1420–1425 *Nucleic Acids Research, 2001, Vol. 29, No. 7*

Enhancement of translation by the epsilon element is independent of the sequence of the 460 region of 16S rRNA

Michael O'Connor* and Albert E. Dahlberg

J. W. Wilson Laboratory, Department of Molecular and Cellular Biology and Biochemistry, Brown University, 69 Brown Street, Providence, RI 02912, USA

Received January 8, 2001; Accepted February 9, 2001

ABSTRACT

The epsilon enhancer element is a pyrimidine-rich sequence that increases expression of T7 gene 10 and a number of *Escherichia coli* mRNAs during initiation of translation and inhibits expression of the *recF* mRNA during elongation. Based on its complementarity to the 460 region of 16S rRNA, it has been proposed that epsilon exerts its enhancer activity by base pairing to this complementary rRNA sequence. We have tested this model of enhancer action by constructing mutations in the 460 region of 16S rRNA and examining expression of epsilon-containing CAT reporter genes and *recF–lacZ* fusions in strains expressing the mutant rRNAs. Replacement of the 460 *E.coli* stem–loop with that of *Salmonella enterica* serovar Typhimurium or a stem–loop containing a reversal of all 8 bp in the helical region produced fully functional rRNAs with no apparent effect on cell growth or expression of any epsilon-containing mRNA. Our experiments confirm the reported effects of the epsilon elements on gene expression but show that these effects are independent of the sequence of the 460 region of 16S rRNA, indicating that epsilon–rRNA base pairing does not occur.

INTRODUCTION

Base pairing between the Shine–Dalgarno (SD) sequence in the 5′ leader regions of most bacterial mRNAs and the 3′ end of 16S rRNA (antiSD) is a critical step in the initiation of translation. This mRNA–ribosome interaction serves to increase the local concentration of ribosomes near the initiation codon (1) and is a major control point in gene expression. However, several bacterial mRNAs lack recognizable SD sequences and mutagenesis experiments of particular mRNAs have also identified other sequence elements both in the 5′ leader and coding regions that are important for controlling initiation efficiency. Moreover, several heterologous mRNAs are translated efficiently in *Escherichia coli* in the absence of any SD–antiSD interaction. In attempting to provide a mechanism for understanding these atypical initiation events both in bacterial and mammalian cells, many rRNA–mRNA base pairing schemes have been invoked that involve other regions of 16S rRNA (reviewed in 2).

The epsilon sequence element was first identified in the phage T7 gene 10 mRNA (3) and was also found to enhance the translational activity of a *lacZ* reporter gene construct. Based on the observation that epsilon was partially complementary to the 460 stem–loop (Fig. 1, helix 17) of 16S rRNA, a model was proposed where epsilon enhanced translation by base pairing to this complementary region of 16S rRNA, the so-called anti-epsilon sequence. Some of the predictions of this model were confirmed by Golshani *et al.* (4) who constructed a series of chloramphenicol acetyltransferase (CAT) reporter genes containing translational initiation regions of varying levels of complementarity to the 460 stem–loop, and who observed that while the original epsilon element enhanced the translation only of mRNAs containing canonical SD sequences (3,4), a CAT construct with an extended epsilon element was able to direct efficient initiation in the absence of any SD element.

Analysis of the elements limiting *recF* expression in *E.coli* identified a sequence similar to epsilon located ~80 nt downstream of the initiation codon, leading to the proposal that helix 17 interacted with initiating and elongating ribosomes (5).

rRNA–mRNA base pairing models, particularly those requiring simultaneous interaction of the mRNA with both the antiSD and other complementary sequence(s) on the rRNA (3,4,6) place considerable constraints on the orientation of mRNA through the ribosome. Despite the support for the epsilon–helix 17 base pairing model from gene expression studies, no mRNA–rRNA crosslinks have been obtained that involve the 460 region (7) Consequently, we have tested this base pairing model by constructing mutations in the 16S rRNA that are predicted to alter radically the base pairing potential of helix 17 with the epsilon enhancer. Our findings are that reversal of all 8 bp in the 460 stem or replacement of the *E.coli* sequence with that of *Salmonella enterica* serovar Typhimurium had no effect on cell growth or expression of reporter gene constructs containing a variety of different epsilon elements. We conclude that while epsilon has considerable influence on translational efficiency, this does not involve a base pairing interaction with the 460 region of 16S rRNA.

*To whom correspondence should be addressed. Tel: +1 401 863 3652; Fax: +1 401 863 1182; Email: michael_o'connor@brown.edu

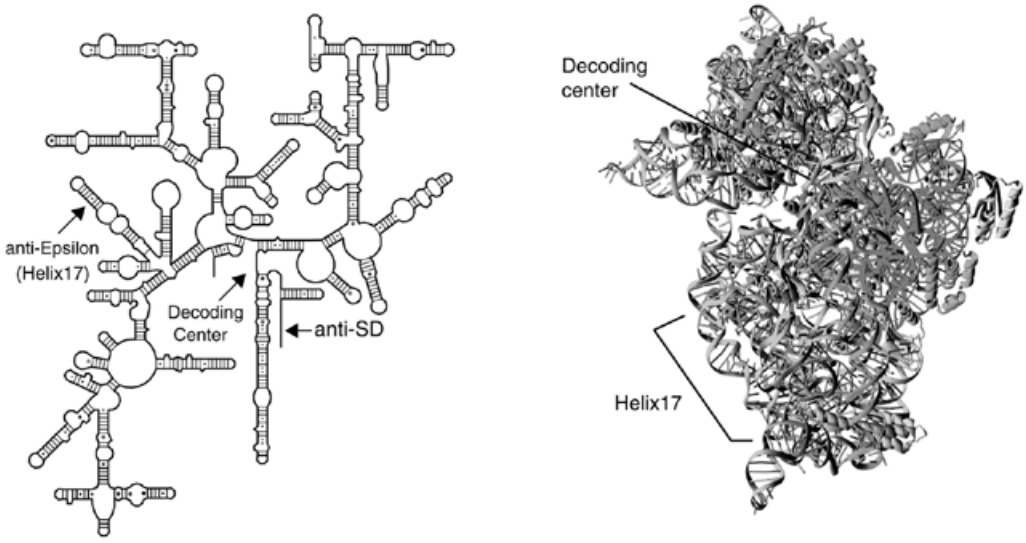

Figure 1. The secondary (left) and tertiary (right) (19) structures of bacterial 16S rRNA with the regions of interest indicated.

Figure 2. Secondary structures of the 460 stem–loop from *E.coli* (left), *S.enterica* serovar Typhimurium (middle) and a mutant containing a reversal of all 8 bp of the *E.coli* stem (right). Nucleotides differing from the *E.coli* wild-type sequence are indicated in bold.

MATERIALS AND METHODS

Bacterial strains and plasmids

Plasmid pMO10 (8) contains an intact wild-type *rrnB* operon and was used to express rRNA in the Δ7 prrn strains. pMOSal460 and pMO460flip are derived from pMO10 and contain the sequence of the *S.enterica* serovar Typhimurium 460 stem–loop or a reversal of all 8 bp in the 460 stem, respectively (Fig. 2). Plasmids pMO28 and pMO30 are derived from pACYC177 (9); pMO28 contains an intact *rrnB* operon and the C1192U spectinomycin resistance mutation while pMO30 contains both the C1192U and 460 Flip rRNA mutations. Growth of strains expressing these rRNAs in the presence of spectinomycin ensures that only plasmid-encoded, spectinomycin-resistant ribosomes are active in translation. TA488 (Δ*rrnE* Δ*rrnB* Δ*rrnG*::*lacZ*$^+$ Δ*rrnH* Δ*rrnA*) carries deletions in five *rrn* operons (10) and was used as a host for the pMO28 and pMO30 plasmids together with the CAT reporter gene plasmids. MC230 is Δ*rrnE* Δ*rrnB* Δ*rrnH* Δ*rrnG*::*cat* Δ*rrnA* Δ*rrnD*::*cat* Δ*rrnC*::*cat recA56*/ pts1192U p70 (11) and was used as a host for pMO10-derived *rrnB* plasmids together with pBR322-derived *recF–lacZ* fusion plasmids. The spectinomycin-resistant *rrnB*-containing plasmid, pts1192U, in MC230 is derived from pSC101 and is incompatible with pMO10-type plasmids. All neomycin-resistant transformants obtained after transformation of MC230 with pMO10-derived plasmids were spectinomycin sensitive. Displacement of pts1192U by pMO10 and its derivatives in MC230 was confirmed by primer extension on total RNA extracted from these strains using the method of Sigmund *et al.* (12) using the oligonucleotide 5′-GCTT-CTTCTGCGGGTAACGTCAAT-3′ complementary to bases 501–478 of 16S rRNA.

The *recF–lacZ* fusion plasmids, pSJS720, pSJS779 and pSJS790 (5) were obtained from Dr Steven Sandler, University of Massachusetts, Amherst. The epsilon-containing CAT plasmids, R6, εI, εII, εSD and SD (4) were obtained from Dr Ashkan Golshani and Dr M.Abou-Haidar, University of Toronto. The sequences of the translational initiation regions of these CAT constructs are indicated in Table 1.

β-Galactosidase and CAT assays

β-Galactosidase was assayed as described by Miller (13). MC230-derived strains containing wild-type or mutant pMO10 plasmids were transformed with the various *recF–lacZ* fusion plasmids and β-galactosidase was measured in cultures grown in minimal medium at 37°C. CAT levels were measured in TA488 transformed with pMO28 and pMO30 and grown in LB medium in the presence of spectinomycin at 37°C. Spectrophotometric assays of CAT activity were carried out as described by Shaw (14) on sonicated extracts. Protein concentrations were determined using the Bradford reagents purchased from Bio-Rad.

Growth rates of MC230 strains containing pMO10 were measured by diluting overnight cultures of these strains into fresh LB medium and following the increases in turbidity thereafter using a Klett–Summerson colorimeter.

Table 1. Sequences of the translational initiation regions of CAT constructs used in this study

Construct	Sequence
εI	UCGAUUUAACUUUAAUUAGCUU**AUG**
εII	UCGAUUUAACUUUAUUUACCUUAUCAGCUU**AUG**
εSD	UCGAUUUAACUUUAAC<u>AAGGAGGU</u>UUCAGCUU**AUG**
SD	UCGAG<u>AAGGAGGU</u>UUAAGCUU**AUG**
R6	UUCGAUUUUUACAAUUACAGCUU**AUG**

Sequences of 5′ leader regions of epsilon-containing CAT constructs described by Golshani *et al.* (4) and used in this study. R6 is functionally equivalent to the ΔεSD construct of Golshani *et al.* (4) and lacks SD and ε elements. The SD sequences are underlined and the AUG initiation codons are indicated in bold.

Mutagenesis

Site-directed mutagenesis of rRNA was carried out on an M13mp18 *Kpn*I–*Xba*I clone carrying the entire (promoterless) coding region 16S rRNA, as described by Kunkel (15). Mutant M13 clones were identified by DNA sequencing and the mutations were transferred into pMO10 by *Hin*dIII fragment exchanges. The 460 flip mutation was transferred from pMO460Flip into pMO28 using a *Pst*I–*Csp*I fragment exchange and the mutant derivative designated pMO30.

RESULTS

Construction and expression of mutations in helix 17 of 16S rRNA

The region of 16S rRNA complementary to the epsilon element (the 460 stem–loop, helix 17; Fig. 1) is a stem–loop of variable sequence. Considerable sequence diversity exists in this helix, even among closely related enteric bacteria such as *E.coli* and *S.enterica* (Fig. 2). Consequently, we reasoned that if the critical feature of this region of 16S rRNA required for ribosome function was a stable stem–loop, reversal of the 8 bp in helix 17 might yield a functional ribosome but with altered base pairing potential to the epsilon enhancer. Similarly, since the 16S, 23S and 5S rRNAs of *S.enterica* are fully functional in *E.coli* (10), we reasoned that the 460 stem–loop of *S.enterica* might also be functional in the context of *E.coli* 16S rRNA. Both mutant rRNAs were constructed by site-directed mutagenesis and expressed from the constitutive P1P2 promoters in the *rrnB*-containing plasmid pMO10, and grew at rates indistinguishable from wild-type when expressed in *E.coli* strains containing intact *rrn* operons. The recent development of a strain of *E.coli*, Δ7 prrn (10), lacking all chromosomal rrn operons, has made it possible to construct strains that express mutant, plasmid-encoded rRNA exclusively. Construction of such strains involves replacement of the resident *rrn* plasmid in the Δ7 prrn strain with the mutant *rrn* plasmids. The Δ7 prrn strain MC230 carries the pSC101-derived, spectinomycin-resistant *rrn* plasmid, pts1192U, which is incompatible with the pSC101-derived, neomycin-resistant plasmid, pMO10. After introduction of pMO10 plasmids carrying wild-type, Sal460 or 460 flip rRNAs into MC230, all neomycin-resistant transformants were spectinomycin sensitive, suggesting that the pMO10 plasmids had displaced pts1192U. Exclusive expression of the mutant Sal460 and 460 flip rRNAs in these transformants was confirmed by primer extension analysis of total RNA from these cells, where only mutant rRNA was detected (data not shown). In addition, strains expressing the Sal460 and 460 flip rRNAs exclusively grew at wild-type rates in rich medium. Strain TA548 carrying the wild-type *rrn* plasmid, pMO10, had a doubling time of 70 ± 3 min, while the isogenic strains expressing the Sal460 and 460 flip rRNAs exclusively had doubling times of 69 ± 3 and 69 ± 4 min, respectively. This suggested that the mutant rRNAs were fully functional in protein synthesis and supports the idea that a stable stem–loop, rather than a specific sequence in the 460 region was required for ribosome function.

Expression of epsilon-containing mRNAs in strains with alterations in helix 17 of 16S rRNA

The original epsilon element derived from the T7 gene 10 initiation region consisted of a nine base sequence UUAACUUUA complementary to nucleotides 458–466 of 16S rRNA and which enhanced the expression of a *lacZ* gene containing a consensus SD sequence (3). However, this nine base epsilon element was unable to promote translation in the absence of a SD element. In a more extensive analysis, Golshani *et al.* (4) confirmed these observations using a CAT reporter gene system. However, they also observed that when the epsilon–16S rRNA complementarity was extended to 16 nt, this enlarged epsilon element was able to promote initiation of translation on its own with an efficiency comparable with that of a consensus SD element. We have used the series of CAT reporter genes constructed by Golshani *et al.* (4) to test the rRNA–mRNA base pairing model for epsilon action by examining CAT activity supported by these constructs in strains expressing wild-type or mutant rRNAs.

The Δ7 prrn strains contain several CAT cassettes used to inactivate chromosomal *rrn* operons (10), so we were unable to use these strains to measure plasmid-dependent CAT activity. Repeated attempts to express *lacZ* from the promoters and translational initiation regions found in the epsilon CAT constructs were unsuccessful, and all plasmids recovered contained frameshifts or various gross rearrangements. This was perhaps due to the high level of expression of *lacZ,* which is toxic to *E.coli* cells. Instead, the altered rRNAs were combined with the C1192U mutation conferring spectinomycin resistance (16) and expressed in strain TA488 which has five of the seven chromosomal *rrn* operons inactivated. Primer extension analysis of total RNA from these cells showed that 80% of the rRNA was of the mutant form. Addition of spectinomycin to cells expressing such rRNAs permits selective utilization of plasmid-encoded rRNAs (17,18). TA488 strains expressing either wild-type or 460 flip rRNAs were transformed with the various epsilon-containing CAT constructs and CAT activity was measured in cells grown in the presence of spectinomycin. As can be seen in Table 2, constructs lacking either SD or epsilon elements (R6) or containing only the original nine base epsilon element (εI) had little CAT activity, and their expression was unaffected by changes in rRNA sequence. CAT constructs containing a consensus SD element (SD) had very high expression levels and a comparable level of activity was obtained with a construct (εII) containing only the extended epsilon element. A CAT construct combining the

Table 2. Expression of epsilon-containing CAT mRNAs in wild-type and 460 flip rRNA mutants

CAT	Wild-type rRNA		460 flip rRNA	
construct	Units of CAT	Relative activity[a]	Units of CAT	Relative activity[a]
SD	17 889 ± 7623	1.00	13 662 ± 2335	1.01
εI	297 ± 70	0.02	269 ± 61	0.02
εII	13 031 ± 5491	0.97	14 913 ± 2159	1.10
εSD	26 353 ± 8247	1.95	23 751 ± 11 064	2.14
R6	1080 ± 206	0.08	793 ± 121	0.06

Units of CAT activity are expressed in micromoles of chloramphenicol acetylated per min per mg of protein.
[a]Expression levels of all constructs are relative to that of the SD construct in wild-type cells which is assigned a value of 1.00.

original epsilon element and the consensus SD sequence (εSD) had a further 2-fold higher level of activity, relative to the SD construct. The relative levels of CAT expression are in good agreement with those originally reported by Golshani *et al.* (4) with the same plasmids. However, despite the radical alterations in rRNA–epsilon pairing potential, the activities supported by all of these CAT mRNAs were completely unaffected by changes in the 460 stem–loop of 16S rRNA. These data indicate that although epsilon has a clear effect on CAT expression, this enhancer activity is completely independent of the sequence of the 460 region of 16S rRNA.

In contrast to its role in promoting initiation, Sandler and Clark (5) have observed a negative effect of an epsilon-like sequence on expression of *recF–lacZ* fusions. A hexapyrimidine tract and an adjacent epsilon element are found within nucleotides 49–146 of the *E.coli recF* gene. Mutagenesis of the hexapyrimidine tract increased expression of the *recF–lacZ* fusion 4-fold and when the hexapyrimidine tract mutant was coupled with a mutant epsilon element that was designed to decrease base pairing to the 460 region of 16S rRNA, expression increased a further 5-fold. The effects of the *recF*-associated epsilon element were examined by measuring expression of wild-type and the mutant *recF–lacZ* fusions constructed by Sandler and Clark (5) in the Δ7 prrn-expressing wild-type or mutant rRNAs exclusively. The data presented in Table 3 show that expression of the wild-type fusion (pSJS779), or a fusion carrying a mutant hexapyrimidine tract (pSJS790) or a fusion with mutations in both the hexapyrimidine tract and epsilon element (pSJS720) were expressed at the same levels in strains containing only wild-type rRNA, or strains expressing only the Sal 460 or 460 flip rRNAs. For unknown reasons, expression of all three *recF–lacZ* fusions was considerably higher in the Δ7 prrn strain than that reported by Sandler and Clark (5) in strain AB1157 and higher than we have measured in a wild-type strain (CSH142) containing intact *rrn* operons. However, the relative expression level of the three fusions in the Δ7 prrn strain is similar to that reported previously, with the double mutant *recF–lacZ* fusion (pSJS720) having a 9–16-fold higher expression level than the wild-type fusion (pSJS779). In summary, our data indicate that although mutagenesis of the hexapyrimidine tract and epsilon element increases expression of a *recF–lacZ* fusion, the level of expression is unaffected by the sequence of the 460 region of 16S rRNA.

Table 3. Effects of mutations in helix 17 of 16S rRNA on expression of *recF–lacZ* fusions

recF–lacZ Plasmid	rRNA		
	Wild-type	460 Sal	460 flip
pSJS720	2205 ± 393	2623 ± 392	2425 ± 517
pSJS779	239 ± 64	238 ± 80	153 ± 17
pSJS790	502 ± 4	563 ± 184	441 ± 193

Numbers represent Miller units of β-galactosidase activity. Each measurement is the mean (±SD) of assays from at least four independent cultures.

DISCUSSION

In this study, we have tested the rRNA–mRNA base pairing model for epsilon activity by altering the proposed anti-epsilon region of 16S rRNA and measuring the expression of epsilon-containing mRNAs under conditions where only the mutant rRNA was functional. In all cases, while we could confirm the influence of the epsilon element on expression, the level of expression was not influenced by the sequence of the 460 region of 16S rRNA. When this study was initiated, the precise location of helix 17 was not known. However, the now-published crystal structure of the 30S ribosomal subunit (19) shows that helix 17 is located on the periphery of the lower body of the 30S subunit, distant from the decoding center and the antiSD sequence (Fig. 1). This in itself renders any models for epsilon–rRNA interaction at initiation unlikely, and models invoking simultaneous interaction of SD and epsilon elements with 16S rRNA untenable. We conclude, therefore, that enhancement of CAT and T7 gene 10 mRNA expression at initiation and the negative effects of epsilon during elongation on *recF* mRNA do not derive from any rRNA–mRNA base pairing interactions and other mechanisms must be sought.

One common characteristic of all epsilon sequences is their high pyrimidine content; the extended epsilon element (εII; Table 1) that functions as an efficient, independent ribosome binding site, contains an additional seven pyrimidines relative to the original nine base epsilon sequence (εI; Table 1). A well-described characteristic of ribosomal protein S1 is its affinity for polypyrimidines (20) and *in vitro* selection experiments have

shown that S1 affinity ligands comprise the majority of the RNAs selected by ribosomes from randomized pools of RNA sequences (21). SD-containing RNAs were selected from the same randomized pool only when ribosomes were first depleted of S1. This suggests that S1–mRNA interactions account for a substantial fraction of the total mRNA–ribosome interactions occurring at initiation. S1-interacting sequences are not limited to polypyrimidine stretches, however, as the S1 affinity ligands selected *in vitro* were purine-rich and all had the potential to assume a pseudoknot structure. Similar sequences were also found in another translational enhancer from *Mycoplasma genitalium* which was also active in *E.coli* (22), as well as in the S1 binding domain of phage Qβ and the ribosome binding site of the autogenously regulated S1 mRNA. Sequences derived from plant RNA viruses consisting of polyU stretches or CAA repeats have also been found to promote efficient initiation in the absence of any SD sequences in *E.coli* in an S1-dependent manner (23). Analysis of initiation complexes formed on such mRNAs by toeprinting has shown that in the presence of IF3, S1–mRNA interactions lead to precise positioning of the 30S subunit on the mRNA (23,24). These observations indicate that S1 can bind a large repertoire of RNA sequences and structural elements and that such interactions are important for selection and positioning of mRNAs by initiating ribosomes. The similarity of described S1-interacting RNAs to the epsilon sequence thus raises the possibility that epsilon may interact with the ribosome by acting as an S1 affinity ligand. According to this model, the extended epsilon element binds S1 on the ribosome with higher affinity than the shorter, nine base sequence, rendering the SD–antiSD interaction dispensable for initiation complex formation. Mutagenesis experiments on the epsilon element indicate that the length of epsilon and its distance from the AUG initiation codon may also contribute to initiation efficiency (25), possibly by influencing the positioning of the 30S subunit on the mRNA relative to the AUG initiation codon. Experiments to test the affinity of isolated S1 for the epsilon element are currently in progress.

The original proposal of the SD–antiSD interaction (26) was based on the analysis of a limited number of phage and bacterial mRNAs. However, this proposal was subsequently supported by a myriad of genetic and biochemical experiments (17,18,27). Similarly, partial complementarity of enhancer elements to discrete regions of rRNA has led to the proposal of other rRNA–mRNA interactions. However, evidence in support of such interactions from biochemistry, mRNA–rRNA co-variation and rRNA mutagenesis is invariably lacking. We have previously demonstrated by rRNA mutagenesis that a widely accepted interaction between the downstream box enhancer and 16S rRNA does not occur (28). Thus, while many of these enhancer elements have demonstrable effects on gene expression, the proposed rRNA–mRNA base pairing models cannot be confirmed and other mechanisms of enhancer action need to be entertained.

ACKNOWLEDGEMENTS

We are indebted to Drs Ashkan Goshani, M.Abou-Haidar and Steven Sandler for providing the various epsilon containing plasmids used in this study, Dr Catherine Squires for providing *rrn* deletion strains, and Dr Steven Gregory for assistance with the figures. We thank George Q.Pennabble for his condign criticisms, and Hugo Strait for support. This work was supported by grant GMS19756 from the US National Institutes of Health to A.E.D.

REFERENCES

1. Gualerzi,C.O. and Pon,C.L. (1990) Initiation of mRNA translation in prokaryotes. *Biochemistry*, **29**, 5881–5889.
2. O' Connor,M., Bayfield,M.A., Gregory,S.T., Lee,W.-C.M., Lodmell,J.S., Mankad,A., Thompson,J.R., Vila-Sanjurgo,A., Squires,C.L. and Dahlberg,A.E. (2000) Probing ribosome structure and function: analyses with RNA and protein mutants. In Garrett,R.A., Douthwaite,S.R., Liljas,A., Matheson,A.T., Moore,P.B. and Noller,H.F. (eds), *The Ribosome: Structure, Function, Antibiotics, and Cellular Interactions*. American Society for Microbiology, Washington, DC, pp. 217–228.
3. Olins,P.O. and Rangwala,S.H. (1989) A novel sequence element derived from bacteriophage T7 mRNA acts as an enhancer of translation of the *lacZ* gene in *Escherichia coli*. *J. Biol. Chem.*, **264**, 16973–16976.
4. Golshani,A., Golomehova,V., Mironova,R., Ivanov,I.G. and Abou-Haidar,M.G. (1997) Does the epsilon sequence of phage T7 function as an initiator for the translation of CAT mRNA in *Escherichia coli*? *Biochem. Biophys. Res. Commun.*, **236**, 253–256.
5. Sandler,S.J. and Clark,A.J. (1994) Mutational analysis of sequences in the *recF* gene of *Escherichia coli* K12 that affect expression. *J. Bacteriol.*, **176**, 4011–4016.
6. Sprengart,M.L. and Porter,A.G. (1997) Functional importance of RNA interactions in selection of translation initiation codons. *Mol. Microbiol.*, **24**, 19–28.
7. Sergiev,P.V., Lavrik,I.N., Wlasoff,V.A., Dokudovskaya,S.S., Dontsova,O.A., Bogdanov,A.A. and Brimacombe,R. (1997) The path of mRNA through the bacterial ribosome: a site-directed crosslinking study using new photoreactive derivatives of guanosine and uridine. *RNA*, **3**, 464–475.
8. O'Connor,M. and Dahlberg,A.E. (1993) Mutations at U2555, a tRNA-protected base in 23S rRNA, affect translational fidelity. *Proc. Natl Acad. Sci. USA*, **90**, 9214–9218.
9. Chang,A.C. and Cohen,S.N. (1978) Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. *J. Bacteriol.*, **134**, 1141–1156.
10. Asai,T., Zaporojets,D., Squires,C. and Squires,C.L. (1999) An *Escherichia coli* strain with all chromosomal rRNA operons inactivated: complete exchange of rRNA genes between bacteria. *Proc. Natl Acad. Sci. USA*, **96**, 1971–1976.
11. O'Connor,M., Lee,W.-C.M., Mankad,A., Squires,C.L. and Dahlberg,A.E. (2000) Mutagenesis of the peptidyltransferase center of 23S rRNA: the invariant U2449 is dispensable. *Nucleic Acids Res.*, **29**, 710–715.
12. Sigmund,C.D., Ettayebi,M., Borden,A., and Morgan,E.A. (1988). Antibiotic resistance mutations in ribosomal RNA genes of *Escherichia coli*. *Methods Enzymol.*, **164**, 673–690.
13. Miller,J.H. (1991) *A Short Course in Bacterial Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
14. Shaw,W.V. (1975) Chloramphenicol acetyltransferase from chloramphenicol resistant bacteria. *Methods Enzymol.*, **43**, 737–755.
15. Kunkel,T.A., Bebenek,K. and McClary,J. (1991) Efficient site-directed mutagenesis using uracil-containing DNA. *Methods Enzymol.*, **204**, 125–139.
16. Mark,L.G., Sigmund,C.D. and Morgan,E.A. (1983) Spectinomycin resistance due to a mutation in an rRNA operon of *Escherichia coli*. *J. Bacteriol.*, **155**, 989–994.
17. Hui,A.S., Eaton,D.H and de Boer,H.A. (1988) Mutagenesis at the mRNA decoding site in the 16S ribosomal RNA using the specialized ribosome system in *Escherichia coli. EMBO J.*, **7**, 4383–4388.
18. Lee,K., Holland-Staley,C.A. and Cunningham,P.R. (1996) Genetic analysis of the Shine–Dalgarno interaction: selection of alternative functional mRNA–rRNA combinations. *RNA*, **2**, 1270–1285.
19. Wimberly,B.T., Broderson,D.E., Clemons,W.M.,Jr, Morgan-Warren,R.J., Carter,A.P., Vonrhein,C., Hartsch,T. and Ramakrishnan,V. (2000) Structure of the 30S ribosomal subunit. *Nature*, **407**, 327–339.
20. Subramanian,A.R. (1983) Structure and functions of ribosomal protein S1. *Prog. Nucleic Acid Res. Mol. Biol.*, **28**, 101–142.
21. Ringquist,S., Jones,T., Snyder,E.E., Gibson,T., Boni,I. and Gold,L. (1995) High-affinity RNA ligands to *Escherichia coli* ribosomes and

ribosomal protein S1: comparison of natural and unnatural binding sites. *Biochemistry*, **34**, 3640–3648.
22. Loechel,S., Inamine,J.M. and Hu,P.C. (1991) A novel translation initiation region from *Mycoplasma genitalium* that functions in *Escherichia coli*. *Nucleic Acids Res.*, **19**, 6905–6911.
23. Tzareva,N.V., Makhno,V.I. and Boni,I.V. (1994) Ribosome-messenger recognition in the absence of the Shine–Dalgarno interactions. *FEBS Lett.*, **337**, 189–194.
24. Boni,I.V., Isaeva,D.M., Musychenko,M.L. and Tzareva,N.V. (1991) Ribosome-messenger recognition: mRNA target sites for ribosomal protein S1. *Nucleic Acids Res.*, **19**, 155–162.
25. Golshani,A., Kolev,V., Abou-Haidar,M.G. and Ivanov,I.G. (2000) Epsilon as an initiator of translation of CAT mRNA in *Escherichia coli*. *Biochem. Biophys. Res. Commun.*, **273**, 528–531.
26. Shine,J., and Dalgarno,L. (1975) Determinant of cistron specificity in bacterial ribosomes. *Nature*, **254**, 34–38.
27. Steitz,J.A. and Jakes,K. (1975) How ribosomes select initiator regions in mRNA: base pair formation between the 3′ terminus of 16S rRNA and the mRNA during initiation of protein synthesis in *Escherichia coli.* *Proc. Natl Acad. Sci. USA*, **72**, 4734–4738.
28. O'Connor,M., Asai,T., Squires,C.L. and Dahlberg,A.E. (1999). Enhancement of translation by the downstream box does not involve base pairing of mRNA with the penultimate stem sequence of 16S rRNA. *Proc. Natl Acad. Sci. USA*, **96**, 8973–8978.