb 20 juhuslikku koodonit. Kuna 3 koodonit 64st on terminaatorkoodonid, siis on 2/3-l avatud lugemisraamidest sisemine terminaatorkoodon. Sellisest raamatukogust erütromütsiiniresistentseid kloone selekteerides selgus, et peptiid peab olema 3 kuni 6 aminohapet pikk. Et esimesest raamatukogust selekteeritud kloonide hulk oli väike, konst-

rueeriti peptiidi järjestusele esitatavate nõuete selgitamiseks ka teine raamatukogu. See on analoogne esimesega, välja arvatud see, et initsiaatorkoodonile järgneb ainult neli juhuslikku koodonit. Sellest raamatukogust selekteeritud järjestuste analüüsimisel selgus, et enamus peptiide sisaldab leutsiini või isoleutsiini jääki kolmandas positsioonis ja hüdrofoobset aminohapet C-terminuses.

Erütromütsiin inhibeerib translatsiooni, blokeerides kasvava polüpeptiidi liikumist. Erütromütsiin ei ihhibeeri esimese peptiidsideme sünteesi ega polüsoome, kus kasvav peptiid on pikem kui viis aminohapet. Seega võib oletada, et erütromütsiini seondumispiirkond kattub selle alaga, kuhu seonduvad ka kasvava peptiidi C-terminusest kolmas, neljas ja viies aminohape. Esimese peptiidsideme sünteesi ajal ei ole kasvav peptiid veel erütromütsiini seondumiskohani jõudnud ja seega inhibeerimist ei toimu. Kui peptiid on üle viie aminohappe pikk, siis katab ta erütromütsiini seondumiskoha ja järelikult ei saa erütromütsiin enam seonduda. Seega võib oletada, et resistentsust põhjustavad peptiidid jäävad ribosoomile pidama ega lase erütromütsiinil seonduda. Järgmine sünteesitav valk kas lükkab peptiidi välja või saab temast kuidagi ümbert mööda.

Eelnevalt oli teada, et erütromütsiiniresistentsust põhjustavad mutatsioonid 23S RNA viiendas domeenis, seal paikneb ka nukleotiid A2058, mille metüülimine põhjustab resistentsust. Peale selle kaitseb erütromütsiin kemikaalide eest kolme viienda domeeni nukleotiidi (A2058, A2059 ja G2505). Seega tundub igati loogiline, et erütromütsiini seondumispiirkond asub viiendas domäänis. Mõni aasta tagasi aga isoleeriti deletsioonid teises domeenis, mis põhjustavad samuti erütromütsiiniresistentsust. Nende funktsioneerimismehhanism sai selgeks siis, kui analüüsiti teise domeeni mutantide mõju pentapeptiidi sünteesile. Erütromütsiiniresistentsust määrava peptiidi lugemisraami ees olev Shine'i-Dalgarno järjestus on tugevas sekundaarstruktuuris ja seega on metsikut tüüpi 23S RNA translatsioon võimatu. 23S RNA fragmentatsioon või mutatsioonid, mis nõrgendavad sekundaarstruktuuri, võimaldavad aga peptiidi translatsiooni ja põhjustavad seega erütromütsiiniresistentsust.

Jääb ebaselgeks, kas 23S RNAd transleeritakse kunagi ka looduslikes tingimustes või oli leitud peptiid lihtsalt juhus. Sellele vaatamata on käesolevas töös kirjeldatud uudset antibiootikumiresistentsuse mehhanismi ning juhitud tähelepanu võimalusele, et ribosoomi tööd võib reguleerida kasvav peptiid.

ACKNOWLEDGEMENTS

I wish to express my gratitude to my supervisor Shura Mankin for his help and support. I want to thank Alexandra DeBlasio, Patti Kloss and Liquin Xiong for their excellent technical assistance and for creating friendly atmosphere in the lab. Discussions with Alexander Neyfakh and Penny Markham helped me a lot.

Jaanus Remme helped to start our collaborative project and his comments and support were very valuable. I would like to thank him also for discussions during preparation of the thesis.

I would like to thank all persons who had access to this computer during preparation of the current thesis for not deleting the files. My special thanks to Ülo Maiväli for not blabbing permanently.

Our work was supported by the Hans Vahlteich Research Award and grant from the National Science foundation. Open Estonia Foundation is acknowledged for providing a travel grant.

PUBLICATIONS

I

Tenson, T., and Mankin, A. (1995) Comparison of functional peptide encoded in the *Escherichia coli* 23S rRNA with other peptides involved in cis-regulation of translation. Biochem. Cell Biol., 73, 1061–1070.

Comparison of functional peptide encoded in the *Escherichia coli* 23S rRNA with other peptides involved in *cis*-regulation of translation

Tanel Tenson and Alexander Mankin

Abstract: A new approach for studying functional rRNA fragments has been developed based on using a plasmid library expressing random fragments of rRNA. A 34 nucleotide long fragment of *Escherichia coli* 23S rRNA has been identified that renders cells resistant to erythromycin, when expressed in vivo. The rRNA fragment contains a five codon long open reading frame, initiating at GUG and terminating at UAA, with a Shine–Dalgarno sequence located at an appropriate distance from the initiator codon. Translation of this mini-gene is required for the observed erythromycin resistance. Experiments with in vitro translated, or synthetic, peptide indicate the ribosome as a likely target for the action of the identified rRNA-encoded peptide, which apparently remains associated with the ribosome after completion of its translation. The known properties of the rRNA-encoded peptide are compared with information about other functionally active short peptides that can be involved in regulation of translation.

Key words: ribosome, rRNA, peptide, translation, erythromycin.

Résumé : Les fragments fonctionnels de l'ARNr ont été étudiés par une nouvelle approche qui utilise une banque de plasmides exprimant des fragments aléatoires d'ARNr. Nous avons identifié un fragment de 34 nucléotides de l'ARNr 23S de *Escherichia coli* qui, lorsqu'exprimé in vivo, rend les bactéries résistantes à l'érythromycine. Le fragment d'ARNr contient un cadre de lecture constitué de cinq codons, débutant à GUG et se terminant à UAA, et une séquence Shine–Dalgarno localisée à une distance adéquate du codon d'initiation. La traduction de ce minigène est requise pour que la résistance à l'érythromycine soit acquise. Des expériences effectuées avec un peptide traduit in vitro ou synthétique indiquent que le ribosome est probablement le site d'action du peptide codé par l'ARNr, celui-ci demeurant apparemment associé au ribosome à la fin de la traduction. Les propriétés du peptide codé par l'ARNr sont comparées à celles d'autres petits peptides actifs qui interviendraient dans la régulation de la traduction.

Mots clés : ribosome, ARNr, peptide, traduction, érythromycine.

[Traduit par la rédaction]

Introduction

Originally, ribosomal RNA was perceived as a messenger RNA that encodes proteins (Crick 1958). This view later underwent a transition: for a time, rRNA was considered to be a scaffold of the ribosome; nowadays, rRNA is more commonly viewed as a functional, maybe even catalytic, constituent of the ribosome (Noller et al. 1990). Still, the fascinating idea that the most abundant cellular RNA may also carry protein-coding information was not abandoned completely. Attempts to find protein genes in the rRNA have been repeatedly undertaken; a few relatively long, suspicious open reading frames have been identified (Brosius et al. 1978; Peng et al. 1992) and the possibility of translation of one of such ORFs has even been demonstrated (Berg et al. 1987). Nevertheless, the translation products of these ORFs were never detected and functions of the putative proteins were never recognized.

Until now, however, the possibility that some smaller open reading frames in rRNA may have functional significance has been essentially ignored. Such short ORFs (4–8 codons), some equipped with reasonable translation initiation signals, are present in almost any long rRNA. It is known that translation of short ORFs plays an important role in several regulatory mechanisms in the cell, the best example of which is attenuation of transcription and translation (Landick and Yanofsky 1987; Lovett 1990). Biological activity of short peptides has been well documented (Alberts et al. 1994), and some antibi-

Received June 20, 1995. Accepted August 17, 1995.

Abbreviations: ORF, open reading frame; uORFs, upstream open reading frames in eukaryotic mRNAs; SRP, signal recognition particle; bp, basepairs; IPTG, isopropyl-β-galactoside.

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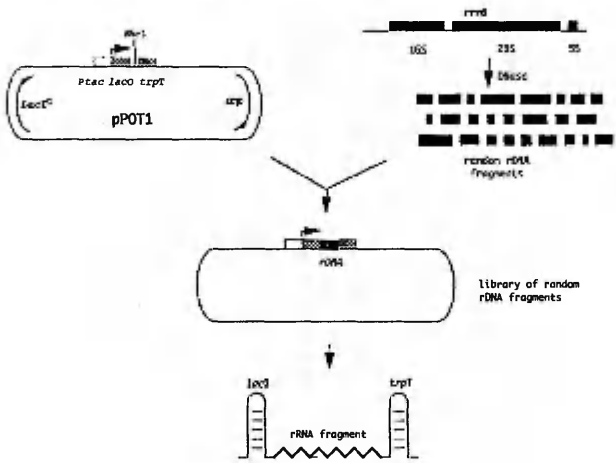
Fig. 1. Construction of random rRNA fragment library. The library was constructed in a specially designed pPOT plasmid vector that contains IPTG-inducible *Ptac* promoter (with *lac*O operator), attached via *Nhe*I linker to *trp*T terminator; *lac*I^q gene ensures efficient repression of the P*tac* promoter under noninduced conditions. For the library construction, *E. coli rrn*B operon rDNA was partially hydrolyzed with DNase I; resulting fragments were ligated with *Spe*I linkers and introduced into the *Nhe*I site of the pPOT vector. Plasmid library was transformed into *E. coli* JM 109 cells and screened for the presence of clones expressing functional rRNA fragments. The bottom part of the figure represents the generalized structure of the rRNA transcripts expressed from the library plasmids. Terminal hairpins correspond to the quasi palindromic sequences of the *lac* operator and *trp* terminator and may increase stability of the transcript in the cell.



otics of peptide nature are capable of specific interaction with the ribosome (Vazquez 1979). Therefore, it would not be senseless to suggest that one or several short ORFs, or encoded peptides, present in rRNA may be functionally meaningful. Unfortunately, it was not clear how to approach the possible function of the encoded peptides.

The primary goal of our studies was not the isolation of protein-coding regions of rRNA. We developed a novel approach that, in our belief, should allow us to isolate various functional segments of rRNA. The idea behind the method is very simple: random fragments of rRNA are expressed in the cell and clones expressing functionally active fragments are selected. Here we use a rather broad operational definition of the word "functional" as we apply this term to any fragment of rRNA that, when expressed in vivo, can modulate ribosome functions in any possible way. Thus, rRNA fragments sequestering one or several ribosomal proteins or translation factors, fragments interfering with inter-domain or subunit interactions, fragments capable of binding antibiotics, fragments competing with the ribosome for binding to tRNA or mRNA, fragments assisting or interfering with ribosome assembly, etc., all will considered functional. What we had not considered possible was that some rRNA fragments may be functional because they can direct the synthesis of the proteins encoded therein. Nevertheless, when in the course of these experiments we identified several fragments of the 23S rRNA that were able to confer erythromycin resistance, it turned out that a short open reading frame present in these fragments (and thus in the 23S rRNA) must be expressed to make cells resistant to the drug. The accumulated data indicate that the ribosome is a likely target for the action of the identified rRNA-encoded peptide, which apparently remains associated with the ribosome after completion of its translation.

In this paper, we compare data obtained in our laboratory on the structural and functional features of the rRNA-encoded peptide with information about other functionally active peptides that can affect translation in *cis*.

Effect of the rRNA-encoded peptide on ribosome function

To understand the functions of individual segments of rRNA, a plasmid library expressing random rRNA fragments was prepared (Fig. 1). Random rDNA fragments ranging in size from ~40 to 500 bp were generated from the *rrn*B operon of *E. coli* and inserted into the newly constructed plasmid vector pPOT where they were transcribed from the IPTG-inducible *tac* promoter. (The details of the library construction will be published elsewhere.) Such a library can be screened in vivo by a variety of functional tests. In our experiments leading to

the finding of the rRNA-encoded peptide, the library was screened for rRNA fragments that rendered cells resistant to ribosome-targeted antibiotics. One of the antibiotics used for screening was erythromycin, which is known to interact with the large ribosomal subunit. Although the exact mechanism of erythromycin action remains largely speculative, results obtained in different laboratories suggest that it may inhibit protein synthesis by sterically hindering growth of the nascent peptide chain (Vester and Garrett 1987).

Screening the rRNA fragment library for erythromycin-resistant clones resulted in the selection of a number of clones that, in the presence of IPTG, were able to grow at erythromycin concentration of 150–200 μg/mL (the growth of the cells transformed with the empty vector was efficiently inhibited at a concentration of 50 μg/mL erythromycin). The rDNA inserts in all selected Ery^r clones were derived from the 23S rRNA gene and encompassed the region from position 1200 to 1300 at the junction of domains II and III of the 23S rRNA (Fig. 2). Subsequent deletion analysis showed that expressions of rRNA fragments as short as 34 nucleotides long (positions 1235–1268) could render cells resistant to erythromycin. This rRNA fragment was named eRNA34 (for effector RNA). Further deletion of 4 nucleotides from the 5′ end or 7 nucleotides from the 3′ end of eRNA34 rendered it inactive. Mutation analysis revealed three regions of eRNA34 that were critical for its activity: GGAGG sequence at the beginning of the eRNA34, GUG sequence 8 nucleotides downstream, and UAA sequence 12 nucleotides further downstream (Fig. 2C). The relative distribution and the sequences of these elements led to the striking possibility that translation of the short open reading frame present in the eRNA34 may be required for its activity in conferring erythromycin resistance. This ORF is equipped with a Shine–Dalgarno sequence GGAGG; it starts at GUG and terminates at the UAA codon and codes for a pentapeptide, Met-Arg-Met-Leu-Thr, which was named e-peptide, for the effector peptide. Fusion of the first 16 nucleotides of eRNA34, from the Shine–Dalgarno region to the first codon of the e-peptide ORF, with the second codon of the chloramphenicol acetyl transferase gene resulted in efficient expression of the reporter gene, indicating that the ribosome binding site of eRNA34 can indeed be used for initiation of translation.

The hypothesis that translation of the e-peptide was necessary for the observed erythromycin resistance was directly tested using an eRNA34 mutant with a point mutation at the second codon, which converted it into the stop codon UGA. The mutant eRNA34 was inactive and cells expressing it remained sensitive to erythromycin. However, introduction into the cell for a UGA suppressor $tRNA^{Arg}$ gene concomitantly with the mutant eRNA34 restored eRNA34 activity and made cells erythromycin resistant. Thus, it is evident that the mechanism of eRNA34 action is mediated by synthesis in the cell of the rRNA-encoded pentapeptide.

Among the different possible targets of e-peptide action, the ribosome appears to be the most probable one. This conclusion comes from in vitro studies of eRNA34 and e-peptide action. The phage MS2 RNA-directed cell-free translation system is sensitive to erythromycin. However, preincubation of the cell-free translation reaction mixture with in vitro transcribed eRNA34, resulting in the e-peptide synthesis, reduced the inhibitory action of erythromycin by 15–25%. Interestingly, in vitro translated peptide remained bound to the ribosome and did not dissociate even during sucrose gradient centrifugation. When synthetic e-peptide was added to the cell-free translation system instead of eRNA, no clear effect on erythromycin sensitivity was observed. Taken together, these data can be interpreted in favor of the *cis* mode of e-peptide action, where the nascent e-peptide translated from the rRNA fragment remains associated with the ribosome (maybe in the form of peptidyl-tRNA) and renders it resistant to erythromycin.

An important question is the actual function of e-peptide in the cell, if any. Indeed, the cell could hardly foresee that its survival would be challenged by exposure to erythromycin. Hence, what is observed as erythromycin resistance is probably nothing but a side effect of a yet obscure activity of the e-peptide related to protein biosynthesis. Interestingly, synthetic e-peptide present at 1–2 mM concentration can efficiently inhibit translation in the cell-free system by interfering with the peptidyl transferase reaction.

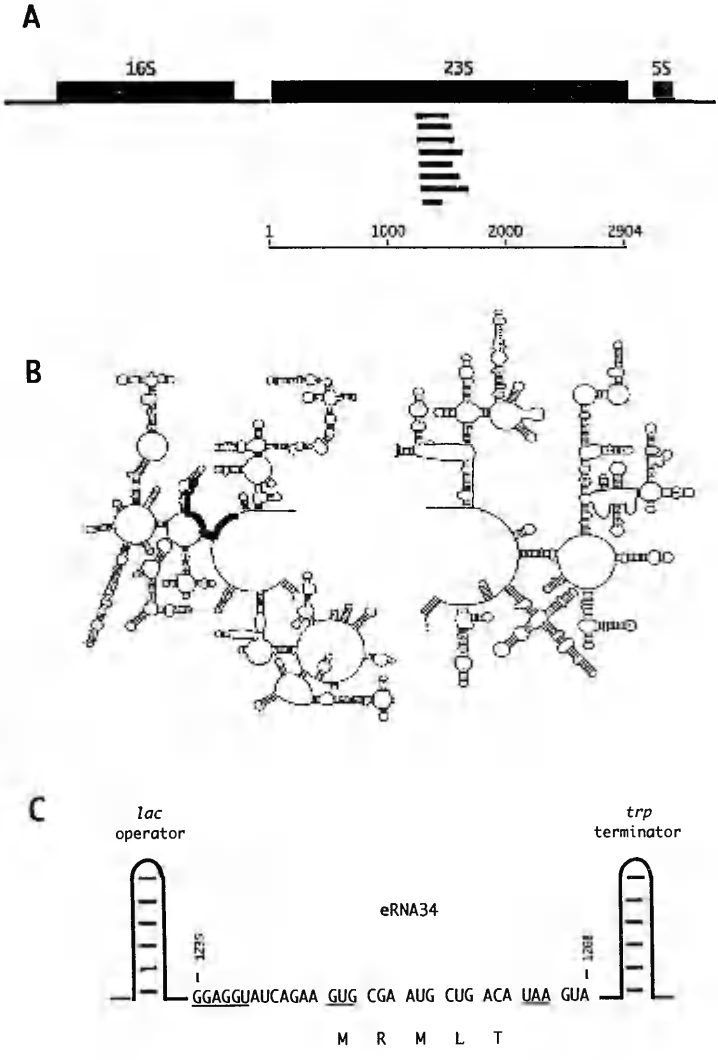
There are several cases described in the literature where ribosome appears to interact in a specific manner with newly translated polypeptide and this interaction has functional consequences for ribosome activity. Many properties of the e-peptide (its small size, possible *cis* mode of action, effect on translation) are similar to those of nascent peptides involved in translation regulation. Therefore, comparing e-peptide with regulatory *cis*-acting peptides may provide leads to understanding its function and mode of interaction with the ribosome. The next section briefly summarizes experimental facts where involvement of *cis*-acting newly synthesized polypeptides is proven or suspected. Discussion of some of these effects can be also found in recent reviews by Lovett (1994) and Geballe and Morris (1994).

Effect of newly synthesized peptides on translation

Nascent peptide-dependent stalling of the ribosome in bacterial antibiotic resistance genes

Translational attenuation is used in bacteria to regulate expression of chloramphenicol-resistance (*cat* and *cmlA*) and erythromycin-resistance (*erm*) genes in response to low concentrations of the corresponding drugs. The ribosome binding sites of these antibiotic resistance genes are sequestered in the RNA secondary structure; ribosome stalling at a specific codon of a short open reading frame preceding the resistance cistron changes the secondary structure of mRNA and permits translation of the downstream main ORF (Lovett 1990). Such stalling occurs at codon 6 of the *cat* leader ORF and at codon 9 of the leader ORFs of *cmlA* and *erm* genes (Fig. 3A). Curiously, ribosome stalling depends on the amino acid sequence of the translated short peptide; missence mutations in the leader ORF changing the nature of the encoded amino acids reduced efficiency of induction of the main cistron translation (Mayford and Weisblum 1989, 1990; Gu et al. 1994*a*). In the stalled ribosome, the nascent peptide is present in the form of peptidyl-tRNA and, thus, is positioned close to the peptidyl transferase center. Synthetic peptides corresponding to the first 5 codons of *cat* or first 8 codons of *cmlA* leader ORFs were able to inhibit in vitro peptidyl transferase reaction (Gu et al. 1993, 1994*b*). Therefore, Lovett and co-workers have suggested that the nascent peptide causes site-specific pausing of translation by inhibiting activity of the peptidyl transferase (Gu et al. 1993); it has been proposed further that the pausing

Fig. 2. Location in *rrn*B operon and structure of the rRNA fragments expressed in erythromycin-resistant library clones. (A) Relative location on the *rrn*B physical map of the inserts from several individual erythromycin-resistant library clones; the scale shows 23S rRNA numeration. (B) Position of the eRNA34 (bold line) in the secondary structure model of the 23S rRNA. (C) Structure of the eRNA34. Terminal hairpins corresponding to the *lac* operator and *trp* terminator are shown schematically. Shine–Dalgarno sequence, initiator GUG codon, and terminator UAA codons are underlined; amino acid sequence of the encoded pentapeptide is shown below and position numbers corresponding to the terminal nucleotides of the eRNA34 in the *E. coli* 23S rRNA are indicated.



ribosome is converted into the stalled state by the inducer, chloramphenicol. However, since little similarity can be found between *cat*, *cml*A, and *erm* leader peptides, it remains unclear what properties of the peptide are important for its regulatory effect.

Effect of eukaryotic upstream open reading frames on translation

Protein synthesis in eukaryotes is usually initiated at the first AUG codon, present in the appropriate context, that is encountered by an initiation complex scanning in the 3′ direction starting from the 5′ end of mRNA (Kozak 1989). However, in about 10% genes, short upstream open reading frames (uORFs) are present in the 5′ leader region of the transcript (Geballe and Morris 1994). Translation of such uORFs may influence expressions of the main cistron. There are several examples when not only the mere presence of an uORF but also the sequence of the encoded peptide is critical for regulation of translation of the main ORF.

Expression of the glutaminase subunit of carbamoyl-phosphate synthase A (*CPA1*) in yeast is controlled posttranscriptionally by the concentration of arginine (Messenguy et al. 1983). The arginine-dependent repression of *CPA1* translation requires the presence of a 25 condon uORF in the 250 nucleotide long 5′ leader region of the *CPA1* mRNA (Werner et al. 1987). Nonsence mutations in the 12th or 20th codons of the uORF, as well as missence mutations in codons 11 and 13, eliminate arginine-dependent regulation of *CPA1* translation and have a *cis*-dominant effect (Fig. 3A); silent mutations that preserved amino acid sequence of the uORF-encoded peptide have little influence on *CPA1* expression (Werner et al. 1987; Delbecq et al. 1994). It seems plausible that the nascent uORF-encoded peptide remains associated with the translating ribosome, causing it to stall and preventing it from proceeding to the initiation codon of the *CPA1* ORF. Interestingly, fusion of the first 23 codons of the uORF in frame with an ORF of a reporter protein made expression of this protein repressible by arginine (Delbecq et al. 1994), indicating that precise termination of *CPA1* uORF translation is not required for the regulatory function of the encoded peptide.

In the previous examples, the role of small cofactors (antibiotics or arginine) for the nascent peptide-mediated translational control seems to be important. However, this does not appear to be a universal rule and involvement of small cofactors has not been demonstrated in a number of the following cases. Efficiency of expression of mammalian *S*-adenosyl methionine decarboxylase (AdoMetDC) correlates with the density of ribosomes on AdoMetDC mRNA (Hill and Morris 1992; Ruan et al. 1994). In T cells, ribosome occupation of AdoMetDC mRNA is inhibited to the extent where AdoMetDC mRNA is present primarily in monosomes. Such inhibition depends on the presence of a 6 codon long uORF in the mRNA leader region (Hill and Morris 1992). The sequence of the encoded hexapeptide is critical for the regulation of the ribosome loading, since missense mutations (but not wobble mutations) in the three carboxyl terminal codons of the uORF relieve translational repression (Hill and Morris 1993). It is not clear whether translation of the uORF causes ribosome stalling, though it seems to be a likely hypothesis, especially taking into account that uORF produces its inhibitory effect in *cis* (Hill and Morris 1993).

The expression of cytomegalovrius glycoprotein gene *gp48* is also modulated at the translational level (Schleiss et al. 1991; Cao and Geballe 1994). Of the three uORFs present in the 5′ leader region of the *gp48* transcript, only the second uORF has an inhibitory effect on the translation of the *gp48* cistron (Schleiss et al. 1991). Analysis of missense and wobble mutations within this 22-codon uORF showed that the sequence of the encoded peptide, not the presence of the ORF per se, was critical for inhibiting downstream translation (Degnin et al. 1993) (Fig. 3A). As in the previous cases, the *cis* dominant character of the uORF mutations has been demonstrated (Degnin et al. 1993).

A similar effect is caused by an upstream ORF present in the 5′ leader region of β_2 adrenergic receptor mRNA (Parola and Kobilka 1994). The presence of this 19 codon long uORF is the cause of 50% repression of translation of the main cistron. The location of the uORF, its size, and, to some extent, the sequence of the encoded arginine-rich peptide is conserved among mammals (Fig. 3A). The synthetic uORF-encoded peptide is active in vitro, where it can repress cell-free translation of several mRNAs. At least three of the arginines present in the C-terminal portion of the peptide sequence are essential for its activity. Replacement of these arginines by alanines alleviates the peptide inhibitory effect in vitro, and mutations of corresponding arginine codons in uORF increases expression of β_2 adrenergic receptor in vivo. Since it has been shown that arginine-rich peptides can bind to RNA in vitro (Calnan et al. 1991) and inasmuch as the mutations in the β_2 adrenergic receptor uORF had a dominant *cis* effect, it has been proposed that the newly synthesized 19 amino acid long arginine rich peptide binds to the same mRNA molecule from which it has been translated and prevents its translation (scanning) by following ribosomes (Parola and Kobilka 1994). An alternative possibility is that the nascent peptide remains associated with the translating ribosome, causing its stalling or preventing reinitiation at the AUG codon of the β_2 adrenergic receptor ORF. Interestingly, one other arginine-rich synthetic peptide (but not several other tested peptides) inhibited in vitro translation in a manner similar to the uORF-encoded peptide.

Why E. coli ribosomes dislike chicken histone H5

The importance of arginines for nascent peptide-mediated inhibition of translation emerges also in another case. The chicken histone H5 is efficiently translated in a rabbit reticulocyte system. However, it cannot be overexpressed in *E. coli* because of accumulation of unfinished nascent peptide intermediates (Gerchman et al. 1994). This results from ribosome stalling in the mRNA region coding for the highly positively charged lysine and arginine-rich C-terminal tail of the protein. Attachment of the same Lys–Arg-rich gene segment to a reporter protein inhibited its translation as well. Interestingly, there were no problems in overexpressing histone H1, which also had highly positively charged C-terminal tail. The main apparent difference in structure of the C-terminal portions of H1 and H5 genes is that basic residues in the H1 tail are mostly lysines with many fewer arginines. Notably, not only a high concentration of arginine residues but also a high proline content is characteristic of both the H5 histone C-terminal tail and the putative inhibitory peptide encoded in the uORF of β_2 adrenergic receptor mRNA (Gerchman et al. 1994; Parola and Kobilka 1994). Thus, it is possible that arginines and prolines

Fig. 3. Examples of short peptides involved in *cis*-regulation of translation. (A) Sequences correspond to the peptides involved in regulation of translation of cistrons coding for bacterial chloramphenicol (*cat* and *cmlA*) and erythromycin (*erm*) resistance proteins, mammalian *S*-adenosyl methionine decarboxylase (*AdoMetDC*), glutaminase subunit of yeast carbamoyl phosphate synthase A (*CPA1*), cytomegalovirus glycoprotein gene (*gp48*), and β_2 adrenergic receptor of different mammals. Also shown are 46 N-terminal amino acids of bacteriophage gene *60* protein. Amino acids in which substitution affects functions of corresponding peptides are underlined. (B) Amino acid sequences of pentapeptides in which expression in *E. coli* cells confers erythromycin resistance. The left column represents the sequence of the 23S rRNA-encoded e-peptide and some of its mutants generated by introducing limited number of mutations into the eRNA34; the right column shows sequences of the active pentapeptides selected from the library where the pentapeptide open reading frame in the eRNA34 was completely randomized.

A

ermC	M G I F S I F V I S T V H Y Q P N K K
cat-86	M V K T D K I S S
cmlA	M S T S K N A D K
AdoMetDC	M A G D I S
gp48	M Q P L V L S A K K L S S L L T C K Y I P P
CPA1	M F S L S N S Q Y T C Q D Y I S D H I W K T S S H
β_2 adrenergic receptor	
murine	M K L P G V R P R P A A P R R R C T R
hamster	M K L P G V C L R P A A P R R R C T R
rat	M K L P G V R P R T A A P H R R C T R
human	M R L P G V R S R P A E P R R G S A R
gene *60*	MKFVKIDSSSVDMKK Y K L Q N N V R R S I K S S S M NYANVAIMTDADHDG-*gap*

B

e-peptide	peptides from the random library
M R M L T	M F Q F V
	M T L F I
	M S I V V
	M L L A V
	M F M E L
	M A V M V
	M R M M V
	M T L K R
mutants of the e-peptide	M V D I G
M R M L I	M N Y L V
M R L L T	M A I H V
M R I M T	M R L S L
M Q M L T	M V N V C
M R M L R	M I K L F
M P M L T	M N F K C
M L I L T	M T I T I
M R M A T	M Y L I S
M R L L I	M N K S V
M R M Q T	M V M W L
	M M L R C

are important for translation inhibition by corresponding peptides.

Interestingly, arginine–proline-rich short peptide ORFs are found in leader regions of early genes of several papovaviruses (Khalili et al. 1987). The presence of these uORFs reduces translation of the main cistrons. Though direct involvement of the papovavirus early leader peptides in translation regulation has not been demonstrated directly, resemblance between these peptides and the Arg–Pro-rich peptides discussed above is highly suggestive of their functional similarity as well.

Ribosome hopping depends on amino acid sequence of the nascent peptide

An exciting example of the influence of nascent peptide on ribosome function has been described by Weiss et al. (1990). Bacteriophage T4 gene *60* mRNA contains an internal 50 nucleotide long untranslated coding gap, separating the first 46 codons of the gene from the last 114 codons. This gap is not excised from the RNA as an intron but rather it is bypassed by elongating ribosomes (Huang et al. 1988). Nascent peptide amino acids 17 to 32 are implicated in activating such ribosome hopping, though the exact requirements for peptide sequence remains obscure. The nascent peptide activates ribosome bypass only in *cis*, which is not surprising since the ribosome reaches the "take-off" site (codon 46) when the functionally active peptide segment, corresponding to amino acids 17–32, is still within the ribosome. It has been suggested that, analogous to the peptide antibiotic edein, the nascent peptide may destabilize P-site tRNA binding and thus trigger the bypass (Weiss et al. 1990). However, in another described example of translational bypass, the nascent peptide sequence is apparently not important (Benhar and Engelberg-Kulka 1993).

Peptidyl transfer and translation termination sensitivity to amino acid context

Reactions catalyzed at the peptidyl transferase center of the ribosome are influenced by the nature of the nascent peptide C-terminal amino acids. In the classic "fragment reaction" experiments (Monro and Marcker 1967), the efficiency of peptidyl transfer correlates with the chemical structure of the donor amino acid residue (Krayevsky and Kukhanova 1979; Chladek and Sprinzl 1985), which structurally an functionally corresponded to the C-terminal amino acid of the nascent peptide. The termination of translation may be influenced by the protein's last two amino acids. Lysine is found more frequently at the C-termini of proteins in which ORF is terminated at UAA, while a similar position is occupied more often by phenylalanine, or serine in case of the UGA stop codon (Arkov et al. 1993). Issakson and co-workers (Mottagui-Tabar et al. 1994) demonstrated that the frequency of read-through of UGA and UAG termination codons depends on the penultimate amino acid. Thus, the C-terminus of nascent peptides may be involved in the fine tuning of protein synthesis.

Nascent peptide interactions outdoors

In many (and maybe all) of the cases discussed above, newly synthesized peptide may exert its effect on translation while still located within the ribosome. However, interaction of the nascent peptides with external factors "outside" of the ribosome is also known to play a role in regulation of translation. Signal recognition particles (SRP), involved in protein targeting to the endoplasmic reticulum, interact with leader regions of the nascent polypeptides and stop or slow down translation until the peptide signal sequence is inserted into the membrane (Wolin and Walter 1988). Other factors that can also interact with leader regions of nascent peptides might control the process of protein synthesis by analogy with SRP (Wiedmann et al. 1994). Interaction of nascent peptides with external factors (chaperones, membranes, etc.) may be used to optimize the translation rate and to allow the nascent peptide time to fold into proper configuration. For example, it has been suggested that temporal arrest of the chloroplast protein D1 translation (the mechanism of which remains unknown) provides additional time for the nascent peptide chain to bind cofactors, such as chlorophyll, before the release of the protein from the ribosome (Kim et al. 1994).

An interesting example of cotranslational interaction of the nascent peptide with external factors outside of the ribosome comes from the studies of regulation of β-tubulin synthesis. Increased concentration of free β-tubulin subunits in the cell triggers the degradation of β-tubulin mRNA in polysomes (Cleveland 1989). The four N-terminal amino acids of β-tubulin nascent peptide (MREI) are essential for such autoregulation (Yen et al. 1988; Backurski et al. 1994). When this segment of the nascent peptide is blocked by antibodies, the tubulin-dependent autoregulation is prevented, suggesting that binding of additional factors (possibly, free β-tubulin subunits) to the nascent peptide N-terminus is necessary for mRNA degradation (Theodorakis and Cleveland 1992).

Discussion

Screening a random rRNA fragment library revealed the presence of clones that became erythromycin resistant of a result of expression of a pentapeptide encoded in 23S rRNA. Our current ideas of the mode of the e-peptide action are based on several experimental facts: (*i*) a short ORF in the *E. coli* 23S rRNA can be translated in vivo and in vitro (at least, from an rRNA fragment); (*ii*) translation of the e-peptide ORF in vivo makes cells resistant to low concentrations of erythromycin; (*iii*) similarly, translation of the e-peptide ORF in vitro decreases erythromycin sensitivity of the cell-free translation system; (*iv*) in vitro translated peptide remains associated with the ribosome; (*v*) synthetic peptide, present in millimolar concentration, inhibits cell-free translation and peptidyl transferase reaction catalyzed by the ribosome; the latter effect is counteracted by erythromycin present at 100 uM concentration. Several assumptions are based on these facts: (*i*) ribosome is the target of the e-peptide action; (*ii*) translated peptide remains associated with, and acts on, the ribosome on which it has been synthesized; (*iii*) the peptide bound to the ribosome may affect its function.

One of the key questions is the location of the e-peptide binding site. Our current model is that the e-peptide remains associated with the nascent peptide channel in the vicinity of the peptidyl transferase center and that when bound there the peptide can modulate ribosome function. This possibility emerges from comparison of the e-peptide with other *cis*-acting peptides discussed in the previous paragraphs. It is likely that many of these peptides affect the ribosome while they are still bound to the nascent peptide channel. This is evident for

the nascent regulatory peptides of the bacterial antibiotic resistance operons. Translation of these peptides is not yet complete when they cause ribosome pausing; therefore, the peptide must still be in the form of peptidyl-tRNA. These peptides are very small (see Fig. 3A) and the amino acid sequence of the peptide immediately adjacent to the putative stalling site is most important for the peptide action (Mayford and Weisblum 1989; Lovett 1994). Thus the "affector" segment of the peptide must be located very close to the peptidyl transferase center. This conclusion is corroborated by the fact that synthetic peptides corresponding to the active portion of the *cat* and *cml*A leader peptides can inhibit peptidyl transferase reaction in vitro, and this effect can be counteracted by erythromycin (Gu et al. 1993, 1994*b*). The e-peptide is reminiscent of the *cat*, *cml*A, and *erm* leader peptides in its size and putative *cis*-mode of action and also in that some in vitro effects found for those peptides can also be reproduced with the e-peptide. For example, similar to *cat* and *cml*A leader peptides, the synthetic e-peptide was able to inhibit peptidyl transferase reaction when present at 1–2 mM concentration and this effect could also be abolished by erythromycin. Therefore, it is likely that all of these short *cis*-acting peptides may interact with the same site in the vicinity of the peptidyl transferase center, probably overlapping with the erythromycin binding site (which would also explain why expression of the e-peptide in vivo results in resistance to erythromycin). In general, it is possible that the ribosomal compartment "used" by short peptides, such as leader peptides of antibiotic resistance operons or e-peptide, may be also used by some other *cis*-acting peptides discussed in the previous section. In spite of the larger size of some of these peptides, their functional segment may be located close to the C-terminus and thus may "fit" into the postulated site.

In the case of e-peptide, its size seems to be important for the functional interaction with the ribosome. A nonsense mutation in the C-terminal codon of the e-peptide ORF, which makes the peptide one amino acid shorter, as well as mutations in the natural stop codon of the e-peptide ORF, which make the translation product longer, abolish e-peptide-dependent erythromycin resistance. However, the sequence specificity of the proposed short peptide binding site on the ribosome is unclear. It appears that this site has certain sequence preferences but does not have strict sequence requirements. In our studies of the mechanism of eRNA34 action, a number of e-peptide mutants with partially altered amino acid sequences were generated. Surprisingly, many of these mutants conferred erythromycin resistance similar to wild-type e-peptide, and when the sequence of the e-peptide ORF in eRNA34 was completely randomized, 10 to 20% of the clones exhibited erythromycin resistance (Fig. 3B). Therefore, it is likely that several different pentapeptides can produce functional effects similar to the e-peptide. Such sequence flexibility makes it difficult to determine if the presence of an active peptide gene in the rRNA is evolutionarily conserved. The e-peptide ORF can be found at the junction of domains II and III of the 23S rRNA in some bacteria, both Gram-positive and Gram-negative, but it is missing in others. Nevertheless, since amino acid sequence of the peptide is not strictly critical for its activity, other short open reading frames that are present in rRNAs of most organisms may serve as a source of active e-peptide analogs. The absence of strict sequence requirement that we observe for the e-peptide was also evident for some other *cis*-acting peptides. Missence mutations that did not affect peptide function have been described for a number of those peptides and there is no apparent sequence similarity between the different peptides listed in Fig. 3A. It is important to note, however, that most e-peptide clones with randomized pentapeptide ORF remained sensitive to erythromycin, even though mutant eRNA was produced in relatively high amount in at least some of the clones. Accordingly, eRNA instability could not account for erythromycin sensitivity in these clones and most probably translated peptides were "inactive." Thus, not every pentapeptide can be "functional."

The primary function of e-peptide remains illusive. As already mentioned, we favor the hypothesis that erythromycin resistance caused by the e-peptide is a side effect of its putative functional interaction with the ribosome. Usually, *cis*-acting peptides function as translational repressors and many (if not all) of the effects discussed in the review section can be explained by the ribosome pausing or stalling on mRNA. It is tempting to speculate that e-peptide can produce a similar effect. This idea is supported by the fact that the e-peptide can inhibit protein biosynthesis in vitro by interfering with the peptidyl tranferase reaction. High peptide concentrations (in the millimolar range) required for the observed in vitro effects can be explained by the difference between co-translation placing of the e-peptide into its binding site versus binding of exogenous peptide. We do not know why the cell "needs" to encode the putative repressor of the ribosome activity in rRNA. It may potentially use this arrangement to inhibit translation under conditions unfavorable for cell growth, which are usually accompanied by ribosome degradation. Fragmentation of rRNA is clearly one way to make expression of the encoded e-peptide possible.

It should be noted that it remains yet unknown if e-peptide can be translated from intact 23S rRNA. The Shine–Dalgarno region of the e-peptide open reading frame is involved in secondary structure interactions in hairpin 46 (Egebjerg et al. 1990) in intact 23S rRNA, and it is unlikely that under normal conditions it can be used efficiently for initiation of the e-peptide translation. However, some spontaneous or engineered mutations in the 23S rRNA, which decrease stability of the hairpin, make expression of e-peptide possible (Douthwaite et al. 1985; T. Tenson and A. Mankin, in preparation). It is conceivable that specific rRNA fragmentation or loosening of the hairpin structure, which may occur under certain physiological conditions, can lead to the synthesis of e-peptide. In any event, occurrence in the rRNA of a gene whose product can act on the ribosome is, at the very least, highly suspicious and deserving of interest.

Acknowledgments

The authors are grateful to Alexandra DeBlasio for invaluable help in experimental work and in preparation of the manuscript. This work was supported by the Hans Vahlteich Research Award and National Science Foundation grant MCB-9420768.

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Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 5641–5646, May 1996
Biochemistry

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

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Communicated by Harry F. Noller, University of California, Santa Cruz, CA, February 8, 1996 (received for review December 15, 1995)

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*111–*Bsp*MI 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, CACTAGTGGAGGTATCAGAAGTGGAGAAAAAAATCAC, 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-

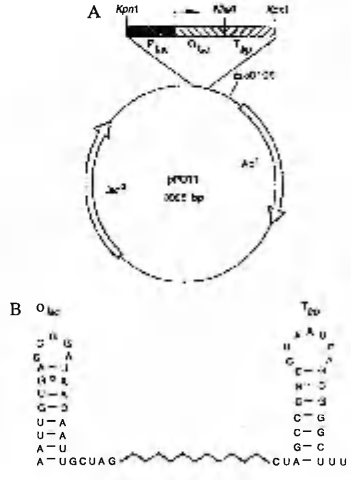

FIG. 1. Random rRNA fragment library. (*A*) The schematic map of the pPOT1 plasmid vector. Relative location and orientation of the $lacI^q$ and β-lactamase (Ap^r) genes are shown by open arrows. An inducible *tac* promoter (P_{tac}) is shown by a black bar; *lac* operator (O_{lac}) and tryptophan terminator (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.

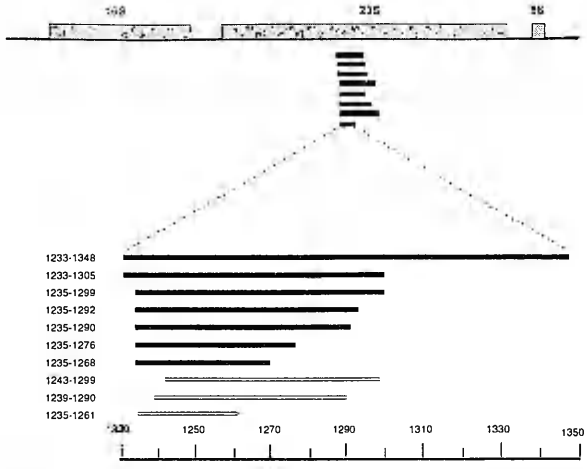

FIG. 2. rRNA fragments expressed in the selected and constructed Ery^r clones. The black bars shown under the schematic map of the rRNA operon correspond to E-RNAs expressed in the originally selected Ery^r clones. The bottom portion shows deletion analysis of the clone that expressed E-RNA corresponding 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.*

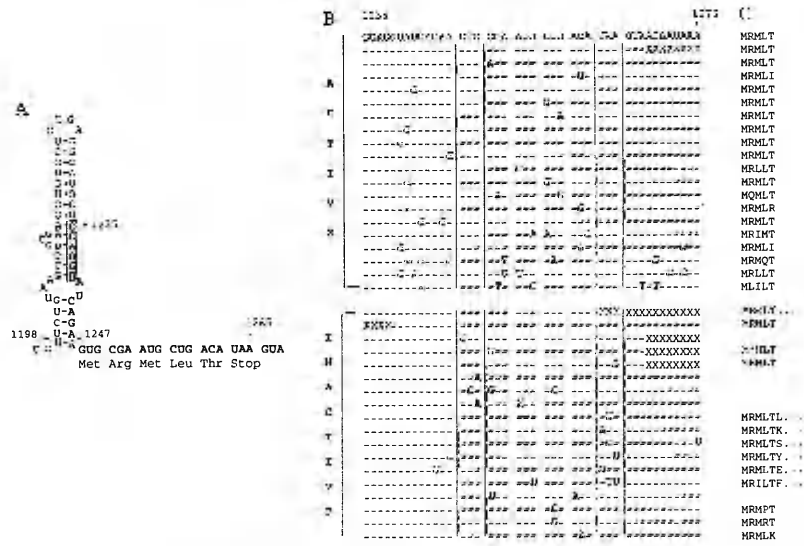

FIG. 3. E-RNA mutants. (*A*) The nucleotide sequence and secondary structure of the *E. coli* wild-type 23S rRNA segment encoding the pentapeptide mini-gene. The minimal rRNA fragment capable of conferring erythromycin resistance (E-RNA34) is shown in boldface. Shine–Dalgarno region (24) of the pentapeptide 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.

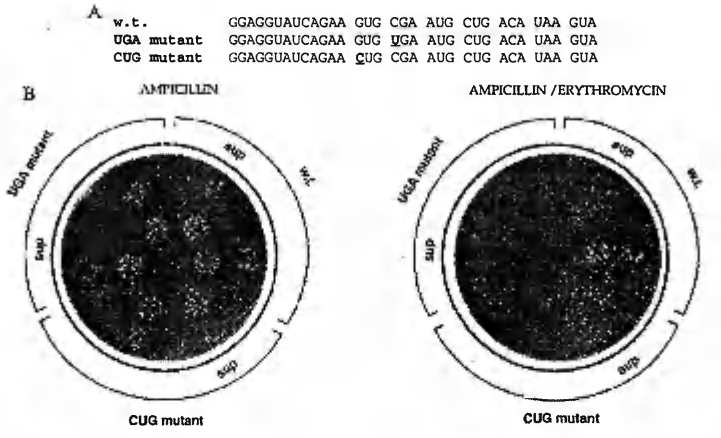

FIG. 4. Effect of the *opal* suppressor tRNA on activity of wild-type and mutant E-RNA34. (*A*) Sequences of the wild-type E-RNA34 and CUG and UGA mutants. (*B*) Effect of erythromycin on growth of cells expressing wild-type and mutant E-RNA34 in the absence or in the presence of the *opal* suppressor $tRNA^{Arg}$. 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

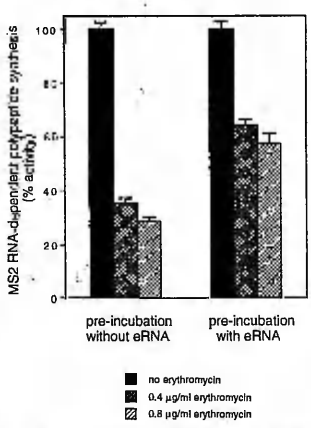

FIG. 5. Effect of the E-RNA translation on erythromycin sensitivity of the cell-free translation system. Ribosomes were preincubated with E-RNA or MS2 RNA and subsequent MS2 RNA-dependent polypeptide synthesis was assessed both in the absence and in the presence of erythromycin. Polypeptide synthesis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

The authors are grateful to A. Neyfakh, N. Vazquez, and J. Remme for encouragement and helpful discussions and to P. Kloss for help in preparing the manuscript. W. McClain is thanked for providing the plasmid with the suppressor tRNA gene. We appreciate the help of B. Bachmann, the curator of the *E. coli* Genetic Stock Center, in choosing isogenic *E. coli* strains. This work was supported by a Hans Vahlteich Research Award and by National Science Foundation Grant MCB 9420768.

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III

Dam, M., Douthwaite, S., **Tenson, T.,** and Mankin, A. S. (1996) Mutations in domain II of 23 S rRNA facilitate translation of a 23 S rRNA-encoded pentapeptide conferring erythromycin resistance. J. Mol. Biol., 259, 1–6.

J. Mol. Biol. (1996) **259**, 1–6

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COMMUNICATION

Mutations in Domain II of 23 S rRNA Facilitate Translation of a 23 S rRNA-encoded Pentapeptide Conferring Erythromycin Resistance

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Mutations in domain II of *Escherichia coli* 23 S rRNA that cause resistance to erythromycin do so in a manner fundamentally different from mutations at the drug binding site in domain V of the 23 S rRNA. The domain II mutations are located in a hairpin structure between nucleotides 1198 and 1247. This is close to a short open reading frame in the 23 S rRNA that encodes a pentapeptide (E-peptide) whose expression *in vivo* renders cells resistant to erythromycin. Therefore, a possible mechanism of resistance caused by domain II mutations may be related to an increased expression of the E-peptide. To test this hypothesis, a range of point mutations was generated in domain II of 23 S rRNA in the vicinity of the E-peptide open reading frame. We find a correlation between erythromycin resistance of the mutant clones and increased accessibility of the ribosome binding site of the E-peptide gene. Furthermore, the erythromycin resistance determinant in the mutants was shown to be confined to a small 23 S rRNA segment containing the coding region and the ribosome binding site of the E-peptide open reading frame. It thus appears that the domain II mutations mediate erythromycin resistance by increasing expression of the 23 S rRNA-encoded E-peptide.

Keywords: ribosome; rRNA; erythromycin; translation; drug resistance

Erythromycin is a potent inhibitor of bacterial protein synthesis, and is thought to act by sterically hindering growth of the nascent peptide chain (Vazquez, 1979). The binding site for erythromycin is located on the large ribosomal subunit in the vicinity of the peptidyl transferase center. Most of the rRNA nucleotide alterations that cause erythromycin resistance are point mutations and base methylation within the central loop of domain V of the 23 S rRNA (for reviews see Weisblum, 1995; Garrett & Rodriguez-Fonseca, 1995; and see Figure 1). Several nucleotides in this region are protected by erythromycin from chemical modification (Moazed & Noller, 1987). This internal loop in domain V of the 23 S rRNA thus appears to be the primary binding site for erythromycin.

It was therefore surprising that a mutation in another region of the 23 S rRNA was also shown to confer erythromycin resistance (Douthwaite *et al.*, 1985). Deletion of 12 nucleotides (positions 1219–1230) within a conserved rRNA hairpin structure between nucleotides 1198 and 1247 (the 1200–1250 helix) in domain II of the *Escherichia coli* 23 S rRNA gene, increased the minimal concentration of erythromycin required to inhibit cell growth three to four times. Other engineered deletions in this region produced similar effects (Douthwaite *et al.*, 1989). Curiously, however, these deletions did not affect binding of erythromycin to the mutant ribosomes, as assayed by footprinting *in vitro* (Douthwaite *et al.*, 1989). This contrasts with a marked reduction in the ribosome–drug interaction caused by point mutations at the central loop in domain V (Douthwaite & Aagaard, 1993). These observations were taken to indicate that the deletion

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Abbreviations used: ORF, open reading frame; S.D., Shine–Dalgarno sequence; E-peptide; 23 S rRNA-encoded pentapeptide, 1200–1250 hairpin, a region of the *E. coli* 23 S rRNA secondary structure encompassing positions 1198 to 1247; IPTG, isopropyl-β-thiogalactopyranoside.

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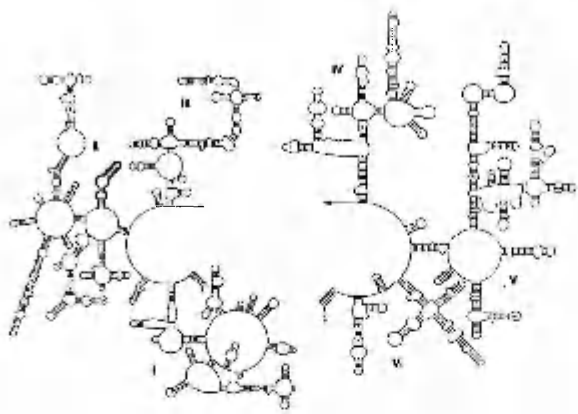

Figure 1. The outline of the 23 S rRNA secondary structure (Noller, 1984; Gutell *et al.*, 1994). The 1200–1250 hairpin in domain II is shown in bold.

mutations disrupted a functional interaction between domains II and V, and thereby suppressed the action of the drug without preventing its binding. However, these observations can now be reinterpreted in the light of new data.

Recently, a novel type of erythromycin resistance has been proposed, the mechanism of which is based on interaction between a specific pentapeptide and the ribosome (Tenson & Mankin, 1995; Tenson *et al.*, 1996). This peptide (the E-peptide) is encoded in a short open reading frame (ORF) between nucleotides 1248 and 1265 at the junction of domains II and III within the *E. coli* 23 S rRNA (Figure 2). A putative Shine–Dalgarno region (S.–D.; Shine & Dalgarno, 1974) for the E-peptide ORF is located at the adjacent sequence 1235 to 1240. The proximity of this ORF to the position of the domain II deletions suggests that resistance to erythromycin might be acquired, owing to enhanced expression of the E-peptide. Here we present data on a range of point mutations that have been introduced into the 1200–1250 helix. These mutations reduce the stability of the helix and render the putative S.–D. sequence more accessible. By correlating the accessibility of the S.–D. sequence with cell resistance to erythromycin, we are able to test the E-peptide hypothesis.

Effects of helix 1200 to 1250 mutations in intact 23 S rRNA

The E-peptide ORF has a canonical S.–D. sequence. In the intact 23 S rRNA, this S.–D. sequence is a part of the 1200–1250 hairpin structure (Figures 1 and 2; and Noller, 1984), and is presumably unavailable to serve as a ribosome binding site for initiation of translation. Nucleotide substitutions were generated in the 1200 to 1250 hairpin to investigate whether altering the stability of the hairpin gives rise to erythromycin resistance. The mutant 23 S rRNAs were expressed *in vivo* from the multicopy plasmid pLK55, which contains a complete rRNA operon under the control of the lambda P_L promoter. Some of the point mutations notably increased cell tolerance to erythromycin (Table 1). To rationalize the effects of individual mutations, the stability of the 1200–1250 hairpin in the mutant 23 S rRNAs was calculated. These results are presented in Table 1, and some of the representative secondary structures are shown in Figure 2.

The data reveal a strong inverse correlation between the stability of the 1200–1250 hairpin and the drug resistance of cells. Out of 35 analyzed mutants only two, pLK55–130 and –136, do not follow this general rule. Mutants 130 and 136 are erythromycin-sensitive, although the over-all stability of the mutant hairpin is weakened (ΔG = –8.9 and –6.5 kcal/mol, respectively, compared to –15.4 kcal/mol for the wild-type helix). However, the 1206A mutation present in these mutants directly stabilizes the interaction between the S.–D. sequence and the opposite side of the hairpin, so that weakening of the apex part of the hairpin may be not sufficient to release the S.–D. region (Figure 3). In addition, the 1236A mutation in pLK55–136 changes the actual S.–D. sequence, reducing its complementarity to the 3' end of 16 S rRNA.

Several mutants carry a 1248A mutation, which changes the rarely used initiation GUG codon of the E-peptide ORF to the prevailing initiator codon AUG (Table 1). The change of initiator codon alone is not sufficient to significantly increase expression of the E-peptide, since mutant 11, having only the 1248A mutation, remains erythromycin-sensitive. However, enhanced drug resistance is observed when the AUG initiator codon occurs in combination with mutations that increase the accessibility of the S.–D. region (mutant 48, Figure 2). The pLK55–48 construct exhibits the highest level of erythromycin resistance of all the point mutants

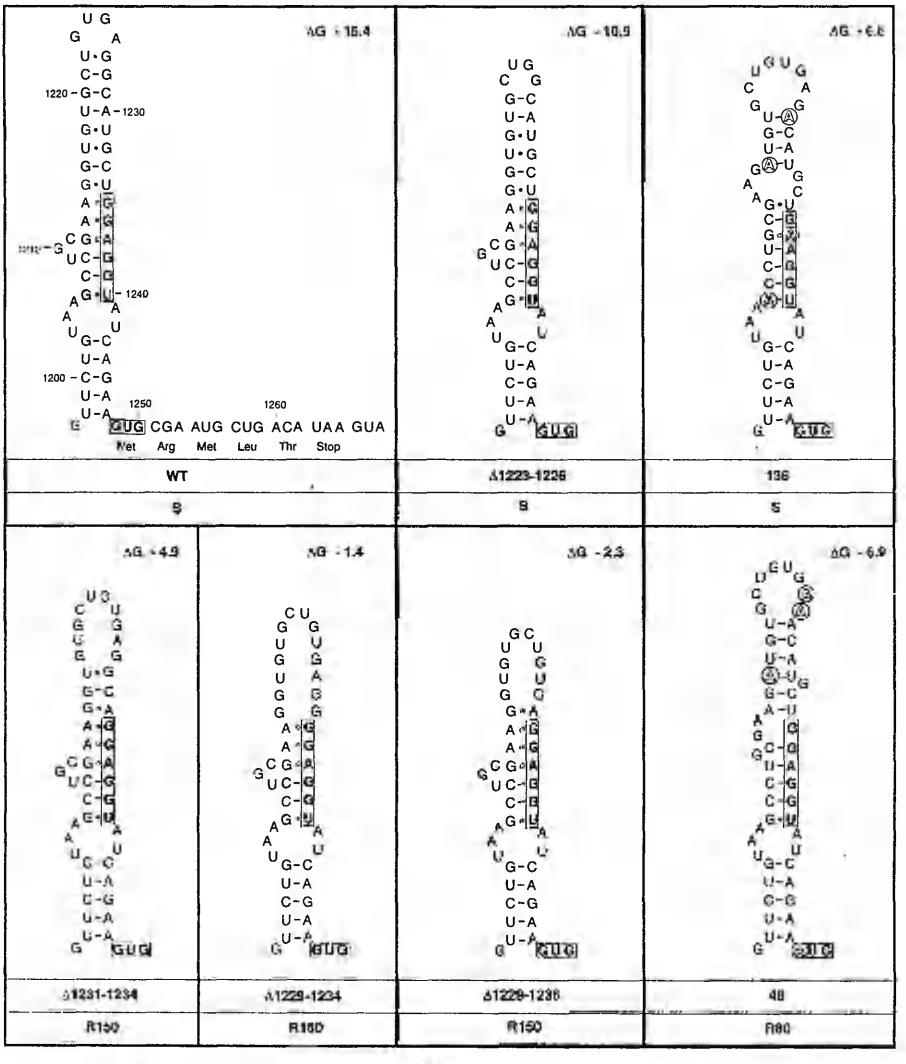

Figure 2. Putative secondary structure of the 1200–1250 helix in wild-type and mutant 23 S rRNAs. The wild-type (WT) structure is shown, together with the adjacent region encoding the E-peptide. The Shine–Dalgarno sequence and the initiator codon of the ORF are boxed. Putative RNA structures resulting from deletions are illustrated, and point mutations in the mutants pLK55–136 and -48 are circled.

analyzed (Table 1). Hence, mutations that may increase expression of the rRNA-encoded E-peptide result in an Ery^r phenotype, while those mutations that would either reduce or leave the peptide expression significantly unchanged do not confer drug resistance.

A similar trend emerges on examination of deletion mutations that also affect the hairpin stability (Douthwaite *et al.*, 1989). Comparison of the secondary structure in these mutants with the wild-type hairpin structure is more complicated, because in many cases the deletions will cause an

Table 1. Effect of point mutations on erythromycin resistance and stability of the 1198-1247 hairpin

Plasmid[a]	Mutations	Phenotype[b]	Hairpin ΔG[c]
pLK55–29	1210A, 1218A, 1248A	S	–15.7
pLK55 (w.t.)	none	S	–15.4
pLK55–11	1248A	S	–15.4
pLK55–157	1235A	S	–15.4
pLK55–152	1215A	S	–15.2
pLK55–15	1206A	S	–15.1
pLK55–114	1236A	S	–15.1
pLK55–21	1227A, 1236A	S	–14.9
pLK55–150	1218A, 1245A	S	–13.7
pLK55–36	1225A	S	–13.4
pLK55–79	1197A, 1202A, 1210A, 1250A	S	–13.4
pLK55–246	1233U	S	–13.0
pLK55–262	1208U	S	–12.8
pLK55–248	1229U	S	–12.8
pLK55–58	1238A	S	–12.8
pLK55–232	1208U, 1211U	S	–12.6
pLK55–204	1207U	S	–12.4
pLK55–244	1221U, 1243U	S	–11.9
pLK55–243	1208U, 1243U	R35	–11.8
pLK55–241	1207U, 1243U	R35	–11.5
pLK55–17	1232A, 1238A	S	–11.4
pLK55–245	1221U, 1233U	R50	–10.5
pLK55–233	1221U, 1229U	R35	–10.4
pLK55–247	1200U, 1208U, 1221U	R35	–9.4
pLK55–130	1206A, 1228A	S	–8.9
pLK55–45	1220A, 1239A	R35	–8.9
pLK55–138	1220A, 1223A, 1228A	R50	–8.9
pLK55–259	1200U, 1207U, 1208U. 1211U, 1221U	R50	–8.3
pLK55–260.	1200U, 1207U, 1208U, 1211U, 1221U, 1243U	R50	–8.3
pLK55–212	1208U, 1221U, 1229U	R50	–7.8
pLK55–264	1206A, 1216A, 1218A, 1223A, 1232A, 1239A	R50	–7.5
pLK55–125	1202A, 1206A, 1212A, 1225A, 1228A, 1235A	R35	–7.1
pLK55–48	1216A, 1227A, 1228A, 1248A	R80	–6.9
pLK55–136	1206A, 1216A, 1228A, 1236A	S	–6.5
pLK55–258	1208U, 1211U, 1221U, 1229U, 1233U, 1243U	R50	–4.2

[a] Mutations were generated in a 1 kb *Sac*I-*Sal*I fragment of the 23 S rRNA gene sub-cloned into bacteriophage M13. DNA heteroduplexes were formed in a manner similar to that described by Rosendahl & Douthwaite (1995), creating a single-stranded window at the sequence from 1197 to 1248. Mutations were introduced by bisulfite deamination of cytosine residues in the single-stranded window (Pine & Huang, 1987). Both strands of the gene fragment were mutagenized to give G → A as well as C → U transitions. *Sac*I-*Sal*I fragments were cloned back into their original *rrn*B context for expression from the lambda P_L promoter in pLK55. Plasmid pLK55 is essentially identical to pLK35 (described by Douthwaite *et al.*, 1989) but contains a phenotypically neutral allele-specific priming site around 23 S rRNA position 1360 (Aagaard *et al.*, 1991). Plasmids pLK35 and pLK55 are pBR322 derivatives containing the entire *E. coli rrnB* operon under control of the lambda P_L promoter (Gourse *et al.*, 1985). Plasmids were propagated in the *E. coli* strain DH1 (Sambrook *et al.*, 1989) for which minimal inhibitory concentration of erythromycin was 25 μg/ml.

[b] Phenotype of the mutants was determined by plating on agar containing ampicillin at 25 μg/ml and different concentrations of erythromycin. S, erythromycin-sensitive mutants; R35, R50 and R80, mutants capable of growing in the presence of 35 μg/ml, 50 μg/ml and 80 μg/ml erythromycin, respectively.

[c] Stability of the wild-type and mutant hairpins (kcal/mol) was determined using energy minimization program (Zuker, 1994) provided on M. Zuker's world wide web homepage (http://ibc.wustl.edu/~zuker/cgi-bin/form1.cgi).

alternative folding of this RNA region. Nevertheless, there is generally a good correlation between accessibility of the S.–D. region of the E-peptide ORF and erythromycin resistance of the mutant. Small deletions that have little effect on hairpin stability do not induce erythromycin resistance (e.g. mutants Δ1223–1226 in Figure 2). However, a similar size deletion, which weakens the hairpin (mutant Δ1231–1234), produces an Ery^r phenotype. Removal of more than four nucleotides significantly destabilizes the hairpin. Thus, mutants lacking 4 to 11 nucleotides to the 3' side from position 1229 are erythromycin-resistant (for example, mutants Δ1229–1234 and Δ1229–1236 in Figure 2). Longer deletions on this side of the helix (12 or more nucleotides 3' to position 1229) start to remove the S.–D. sequence resulting in loss of resistance. Deletions of 9 to 17 nucleotides 5' to the position 1232 destabilize the hairpin, while leaving the S.–D. sequence intact and also confer drug resistance. All these observations are completely consistent with the prediction that an accessible S.–D. sequence is required for translation of the E-peptide. However, it is not immediately obvious why mutants carrying 23 S rRNA lacking 17 to 24 nucleotides 5' to the position 1232 are not resistant to erythromycin.

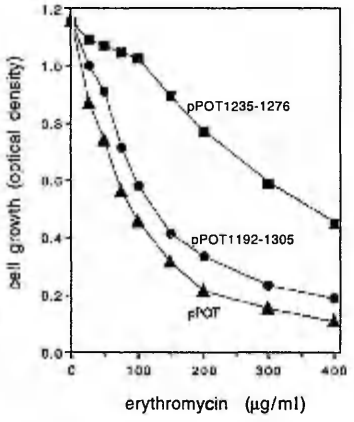

Figure 3. Erythromycin inhibition of cell growth. Cells transformed with an empty pPOT vector (▲), or vector, expressing the 23 S rRNA fragment 1192–1305 (●) or the 1235–1276 fragment (■) were grown overnight in LB medium containing ampicillin at 100 µg/ml. Samples (50 µl) of overnight cultures were inoculated into 2 ml LB medium containing ampicillin at 100 µg/ml, 1 mM IPTG and different concentration of erythromycin. Cultures were grown for 3.5 hours at 37°C with constant shaking, and the absorbance was measured at 600 nm.

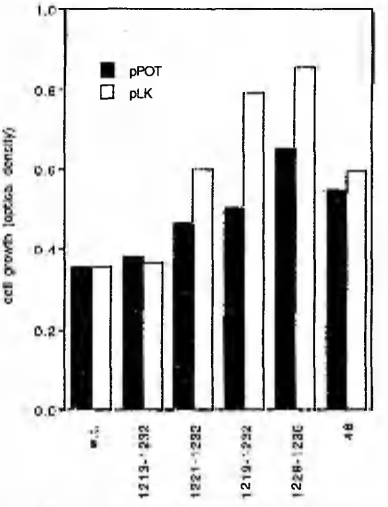

Figure 4. Growth of cells expressing complete mutant 23 S rRNA (open bars) or mutant rRNA fragments (black bars) in the presence of a subinhibitory concentration of erythromycin. Mutant 23 S rRNA was expressed from a multicopy pLK 55 plasmid under the control of lambda P_L promoter. Fragments from positions 1192 to 1305 were PCR amplified from wild-type (w.t.) and mutant 23 S rRNA alleles and introduced into the pPOT1 vector (Tenson *et al.*, 1996), where they were expressed from the tac promoter. Wild-type and mutant rRNA fragments were expressed in approximately the same amounts as revealed by Northern blotting. All the plasmids were propagated in JM109 cells (Sambrook *et al.*, 1989). Samples (10 µl) of overnight cultures were inoculated into 2 ml LB medium containing 100 µg/ml ampicillin and 1 mM IPTG with or without 100 µg/ml erythromycin. (Minimal inhibitory concentration of erythromycin for untransformed *E. coli* JM109 cells was 150 µg/ml). Cultures were grown for five hours at 37°C, and their absorbance at 600 nm was measured. Growth of the cells in the presence of erythromycin was normalized according to their growth in the absence of erythromycin.

Possibly, the mutant rRNA folds up differently and prevents access to the E-peptide ORF S.–D. or, alternatively, these deletions could enhance degradation of the rRNA. In spite of these exceptions, the effects of the majority of Ery^r point mutations and deletions can be explained by weakening the hairpin structure and making the S.–D. region of the E-peptide ORF more accessible.

Small rRNA fragments derived from mutant 23 S rRNA can confer erythromycin resistance

The mechanism of erythromycin resistance conferred by domain II mutations was originally thought to involve a change in the 23 S rRNA function within the ribosome (Douthwaite *et al.*, 1985). If this were the case, intact mutant 23 S rRNA would be required for assembly into functionally active ribosomes. However, if our new model is correct and the Ery^r effect of domain II mutations is mediated by translation of the E-peptide, the presence of the intact mutant 23 S rRNA would be unnecessary, and smaller rRNA fragments should be sufficient to confer resistance.

As the first step to discriminating between the two models, the erythromycin tolerance of cells expressing the wild-type 23 S rRNA fragment 1192–1305 (containing the complete 1200–1250 hairpin) was compared with the tolerance of cells expressing the fragment 1235–1276 where only 3' strand of the hairpin was present. These rRNA gene fragments were PCR amplified from the *E. coli rrn*B operon and introduced into the pPOT1 vector, where they were expressed under the control of IPTG-inducible *tac* promoter (Tenson *et al.*, 1996). The cells expressing the 1235–1276 rRNA fragment exhibited significantly lower resistance to erythromycin compared to the cells expressing 1192–1305 fragment (Figure 3). This again indicates that expression of the E-peptide is prevented when the 1200–1250 hairpin remains intact.

Next, gene fragments of the mutant 23 S rRNAs, containing the entire 1200–1250 hairpin and E-peptide ORF, were expressed *in vivo* from the *tac* promoter of the pPOT1 vector (Tenson *et al.*, 1996). Cell growth in the presence of subinhibitory concentrations of erythromycin was measured. For the five mutations tested, a close correlation was

observed between their ability to confer erythromycin resistance when expressed in the context of intact 23 S RNA and in the context of small rRNA fragments (Figure 4). These results demonstrate that the resistance determinant in the tested mutants is confined to the 23 S rRNA region containing the 1200–1250 hairpin and the adjacent E-peptide ORF, and thus provides further support to the hypothesis that erythromycin resistance is mediated by an increased expression of the E-peptide.

Conclusions

23 S RNA domain II deletions and point mutations that confer erythromycin resistance do so by a completely different mechanism than other Ery^r mutations located at the drug binding site in domain V. Ery^r mutations in domain II affect the stability of a hairpin in which the S.–D. region of the rRNA-encoded E-peptide ORF is sequestered. Since accumulation of the E-peptide affords erythromycin resistance (Tenson & Mankin, 1995; Tenson *et al.*, 1996), it is most probable that the effect of Ery^r mutations in domain II is to mediate activation of expression of the E-peptide, which is apparently not translated from the intact wild-type 23 S rRNA. Furthermore, it is possible that the peptide-mediated mechanism of erythromycin resistance accounts for Ery^r phenotypes of some clinical bacterial isolates.

Acknowledgements

Authors are grateful to A. DeBlasio for invaluable help and to P. Kloss for help in preparing the manuscript. This work was supported by NSF grant MCB 9420768 and Vahlteich Research Award (to A.S.M.), a Carlsberg Foundation scholarship (to M.D.) and grants from the Carlsberg Foundation and the Danish Natural Sciences Research Council (to S.D.)

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Edited by D. E. Draper

(Received 11 January 1996; received in revised form 27 February 1996; accepted 4 March 1996)

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Tenson, T., Xiong, L., Kloss, P., and Mankin, A. S. (1997) Erythromycin resistance peptides selected from random peptide libraries. J. Biol. Chem., 272: 17425–17430.

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 272, No. 28, Issue of July 11, pp. 17425–17430, 1997
Printed in U.S.A.

Erythromycin Resistance Peptides Selected from Random Peptide Libraries*

(Received for publication, April 10, 1997)

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Translation of a 5-codon mini-gene encoded in *Escherichia coli* 23 S rRNA was previously shown to render cells resistant to erythromycin (Tenson, T., DeBlasio, A., and Mankin, A. S. (1996) *Proc. Natl. Acad. Sci. U. S. A.* 93, 5641–5646). Erythromycin resistance was mediated by a specific interaction of the 23 S rRNA-encoded pentapeptide with the ribosome. In the present study, peptides conferring erythromycin resistance were selected from *in vivo* expressed random peptide libraries to study structural features important for peptide activity. Screening of a 21-codon mini-gene library (the general structure ATG $(NNN)_{20}$ TAA) demonstrated that only short peptides (3–6 amino acids long) conferred erythromycin resistance. Sequence comparison of erythromycin resistance peptides isolated from the 5-codon library (ATG $(NNN)_4$ TAA) revealed a strong preference for leucine or isoleucine as a third amino acid and a hydrophobic amino acid at the C terminus of the peptide. When tested against other antibiotics, erythromycin resistance peptides rendered cells resistant to other macrolides, oleandomycin and spiramycin, but not to chloramphenicol or clindamycin. Defining the consensus amino acid sequence of erythromycin resistance peptides provided insights into a possible mode of peptide action and the nature of the peptide binding site on the ribosome.

It was assumed for a long time that the ribosome is indifferent to the sequence of the polypeptide it is synthesizing. New evidence, however, indicates that nascent or newly synthesized polypeptides can affect functions of the ribosome in *cis*. In a number of cases, the newly translated peptide exerts its effect on translation while still being located within the ribosome. For example, short nascent peptides regulate stalling of the ribosome on mRNA, which is required for inducing the expression of chloramphenicol resistance (*cat* and *cmlA*) and erythromycin resistance (*erm*) genes (1, 2). Ribosome stalling depends on the amino acid sequence of the nascent peptide rather than on the nucleotide sequence of mRNA and occurs when the nascent peptide is only several amino acids long and should be located within the ribosome. Other examples include: translational bypass of the coding gap in bacteriophage T4 gene *60* mRNA (ribosome "hopping"), which depends on the amino acid sequence of the nascent peptide (3); dependence of termination efficiency on the amino acid sequence of the nascent peptide (4); attenuation of eukaryotic gene expression by short upstream open reading frames, which depends on the encoded amino acid sequences but not the mRNA sequence; and others (5–7). Despite a growing number of cases where *cis*-action of the newly synthesized peptide on the ribosome has been either demonstrated or suspected, almost nothing is known about molecular mechanisms of interaction between the ribosome and regulatory *cis*-acting peptides. The nature and location of the peptide-responsive site remains obscure.

A new example of a *cis*-acting peptide is represented by a pentapeptide encoded in *Escherichia coli* 23 S rRNA (6). It was demonstrated that production in *E. coli* cells of a 34-nucleotide-long segment of 23 S rRNA, positions 1235–1268 (8), renders cells resistant to the ribosome-targeted antibiotic erythromycin (8, 9). Curiously, erythromycin resistance was mediated by translation of a pentapeptide (E-peptide)[1] encoded in the rRNA fragment. Mutations that affected translation initiation signals of the E-peptide mini-gene (Shine-Dalgarno region and initiator GUG codon) abolished erythromycin resistance. Interestingly, mutations at the terminator UAA codon, as well as some missense mutations, also interfered with peptide activity, suggesting that the size of the peptide and its amino acid sequence are essential for its functions. Translation of the E-peptide mRNA in the cell-free system rendered ribosomes resistant to erythromycin. However, addition of the synthetic E-peptide to the translating ribosome *in vitro* did not confer any erythromycin resistance (8). Thus, it appears that the E-peptide acts in *cis* so that only the ribosome on which the peptide has been translated becomes resistant to the drug. The single binding site of erythromycin is located on the large ribosomal subunit in the vicinity of the peptidyltransferase center. Accordingly, one possible mechanism of the E-peptide action is that the newly translated peptide remains bound to the ribosome and occupies the erythromycin binding site, thus preventing drug binding. However, fundamental questions of how and where such interaction may occur remains unanswered. This is due in part to a lack of information about structural features of the E-peptide that are important for its function.

To gain a better understanding of the size and sequence requirements for E-peptide activity, a library of *in vivo* expressed random peptides was constructed from which a collection of erythromycin resistance peptides was isolated. Comparison of their sizes and sequences revealed structural features that are important for the activity of erythromycin resistance peptides and their interaction with ribosome.

* This work was supported by National Institutes of Health Grant GM53762. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: E-peptide, erythromycin resistance pentapeptide; PCR, polymerase chain reaction; IPTG, isopropyl-1-thio-β-D-galactopyranoside; MIC, minimal inhibitory concentration.

EXPERIMENTAL PROCEDURES

Strains and Materials—*E. coli* JM109 strain (10) (*endA*1, *recA*1, *gyrA*96, *thi*, *hsdR*17 (r_K^-, m_K^+), *relA*1, *supE*44, Δ(*lac-proAB*), [F', *traD*36, *proAB*, $lacI^qZ$DM15] was used for most of the cloning experiments. For the library construction, the ligation mixtures were originally transformed into ultracompetent *E. coli* cells XL2-Blue MRF' (Δ(*mcrA*)183, Δ(*mcrCB-hsdSMR-mrr*)173, *endA*1, *sup*E44, *thi*-1, *recA*1, *gyrA*96, *rel*A1, *lac* [F' *proAB*, $lacI^q$, ZΔM15, Tn*10*(tet^r)] Amy, Cam^r) (Stratagene). Synthetic oligonucleotides were from DNAgency. Enzymes were from Promega and New England BioLabs. Chemicals and antibiotics were from Fisher or Sigma.

Library Construction—pPOT1 vector described previously (8) was used for the construction of random peptide libraries. The original vector, which contains a P*tac* promoter-T*trp* terminator expression cassette with a single *Nhe*I cloning site, was modified by introducing sites for restriction nucleases *Eco*RI and *Afl*II between the P*tac* promoter and *trp* terminator. The resulting vector, pPOT1AE (see Fig. 1), was cut with *Eco*RI and *Afl*II, and the linear plasmid was gel-purified.

Random peptide mini-gene DNA was prepared from synthetic oligonucleotides d(GGCTTAAGGAGGTCACATATG$(N)_{19}$TAACTAGCTGAATTCCG) or d(GGCTTAAGGAGGTCACATATG$(N)_{60}$TAACTAGCTGAATTCCG). The oligonucleotides were PCR-amplified from a pair of primers, d(CGGAATTCAGCTAGTTA) and d(GGCTTAAGGAGGTCAC). The PCR products were cut with *Eco*RI and *Afl*II, gel purified, and ligated with linearized pPOT1AE vector.

Plasmid libraries were transformed into *E. coli* XL2-Blue MRF' ultracompetent cells (Stratagene). An aliquot of transformed cells from each library was plated onto agar plates to estimate the number of clones in each library, whereas the rest of the cells were grown in 100 ml of LB medium containing 100 μg/ml ampicillin. When culture densities reached A_{600} = 0.8, cells were harvested, and plasmid libraries were isolated. The 5- and 21-codon libraries contained ~5 × 10^5 and ~1 × 10^6 clones, respectively.

Selection of Erythromycin Resistance Peptides—*E. coli* JM109 competent cells were transformed with the random mini-gene plasmid libraries and plated onto LB agar plates containing 100 μg/ml ampicillin, 150 μg/ml erythromycin, and 1 mM IPTG. Plates were incubated overnight at 37 °C. Colonies that appeared on plates were streaked on plates containing 100 μg/ml ampicillin and 150 μg/ml erythromycin or 100 μg/ml ampicillin, 150 μg/ml erythromycin, and 1 mM IPTG. Colonies growing in the presence but not in the absence of IPTG were taken for further analysis. Plasmids were isolated from all selected clones and retransformed into fresh competent cells, and phenotypes of the secondary transformants were checked by replica plating onto ampicillin/erythromycin or ampicillin/erythromycin/IPTG plates. Peptide minigenes from the plasmids conferring retransformable IPTG-dependent erythromycin resistance were sequenced.

Selection of clones resistant to a higher concentration of erythromycin was performed in essentially the same way except that the selective plate contained 1 mg/ml instead of 150 μg/ml erythromycin.

Comparing Erythromycin Resistance of Cells Expressing Different E-peptides—Overnight cultures of cells expressing different E-peptides were grown in LB medium containing 100 μg/ml ampicillin. Cultures were diluted with LB medium containing 100 μg/ml ampicillin and 2 mM IPTG to the final density of A_{650} = 0.005. 3 ml of each culture was placed into two 15-ml tubes, and tubes were incubated at 37° C with constant shaking. After a 1 h incubation, 10 μl of erythromycin solution (30 mg/ml) was added to one of the two tubes in each parallel trial, and cells were grown until the optical density of the control cultures reached ~A_{650} = 1. At this time, optical densities of all cultures were measured. Absorbance of cultures grown in the presence of erythromycin was divided by the absorbance of cultures grown in the absence of erythromycin, and the results were plotted.

Testing Antibiotic Resistance of E-peptide-expressing Cells—Overnight cultures were grown from cells transformed either with the empty pPOT1AE vector, a plasmid isolated from a randomly picked unselected clone expressing pentapeptide MDVEQ or a plasmid from Ery^r clone expressing E-peptide MSLKV. Cultures grown in LB medium containing 50 μg/ml ampicillin were diluted with fresh medium containing 50 μg/ml ampicillin and 1 mM IPTG to A_{600} = 0.008. Erythromycin, oleandomycin, spiramycin, chloramphenicol, or clindamycin was then added to concentrations of 100, 1000, 200, 1, and 50 μg/ml, respectively. Cultures were grown until optical density of the control culture, grown only in the presence of ampicillin and IPTG, reached A_{600} = 1. At this time, optical densities of all cultures were measured and normalized relative to the control culture.

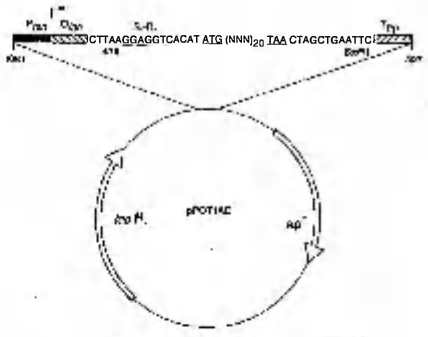
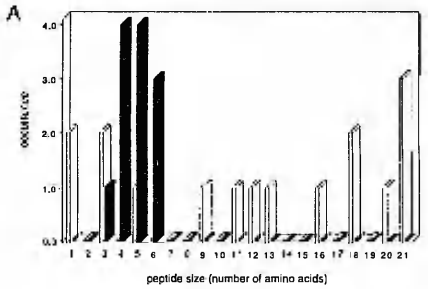


FIG. 1. **A 21-codon mini-gene library in pPOT1AE vector.** pPOT1AE is identical to the pPOT1 vector described previously (8) except that *Afl*II and *Eco*RI cloning sites were introduced into a single cloning *Nhe*I site of pPOT1. IPTG-inducible P*tac* promoter is shown as a *black bar*, and the *lac* operator (*Olac*) and trp terminator (*Ttrp*) are shown as *hatched bars*. The transcription start site is indicated by an *arrow*. *Afl*II and *Eco*RI sites used for cloning of the mini-gene library are shown. The Shine-Dalgarno sequence (*S.-D.*), initiator AUG codon, and terminator UAA codon of the mini-gene are *underlined*. Positions of β-lactamase gene (Ap^r) and lac I^q genes in the plasmid are shown by *open arrows*.



B

M C F M K L
M V L R L G
M I V K V G
M V W T L
M A W I V
M R L A I
M F I I S
M I M L
M I K S
M L R T
M M H V
M R T

FIG. 2. **Size distribution and amino acid sequences of erythromycin resistance peptides isolated from the 21-codon library.** *A*, distribution of peptide sizes (number of amino acids) encoded in minigenes in randomly picked unselected clones (*open bars*) and erythromycin-resistant clones (*shaded bars*). The y axis represents the number of sequenced clones that encoded peptides of a particular size. *B*, amino acid sequences of peptides encoded in mini-genes in erythromycin-resistant clones. Peptide sequences are aligned relative to the C-terminal amino acid.

Construction of Random Mini-gene Libraries—Two random mini-gene plasmid libraries were constructed for isolation of peptides whose expression renders cells resistant to erythromycin. Random mini-genes were generated by PCR amplification of oligonucleotides containing initiator and terminator codons separated by 12 (for 5-codon library) or 60 (for 21-codon library) random nucleotides (Fig. 1). The initiator codon was preceded by an optimized Shine-Dalgarno sequence (11, 12) to ensure efficient translation of the mini-gene. The PCR-amplified mini-gene library was introduced unidirectionally in the pPOT1AE vector (8), where transcription of the mini-gene was controlled by a strong IPTG-inducible *Ptac* promoter. Sequencing mini-genes from a number of randomly picked unselected clones from both libraries showed no significant bias in nucleotide composition in the randomized segment of the mini-gene. The 5-codon library contained ~500,000 clones. Since the total number of various pentapeptides (with fixed methionine in the first position) is 20^4 = 160,000, it was assumed that most possible pentapeptides were encoded in the 5-codon library. The 21-codon library had ~10^5 clones. Naturally, only a relatively small segment of the sequence space corresponding to all possible peptides encoded in 21-codon-long open reading frames were represented in this library.

Isolation of Erythromycin-resistant Clones from 21-codon Random Mini-gene Library—Due to an occasional presence of stop codons in a random open reading frame, the 21-codon library can encode peptides ranging in size from 1 to 21 amino acids. This library was used primarily to determine the predominant size of erythromycin resistance peptides. Clones that became erythromycin-resistant due to expression of peptide mini-genes were selected by plating the 21-codon library on agar medium containing ampicillin, erythromycin, and IPTG. For most clones that appeared on the plate, the Ery^r phenotype was retransformable with the plasmid and depended on the presence of IPTG in the medium, indicating that expression of the peptide mini-gene was necessary for the resistance. Plasmids from 12 Ery^r clones were sequenced alongside of plasmids isolated from several unselected, randomly picked clones. Whereas mini-genes in unselected clones showed a broad distribution of sizes of the encoded peptides (*open bars* in Fig. 2), the mini-genes in 12 isolated Ery^r clones encoded only short peptides in a very narrow range of sizes, from 3 to 6 amino acids (*filled bars* in Fig. 2). Thus, it appears that only short peptides can confer resistance to erythromycin.

Erythromycin Resistance Peptides from 5-codon Mini-gene Library—The experiment with the 21-codon library showed that expression of predominantly short peptides can render cells erythromycin resistance. Furthermore, the first described erythromycin resistance peptide (E-peptide) is encoded in a 5-codon-long open reading frame in the *E. coli* 23 S rRNA (8). Therefore, the next selection and all subsequent experiments were done with a plasmid library where random mini-genes contained only 5 codons. By analogy with the rRNA-encoded E-peptide, the erythromycin resistance pentapeptides selected from the library are referred to as E-peptides.

More than 100 Ery^r clones were selected from the 5-codon library on ampicillin/IPTG plates containing 150 μg/ml erythromycin. The relation of the Ery^r phenotype to the expression of plasmid-encoded peptides was confirmed by IPTG-dependence of erythromycin resistance and by its co-transference with the plasmid. Peptide mini-genes from >50 Ery^r clones were sequenced. Only 1 of these clones had an in-frame stop codon in the mini-gene that coded for a tetrapeptide MILV; pentapeptides were encoded in all the rest of the clones. Sequences of E-peptides expressed in Ery^r clones showed significant deviation from sequences of peptides from unselected clones (Fig. 3, *A* and *C*) and exhibited a clear preference for certain amino acids in positions three and five. More than two-thirds of selected peptides had either leucine or isoleucine in the third position, and most E-peptides had a hydrophobic amino acid, predominantly valine, at the C terminus. A similar trend was observed by S. Douthwaite, who isolated erythromycin resistance peptides from a slightly different library.[2] Altogether, Leu/Ile in the third position or a nonpolar amino acid in the fifth position could be found in 48 out of 52 E-peptide sequences. Only four of the E-peptides isolated from the library, MVQLR, MNWKR, MINQT, MYMLT, together with the rRNA-encoded E-peptide, MRMLT, do not conform to the consensus, though three of these peptides, MVQLR, MYMLT and MRMLT, have Leu in the fourth position, which might possibly compensate for the lack of Leu or Ile in the third position.

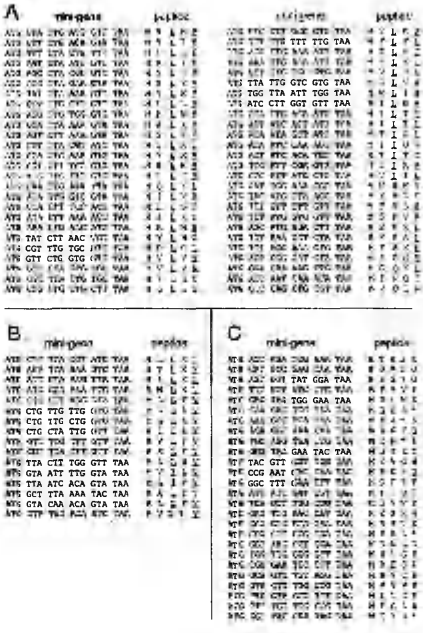


FIG. 3. **Nucleotide sequences of mini-genes and amino acid sequences of the encoded peptides expressed in erythromycin-resistant clones isolated on plates with 150 μg/ml erythromycin (*A*), 1 mg/ml erythromycin (*B*), and randomly picked unselected clones (*C*) from the 5-codon library.** Conserved Leu and Ile in the third position and hydrophobic amino acids in the C-terminal position of the peptide sequence are *underlined*. *Asterisks* in the peptide sequences correspond to stop codons in peptide mini-genes.

The relative efficiency of various E-peptides was assessed by comparing growth of the clones in liquid culture in the presence of a subinhibitory concentration of erythromycin (Fig. 4). A good correlation was observed between growth rate of clones in the presence of the drug and the presence of Leu or Ile in the third position and a hydrophobic amino acid at the C terminus

[2] S. Douthwaite, personal communication.

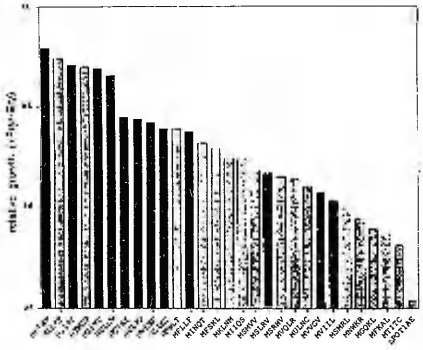


FIG. 4. **Growth of clones expressing various E-peptides in the presence of a subinhibitory concentration of erythromycin.** The relative growth is expressed as a ratio of optical density (at 650 nm) of cultures grown in the presence of 100 μg/ml ampicillin, 2 mM IPTG, and 100 μg/ml erythromycin to the optical density of cultures grown in the presence of only ampicillin and IPTG (see "Experimental Procedures" for details). The amino acid sequences of peptides expressed in the clones is shown at the *bottom*. *Black bars* correspond to peptides having Leu or Ile in the third position and a hydrophobic amino acid at the C terminus. The peptide MRMLT was encoded in a 34-nucleotide fragment of the 23 S rRNA and was expressed from the pPOT1 vector (8). The *bar* at the very *right* shows the growth of cells transformed with an empty pPOT1AE vector.

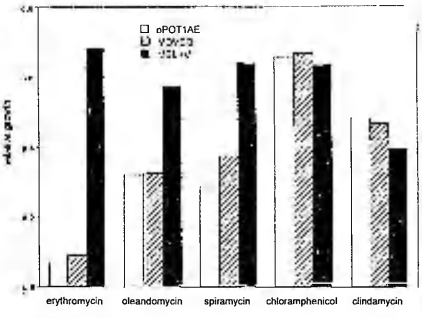


FIG. 5. **Sensitivity of cells transformed with an empty vector (*open bars*), an unselected plasmid-encoding peptide MDVEQ (*striped bars*), and a plasmid isolated from Ery^r cells expressing E-peptide MSLKV (*black bars*) to different antibiotics.** Sensitivity is expressed as a ratio of optical density (at 650 nm) of cultures grown in the presence of 100 μg/ml erythromycin, 1 mg/ml oleandomycin, 200 μg/ml spiramycin, 1 μg/ml chloramphenicol, or 50 μg/ml clindamycin to cultures grown in the absence of the drug. All cultures nevertheless contained 100 μg/ml ampicillin and 2 mM IPTG.

of the peptide. Peptides that have both of these features group at the top of the activity histogram on Fig. 4. Of the 10 clones that show the best growth in the presence of erythromycin, 8 peptides conform to this rule. Conversely, peptides with lower activity tend to lack either Leu or Ile in the third position or a hydrophobic amino acid at the C terminus. Thus, the nature of these two positions in the E-peptide structure appear to be important for peptide activity.

This conclusion was further corroborated when Ery^r clones were selected on ampicillin/IPTG plates containing very high concentrations of erythromycin (1 mg/ml). From 16 different mini-gene sequences found in such clones, 15 encoded pentapeptides that had Leu or Ile in the third position; 14 such mini-genes also encoded a hydrophobic amino acid (predominantly Val) in the fifth position (Fig. 3*B*).

To rule out a possible strain specificity of E-peptide action, the effect of one of the E-peptides, MSLKV (Fig. 4), was compared in three *E. coli* strains differing in their sensitivity to erythromycin. Erythromycin-supersensitive strain DB10 (13) (erythromycin MIC 1 μg/ml), wild type strain MRE600 (14) (MIC 8 μg/ml), and intrinsically erythromycin-tolerant JM109 (10) (MIC 100 μg/ml) were transformed with the plasmid coding for the MSLKV E-peptide. As a control, all strains were transformed with plasmid isolated from a randomly picked, unselected clone coding for peptide MDVEQ. Transformation with the control plasmid did not change erythromycin sensitivity of any of the strains, whereas expression of the E-peptide in any of the three strains increased erythromycin MIC 3–4-fold (data not shown). Thus, E-peptide can confer erythromycin resistance in different *E. coli* strains.

Resistance to Different Antibiotics—To investigate whether E-peptide expression affects sensitivity to antibiotics other than erythromycin, cells transformed with the plasmid coding for the E-peptide MSLKV were grown in the presence of several antibiotics known to interact with the large ribosomal subunit (Fig. 5). Expression of the E-peptide increased cell resistance not only to erythromycin but also to two other macrolide antibiotics, spiramycin and oleandomycin, whereas sensitivity to chloramphenicol and clindamycin was not affected.

DISCUSSION

In the present study we asked the question, Which properties of a peptide make possible its functional interaction with the ribosome resulting in resistance to erythromycin? To answer this question, we used random mini-gene libraries for isolation of a variety of erythromycin resistance peptides. The use of mini-gene expression libraries has a number of advantages compared with the other combinatorial methods exploiting libraries of synthetic peptides (15) or phage display libraries (16). First, it is much easier to synthesize a random DNA sequence of the peptide gene than a random amino acid sequence of the peptide itself, leading to better representation of a random peptide sequence space in a mini-gene library. Second, phenotypic selection permits not only screening of hundreds of thousands of peptide sequences in a single experiment but also amplification of the "signal" (the selected sequences) by growing cells that passed the selection. Third, in contrast to phage display libraries where a random amino acid sequence is expressed as a segment of a larger protein, the mini-gene library peptides are expressed in their free form, which can be critical for assessing functionality of the peptide. Because of these advantages, random mini-gene libraries can be used for isolation of different functional peptides including enzyme cofactors, inhibitors, etc.

In our experiments, a number of clones expressing erythromycin resistance peptides were isolated from 21- and 5-codon libraries. Comparison of peptide sequences allowed us to draw the first conclusions about the sequence and size requirements for peptide activity. Thus, screening of the 21-codon library primarily revealed the preferred size of erythromycin resistance peptides. Each of the random codons in the library minigene can be either 1 out of a possible 61 sense codons or 1 of the 3 stop codons. The probability that, out of 20 random codons, none will be a terminator codon is $(61/64)^{20} = 0.38$; therefore, about two-thirds of the clones in the 21-codon library are expected to have in-frame stop codons. Indeed, as expected, a broad distribution of sizes of the encoded peptides were found

in unselected, randomly picked clones. In contrast, the majority of peptides expressed in Ery^r clones fell within an amazingly narrow size range; 11 out of 12 peptides were 4, 5, or 6 amino acids long. Though it is possible that more extensive screening could reveal some functional peptides larger than hexapeptides, this experiment showed a clear tendency of erythromycin resistance peptides to be 4–6 amino acids long. In agreement with this finding, the originally described rRNA-encoded E-peptide was 5 amino acids long (8).

Previously it had been demonstrated that any mutation eliminating the stop codon of the rRNA-encoded E-peptide abolished erythromycin resistance (8). This showed that a mere presence of the E-peptide sequence at the N terminus of a longer polypeptide could not render ribosomes resistant to erythromycin. The results of screening a 21-codon library not only confirmed this observation but also indicated that the E-peptide sequence is not functional when present at the C terminus of a longer oligopeptide (otherwise we could isolate clones coding for long peptides where a critical sequence would be located close to the C terminus). Thus, we can conclude that an erythromycin resistance peptide cannot be part of a longer protein and that the size of the peptide is essential for its activity. The strict size limitation may mean that the peptide binding site cannot accommodate a longer polypeptide.

If analysis of clones isolated from the 21-codon library revealed peptide size preference, then screening the 5-codon library provided clues to the sequence features that are important for E-peptide activity. Comparison of pentapeptide sequences found in Ery^r clones selected at 150 μg/ml erythromycin showed a strong tendency of E-peptides to have Leu or Ile in the third position and a hydrophobic amino acid in the C-terminal position. Not only did these sequence signatures appear in the majority of isolated E-peptides (Fig. 3*A*), but there is also a correlation between the degree of peptide activity and the presence of Leu or Ile in the third position and a hydrophobic amino acid at the C terminus (Fig. 4). Peptides expressed in clones growing at a very high concentration of erythromycin (1 mg/ml) show even stronger selectivity at positions 3 and 5; most of such peptides (with only one exception) have Leu or Ile in the third position, and all peptides but one have a hydrophobic amino acid, most commonly Val, at the C terminus (Fig. 3*B*). In addition, peptides expressed in the highly resistant cells frequently have hydrophobic amino acids at the second and fourth positions: 14 out of 16 clones resistant to 1 mg/ml erythromycin express peptides with a hydrophobic amino acid in the second position, and in 8 of these peptides, a hydrophobic amino acid is present also at the fourth position. As a result, most of the peptides isolated from highly resistant clones are very hydrophobic, suggesting that the peptide binding site is also of a hydrophobic nature and presumably not exposed to the solvent.

The ribosome appears to be the primary target of action of E-peptides since translation of the E-peptide mRNA *in vitro* rendered ribosomes resistant to erythromycin (8). At the same time, synthetic E-peptide did not affect sensitivity of the cell-free translation system to erythromycin. This led to a hypothesis that E-peptide enters the site of its action co-translationally and acts in *cis*, affecting properties only of that ribosome on which it has been translated. The simplest way in which E-peptide can render the ribosome resistant to erythromycin is by direct blocking of the drug binding site on the ribosome. This hypothesis is in a good agreement with the known mode of erythromycin action and the *cis* nature of the E-peptide effect. Erythromycin interacts with a vacant ribosome in the vicinity of the peptidyltransferase center and inhibits protein synthesis by sterically hindering growth of the nascent peptide (17). *In*

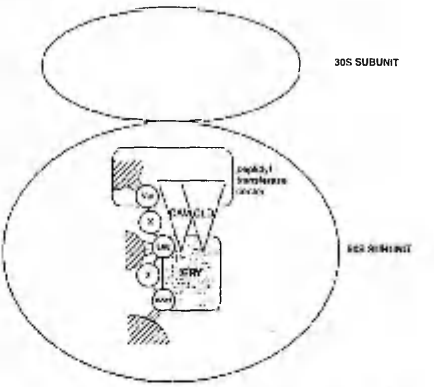


FIG. 6. **Proposed model of E-peptide action.** Erythromycin binding site (*ERY*) is shown in *gray*, and the binding sites of chloramphenicol (*CAM*) and clindamycin (*CLD*) are shown as *open triangles*. The third position of the peptide, commonly represented by Leu (as shown in the figure) or Ile, is assumed to overlap with the erythromycin binding site. The conserved amino acids (N-terminal formyl methionine, the third Leu or Ile, and C-terminal hydrophobic amino acid commonly represented by Val, as shown in the figure) may form specific contacts with rRNA or ribosomal proteins.

vitro, the antibiotic does not inhibit formation of the first peptide bond, but it can inhibit the peptidyltransferase reaction when the donor substrate becomes 2 or more amino acids long (7, 18); nascent peptide chains longer than 2–5 amino acids (depending on the nature of polymerized amino acids) prevent erythromycin binding (19–21). Therefore, in the cell, erythromycin can bind only to the vacant ribosome that has already released a newly synthesized protein but before several amino acids of a newly initiated protein are polymerized. If translated E-peptide does not leave the ribosome and remains tightly bound, the erythromycin binding site will be blocked, and the ribosome will be immune to erythromycin. A newly initiated nascent peptide may possibly go "around" the bound E-peptide or, alternatively, displace it.

A model of E-peptide-ribosome interaction is shown in Fig. 6. The binding site of E-peptide is located most probably in the large ribosomal subunit, in or immediately near the nascent peptide channel, and overlaps with the erythromycin binding site (shown in *shading* in Fig. 6). Erythromycin starts to inhibit protein synthesis at a step when the third amino acid is added to the growing nascent peptide (22); therefore, if the C-terminal peptide residue is positioned in the ribosomal P-site, then the third residue from the C terminus would be located very close to the hypothetical erythromycin binding site. The bulky hydrophobic side chain of leucine or isoleucine may interfere with interaction of erythromycin with its binding site in the vicinity of the peptidyltransferase center. The peptide may enter its binding site co-translationally from the side of the peptidyltransferase center; this would explain a *cis*-mode of E-peptide action. E-peptide binding is probably stabilized by the interaction of the essential amino acids with the ribosome components, rRNA or proteins. Three amino acid positions in the peptide appear to be primarily important. Besides Leu or Ile in the third position and a hydrophobic residue at the C terminus, the N-terminal formyl methionine may be also critical for peptide binding. Though importance of fMet is difficult to assess since by default it is present in all library-coded E-peptides, the

fact that E-peptide cannot be part of a longer protein suggests that the position or formylation of the N-terminal methionine is crucial for peptide activity.

Expression of E-peptide rendered cells resistant to other macrolide antibiotics: oleandomycin, which is similar to erythromycin and has a 14-atom lactone ring, and spiramycin, a macrolide with a 16-atom ring. At the same time, E-peptide did not affect cell sensitivity to structurally different chloramphenicol and clindamycin. All drugs tested compete for binding to the ribosome (23); however, the binding sites of chloramphenicol and clindamycin do not precisely coincide with the binding site of macrolides, as demonstrated by RNA footprinting and the difference in the mode of action of these drugs (17, 24, 25). Thus, the site of E-peptide action probably overlaps specifically with the binding site of macrolides but not with that of other antibiotics interacting with the ribosome in the vicinity of the peptidyltransferase center.

In the proposed model, E-peptide is assumed to interact with the large ribosomal subunit in the vicinity of the peptidyltransferase center (Fig. 6). A similar site of action was proposed for the *cis*-acting peptides regulating expression of *erm*, *cat*, and *cmlA* antibiotic-resistant genes (2, 26). These peptides, acting in a form of peptidyl tRNA, cause ribosome stalling on mRNA in the presence of low, noninhibitory concentrations of erythromycin (27) or chloramphenicol (28). It is conceivable that erythromycin resistance E-peptides and regulatory *cis*-acting peptides may utilize a basically similar mechanism where tight binding of a peptide to the ribosome in the vicinity of the peptidyltransferase center causes erythromycin resistance in the case of E-peptide or ribosome stalling in the case of regulatory peptides of *erm*, *cat*, and *cmlA* genes. The lack of apparent similarity between the consensus sequence of E-peptide and sequences of other *cis*-acting peptides may be related to the fact that stalling peptides become active only in the presence of low concentrations of chloramphenicol or erythromycin. Application of a random library approach, which proved useful in the E-peptide studies, may provide insights into functionally important features of other *cis*-acting peptides and may eventually lead to a better understanding of how the ribosome "talks" to the protein it is synthesizing.

Acknowledgments—We thank Drs. B. Weisblum (University of Wisconsin), P. Lovett (University of Maryland), J. Menninger (University of Iowa), and L. Katz (Abbott Laboratories) for stimulating discussions, Dr. Douthwaite (University of Odense) for communicating his unpublished results, and Dr. J.-P. Pernodet (University of Orsay) for providing spiramycin.

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ISSN 1024–6479
ISBN 9985–56–263–1