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Alterations in the peptidyltransferase and decoding domains of ribosomal RNA suppress mutations in the elongation factor G gene

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ABSTRACT

The translocation stage of protein synthesis is a highly conserved process in all cells. Although the components necessary for translocation have been delineated, the mechanism of this activity has not been well defined. Ribosome movement on template mRNA must allow for displacement of tRNA–mRNA complexes from the ribosomal A to P sites and P to E sites, while ensuring rigid maintenance of the correct reading frame. In *Escherichia coli*, translocation of the ribosome is promoted by elongation factor G (EF-G). To examine the role of EF-G and rRNA in translocation we have characterized mutations in rRNA genes that can suppress a temperature-sensitive (ts) allele of *fusA*, the gene in *E. coli* that encodes EF-G. This analysis was performed using the ts *E. coli* strain PEM100, which contains a point mutation within *fusA*. The ts phenotype of PEM100 can be suppressed by either of two mutations in the decoding region of the 16S rRNA when present in combination with a mutation at position 2058 in the peptidyltransferase domain of the 23S rRNA. Communication between these ribosomal domains is essential for coordinating the events of the elongation cycle. We propose a model in which EF-G promotes translocation by modulating this communication, thereby increasing the efficiency of this fundamental process.

Keywords: EF-G mutants; ribosomes; rRNA mutants; translocation

INTRODUCTION

The central step of protein synthesis, peptide elongation, involves the sequential addition of amino acids to the growing peptide chain, as directed by the codon sequence on the mRNA template. Following successful initiation, each aminoacyl-tRNA (aa-tRNA) is delivered to the ribosomal A site as part of a ternary complex with elongation factor Tu (EF-Tu) and GTP. Following GTP hydrolysis, EF-Tu and GDP are released, allowing the formation of a peptide bond between the neighboring amino acids. According to the "hybrid-states" model of translocation (Moazed & Noller, 1989; Wilson & Noller, 1998b), peptide bond formation is accompanied by the simultaneous movement of the acceptor ends of the aa-tRNAs relative to the large ribosomal subunit, creating a hybrid state in which the anticodon ends of the aa-tRNAs are bound to the ribosomal P and A sites, whereas the acceptor ends are bound to the E and P sites, respectively (the so-called P/E and A/P states). EF-G-GTP then binds to the ribosome at a site that overlaps both the A site and the ternary complex binding site, leading to translocation of the ribosome by one codon (Moazed et al., 1988; Richman & Bodley, 1972; Stark et al., 1997; Wilson & Noller, 1998a). Translocation allows the dissociation of the empty tRNA via the E site, restores the P/P binding state of the peptidyl-tRNA and renders the A site empty. Following GTP hydrolysis and the subsequent release of EF-G, the ribosome is in a state whereby it has translocated along the mRNA by precisely one codon, and it is ready to accept another aa-tRNA from the ternary complex.

Although translocation can occur in vitro independently of EF-G, the rate of this factor-free elongation is not sufficient to support cell growth (Pestka, 1969; Gavrilova & Spirin, 1971). This suggests that the pro-

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Abbreviations: EF-G: elongation factor G; LB: Luria Bertani; ts: temperature sensitive

cess of translocation is intrinsic to the ribosome, and the role of EF-G is to increase the efficiency with which it occurs. The present study was undertaken to further elucidate the role of EF-G and rRNA in the elongation stage of protein synthesis. Of particular interest were the interactions between the functional domains of rRNA and the association of EF-G with these domains during the elongation cycle. To date there have been very few genetic studies of these complex interactions (Hou et al., 1994b), despite the large volume of structural and biochemical information that is now becoming available (Ævarsson et al., 1994; Czworkowski et al., 1994; Czworkowski & Moore, 1997; Munishkin & Wool, 1997; Nyborg & Liljas, 1998; Wilson & Noller, 1998a; Ban et al., 1999; Cate et al., 1999; Clemons et al., 1999). The present study aims to address this problem by contributing genetic data to complement this plethora of biochemical and structural data, and to integrate this information to describe the intricate process of elongation as it occurs in vivo.

Previously we have described the isolation of *Escherichia coli* strain PEM100, which harbors a missense mutation in *fusA* (*fusA100* allele), the gene that encodes EF-G (Hou et al., 1994b). The resulting gene product (EF-G G502D) is deficient in ribosome binding, particularly at elevated temperatures, to the extent that the protein is nonfunctional at 42 °C (Hou et al., 1994a). This causes PEM100 to display a ts phenotype. The present study examined the suppression of this phenotype by mutations in 16S and 23S rRNA. Our results support a model whereby EF-G promotes translocation by modulating the communication between the peptidyltransferase domain of the 23S rRNA and the decoding region of the 16S rRNA during elongation.

RESULTS

Suppression of the temperature-sensitive phenotype of PEM100 by rRNA mutations

In this study we have isolated several mutations in rRNA genes that are able to restore the growth of PEM100 at 42 °C on solid media. The plasmids pKK3535 (containing the entire *rrnB* operon) and pSTL102 (a derivative of pKK3535; see Materials and methods) were initially used as wild-type templates for in vitro hydroxylamine mutagenesis to isolate mutations in rRNA genes that could suppress the ts phenotype of PEM100 in *trans*. Unexpectedly, however, the C → U substitution at position 1192 of 16S rRNA (C1192U) and the A → G substitution at position 2058 of 23S rRNA (A2058G) in pSTL102 were together able to restore the growth of PEM100 at 42 °C (Table 1). The wild-type rRNA genes carried by pKK3535 were unable to restore growth at the nonpermissive temperature, even after extended incubation.

To determine whether one or both of these mutations were necessary for suppression, PEM100 was transformed with plasmid pKK1192U (containing C1192U) and pKK2058G (containing A2058G). Neither plasmid was capable of restoring growth at 42 °C (Table 1). To search for other combinations of 16S and 23S rRNA mutations that could suppress the ts *fusA* allele, plasmids pKK1192U and pKK2058G were treated with hydroxylamine. After screening approximately 10,000 colonies, no additional suppressor mutations were isolated from pKK1192U, but a similar screening yielded a C1400U mutation in pKK2058G, which, in combination with A2058G, restored growth of PEM100 at 42 °C. To eliminate the possibility of additional mutations during hydroxylamine treatment, plasmid pKK1400U/2058G was constructed by site-directed mutagenesis and shown to suppress the ts mutation in PEM100 at 42 °C (Table 1). Plasmid pKK1400U (C1400U) was not able to suppress PEM100. Therefore, both a 16S and a 23S mutation were necessary to restore growth, as was demonstrated with pSTL102. Several other plasmids containing various rRNA mutations in the decoding region of 16S or the peptidyltransferase or GTPase regions of 23S rRNA were tested for their ability to suppress the ts phenotype of PEM100 (Table 1). None of these restored growth at 42 °C, and it was not even possible to isolate transformants at 30 °C of plasmids with mutations at EF-G binding sites in 23S rRNA (positions 1067 and 2661).

To determine the degree to which the suppressor mutations were able to restore the growth of PEM100 at 42 °C, and whether these rRNA mutations had any effect on the growth rate of PEM100 and the parent strain JM83 under permissive conditions, doubling times were determined for cells containing pKK3535, pSTL102, and pKK1400U/2058G at both 30 °C and 42 °C in liquid media (Table 2). As observed previously, the EF-G mutation was not silent at 30 °C (Hou et al., 1994b), and for both wild-type EF-G (JM83) and EF-G G502D (PEM100) cells, doubling times increased at 30 °C in the presence of plasmids in contrast to cells that did not carry suppressor plasmids. In both PEM100 and its parent strain JM83, the increase in doubling time was greatest for cells containing pKK1400U/2058G. After shifting to 42 °C, PEM100 cells that did not carry suppressor plasmids ceased to grow after only 90 min (Hou et al., 1994b), whereas PEM100 containing suppressor plasmids continued to grow for about 3 h. The doubling times for PEM100 pSTL102 and PEM100 pKK1400U/2058G at 42 °C were determined for the period immediately following the shift to the nonpermissive temperature until the point at which the optical density ceased increasing. To allow for comparisons to be made, the value determined for PEM100 (without suppressor plasmids) at 42 °C was calculated for the same period. During the period following the shift to 42 °C, the doubling time of PEM100 increased almost

TABLE 1. Suppression of the ts phenotype of PEM100.[a]

Plasmid	rRNA	Alleles	Suppression[b]	Comments	References
pKK3535	16S	wt[c]	no	wild-type rrnB	Brosius et al., 1981
	23S	wt		operon	
pSTL102	16S	C1192U	yes	decoding region	Triman et al., 1989
	23S	A2058G		peptidyltransferase	
pKK1192U	16S	C1192U	no	decoding region	Makosky & Dahlberg, 1987
	23S	wt			
pKK2058G	16S	wt	no		Douthwaite, 1992
	23S	A2058G		peptidyltransferase	
pKK1400U/2058G	16S	C1400U	yes	decoding region	this work
	23S	A2058G		peptidyltransferase	
pKK1400U	16S	C1400U	no	decoding region	Thomas et al., 1988[d]
	23S	wt			
pKK1491U/2058G	16S	G1491U	no	decoding region	De Stasio et al., 1989;
	23S	A2058G		peptidyltransferase	De Stasio & Dahlberg, 1990; O'Connor et al., 1991
pKK1491C/2058G	16S	G1491C	no	decoding region	De Stasio et al., 1989;
	23S	A2058G		peptidyltransferase	De Stasio & Dahlberg, 1990; O'Connor et al., 1991
pKK1199C	16S	U1199C	no	decoding region	Moine & Dahlberg, 1994
	23S	wt			
pKK1202C	16S	U1202C	no	decoding region	Moine & Dahlberg, 1994
	23S	wt			
pKK1067G	16S	wt	no[e]		Thompson et al., 1988
	23S	A1067G		GTPase center	
pKK1067U	16S	wt	no[e]		Thompson et al., 1988
	23S	A1067U		GTPase center	
pKK1067C	16S	wt	no[e]		Thompson et al., 1988
	23S	A1067C		GTPase center	
pKK2661C	16S	wt	no[e]		Tapprich & Dahlberg, 1990
	23S	G2661C		GTPase center	

[a] The plasmids listed were transformed into PEM100 and equal aliquots of the transformation mixture were plated onto two LB plates; One was incubated at 30 °C and one at 42 °C. The different alleles of the 16S and 23S rRNA genes are indicated.

[b] Yes: the plasmid could support growth on plates at 42 °C. In all cases the rate of colony growth was significantly slower (40-h incubation) compared to cells harboring a wild-type copy of *fusA* (24-h incubation). No: colonies were not observed even after extended incubation (>84 h).

[c] wt: wild-type.

[d] Originally designated pEXP145, but redesignated pKK1400U for clarity and consistency.

[e] No transformants at either 30 °C or 42 °C.

TABLE 2. Doubling times for JM83 (parental wild-type EF-G strain) and PEM100 (ts EF-G mutation) with and without plasmids.[a]

	Doubling time (min)	
Strain	30 °C	42 °C
PEM100	49 ± 1	462 ± 20[b]
PEM100 pKK3535	49 ± 3	479 ± 16[b]
PEM100 pSTL102	65 ± 3	126 ± 7
PEM100 pKK1400U/2058G	72 ± 2	151 ± 8
JM83	41 ± 1	31 ± 1
JM83 pKK3535	49 ± 1	33 ± 1
JM83 pSTL102	50 ± 1	33 ± 2
JM83 pKK1400U/2058G	56 ± 2	42 ± 2

[a] Growth was measured in LB medium and doubling times are the average of four experiments.

[b] Extrapolated from data points during 3 h after shift from 30 °C to 42 °C.

10-fold, from 49 min to 462 min. Although doubling times also increased when PEM100 pSTL102 and PEM100 pKK1400U/2058G were shifted from 30 °C to 42 °C, the extent of these increases was only approximately twofold, although the cells ultimately stopped growing. These results in liquid culture are consistent with those showing colony growth on plates and demonstrate the ability of the mutations to suppress the ts phenotype of PEM100. Why the cells continue to grow on plates but not in liquid media is not known.

Distribution of mutant (suppressor) and wild-type rRNA

Because the cells contain both wild-type (chromosomal) rRNA and mutant (plasmid) rRNA, the pool of available

ribosomes is heterogeneous. To ascertain how the suppressor mutations might restore growth of PEM100 at the nonpermissive temperature, the distribution of the mutant rRNA species was determined. The proportion of mutant and wild-type rRNA in each of the cellular rRNA fractions (30S subunits, 50S subunits, 70S ribosomes, and polysomes) and in the total cellular rRNA was determined by primer extension analysis of rRNA from cell lysates and sucrose density gradient fractions. Both 16S and 23S rRNA were measured in PEM100 pSTL102 and PEM100 pKK1400U/2058G grown at both 30 °C and 42 °C (Fig. 1). Control analyses were also conducted with JM83 containing the same plasmids. Between three and six replicates were performed for each strain. It is apparent from these results that mutant 23S rRNA, much more than 16S rRNA, was enriched in PEM100 pSTL102 and PEM100 pKK1400U/2058G at 42 °C. Most significantly, in the absence of functional EF-G (at 42 °C) there is a significant preferential incorporation of the mutant 23S rRNA species into polysomes (Fig. 1B,D). In contrast, in no case was there a temperature-dependent selection of mutant rRNA into any rRNA fraction in the JM83 control cells.

Cellular EF-G content

We have shown previously that EF-G G502D produced from the *fusA100* allele in PEM100 is not stable at 42 °C in vivo in comparison to wild-type EF-G (Hou et al., 1994b). An important question is whether suppression of the ts growth defect affects the stability of mutant EF-G. Figure 2 demonstrates that there is a direct correlation between suppression of the ts growth defect and stabilization of EF-G G502D at 42 °C. Although wild-type EF-G is stable at both 30 °C and 42 °C when only wild-type rRNA is present (Fig. 2, lanes 1, 2, and 9), EF-G G502D clearly is not detectable in PEM100 at 42 °C (Fig. 2, compare lanes 3 and 4 and lanes 16 and 17). Mutant EF-G G502D is stable in PEM100 at 42 °C only in the presence of suppressor plasmids pSTL102 and pKK1400U/2058G (Fig. 2, lanes 7 and 8 and lanes 10 and 11). The single mutations in either 16S or 23S rRNA (C1400U, C1192U, or A2058G) do not confer stability to EF-G G502D at the nonpermissive temperature (Fig. 2, lanes 5 and 6, 12 and 13, and 14 and 15). These results show that mutations in both 16S and 23S rRNA are required to prevent degradation of EF-G G502D at 42 °C, presumably by maintaining effective EF-G–ribosome interactions during elongation.

Previously we have measured the rate of disappearance of EF-G in PEM100 following a shift to 42 °C (Hou et al., 1994b). By 30 min postshift to 42 °C, the cellular levels of EF-G was reduced approximately twofold, by 90 min EF-G protein was barely detectable, and by 180 min EF-G was not detectable. The rate of disappearance was not examined for samples shown in Figure 2.

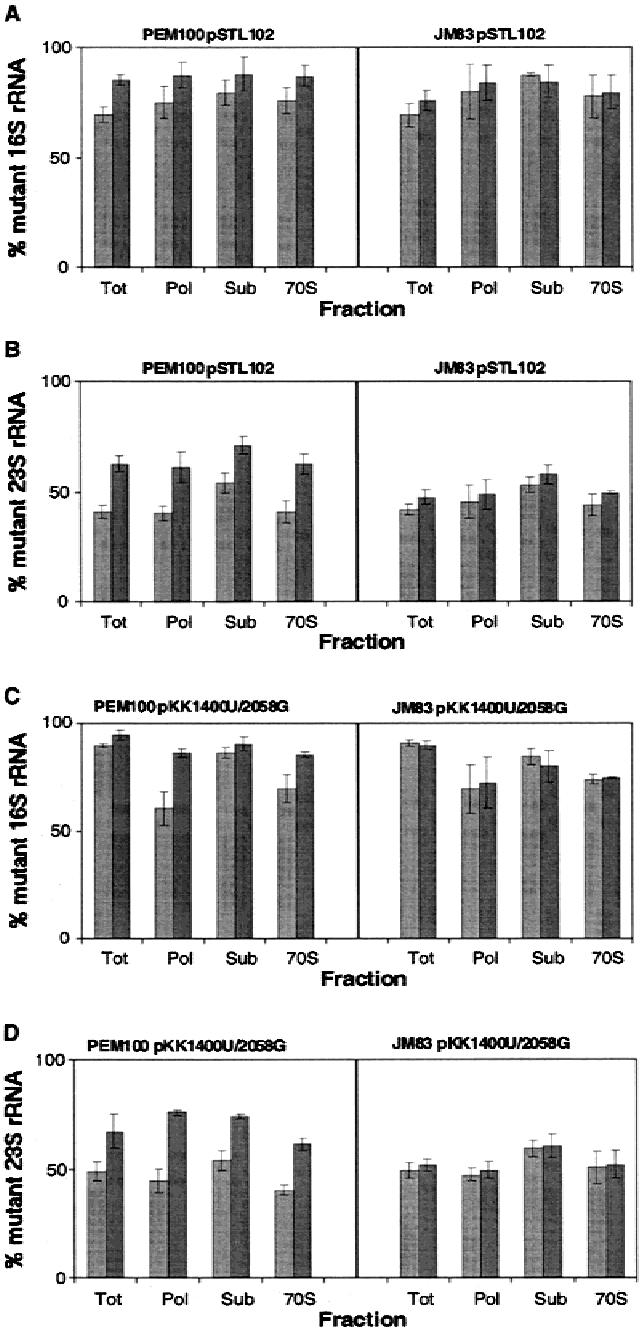

FIGURE 1. Percentage of mutant rRNA in the total cellular rRNA and the rRNA fractions of PEM100 pSTL102, JM83 pSTL102, PEM100 pKK1400U/2058G, and JM83 pKK1400U/2058G when grown at 30 °C (light gray) and 42 °C (dark gray) as determined by primer extension analysis. Values are given for 16S rRNA (**A** and **C**) and 23S rRNA (**B** and **D**) in the total cellular rRNA pool (Tot), polysome fraction (Pol), free-subunit fraction (Sub) and 70S monosome fraction (70S).

DISCUSSION

Here we show that the ts phenotype of the EF-G mutant G502D can be suppressed by specific mutations in 16S and 23S rRNAs (Table 1). Plasmids pSTL102 and pKK1400U/2058G maintained the growth of PEM100 after the temperature was raised to 42 °C (Table 2). A

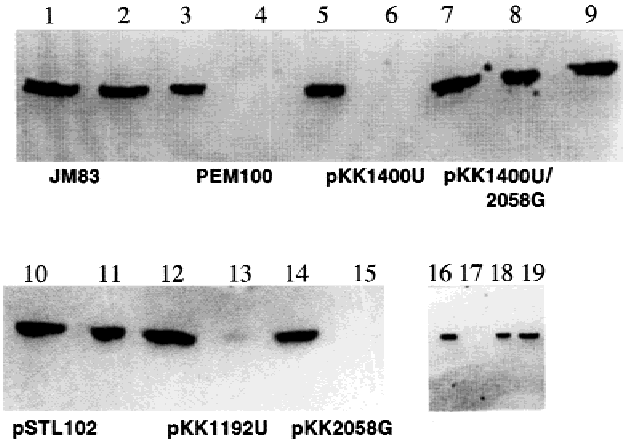

FIGURE 2. Determination of cellular EF-G content by immunoblotting. Protein samples were prepared as described in Materials and methods. Equal amounts of total cell protein were loaded into each lane of a polyacrylamide gel and subjected to SDS-PAGE. Following electrophoresis, protein was transferred to nitrocellulose and then subjected to immunoblotting as described in Materials and methods. Lysates derived from JM83 (lanes 1 and 2) and PEM100 (lanes 3 and 4) as well as PEM100 containing pKK1400U (lanes 5 and 6), pKK1400U/2058G (lanes 7 and 8), and pECEG, which expresses wild-type EF-G (lane 9) are shown in the top panel. Lysates from 30 °C cultures are shown in lanes 1, 3, 5, and 7; lysates from 42 °C cultures are shown in lanes 2, 4, 6, 8, and 9. Lysates derived from PEM100 containing pSTL102 (lanes 10 and 11), pKK1192U (lanes 12 and 13), and pKK2058G (lanes 14 and 15) are shown in the bottom left panel. Lysates from 30 °C cultures are shown in lanes 10, 12, and 14; lysates from 42 °C cultures are shown in lanes 11, 13, and 15. Control lysates derived from PEM100 pKK3535 (lanes 16 and 17) as well as PEM100 pKK1400U/2058G for comparison on the same gel (lanes 18 and 19) are shown in the bottom right panel. Lysates derived from 30 °C cultures are shown in lanes 16 and 18; lysates derived from 42 °C cultures are shown in lanes 17 and 19.

major determinant of growth rate under optimal conditions is cellular protein–synthetic capacity (Nomura et al., 1984; Lindahl & Zengel, 1986). The increases in doubling time observed in a wild-type EF-G background (i.e., in PEM100 at 30 °C or in JM83 at either 30 °C or 42 °C) reflect a reduced translational efficiency due either to overloading of ribosomes per cell by plasmid-encoded *rrn* operons (pSTL102) (Gourse et al., 1982) or to a defect in the decoding region of 16S rRNA (C1400U mutation). However, despite the detrimental effect on growth rate, both plasmids effectively suppress the ts EF-G mutation at 42 °C (Table 2).

The sites of both suppressor mutants in 16S rRNA are located in the decoding region of the 30S subunit (Dahlberg, 1989). The site of the EF-G mutation, domain IV, binds to the ribosome in close proximity to C1400 (Agrawal et al., 1998; Wilson & Noller, 1998a), and UV crosslinking experiments have revealed that position 1400 associates with the anticodon loop of the aa-tRNA in the ternary complex with EF-Tu-GTP (Prince et al., 1982), of which EF-G domain IV is a structural mimic (Nissen et al., 1995). Position 1192 in 16S rRNA is located in helix 34, which has also been placed in the decoding region of the ribosome (Dontsova et al., 1992; Moine & Dahlberg, 1994). The resistance to spectinomycin that is conferred by the C1192U substitution suggests that this nucleotide is involved in the translocation step of elongation, as spectinomycin is believed to inhibit EF-G-ribosome interactions (O'Connor et al., 1995). Furthermore, the spectinomycin-resistance phenotype conferred by pKK1192U can be suppressed by a mutation in EF-G (Johanson & Hughes, 1994). Interestingly, mutants in 16S rRNA alone are not sufficient to suppress the ts EF-G mutant; there is a need for the 23S rRNA mutation A2058G, which resides in the peptidyltransferase loop of domain V (Douthwaite, 1992). Both of these functional centers on the ribosome are directly involved in the elongation cycle (Wilson & Noller, 1998b), but mutations at the two known EF-G binding sites in 23S rRNA, 1067 and 2661, did not give suppression and, in fact, were incompatible with the EF-G mutation at any temperature (Table 1).

The preferential incorporation of mutant 16S and 23S rRNA into the polysomes of PEM100 pSTL102 and PEM100 pKK1400U/2058G at 42 °C (Fig. 1) suggests the suppressor mutations are more functional than wild-type rRNAs at the restrictive temperature. It is clear, however, that the polysome fractions are not composed exclusively of mutant rRNA, but do contain some wild-type rRNA. Suppression of the ts EF-G defect must be achieved by ribosomes containing either a 16S or a 23S rRNA mutation. Mutations in both 16S and 23S rRNA may be required to increase the percentage of ribosomes with at least one suppressor mutation in cells containing a heterogeneous ribosome population (with both chromosomal and plasmid-encoded rRNA). The disparity in proportion of total mutant 23S and total mutant 16S rRNA in the cells (Fig. 1) could reflect differences in primer extension efficiency in the assay (at sites in 16S and 23S rRNA) or differences in ribosome stability.

Analysis of the distribution of C1400U mutant 16S rRNA in cells containing pKK1400U/2058G (Fig. 1C) reveals an interesting trend. In a wild-type EF-G background, the proportion of mutant rRNA in the 70S monosomes and polysomes is lower than that found in the free subunit pool or in the total cellular rRNA pool. This suggests that permissive conditions are selective against the C1400U substitution, indicating that it may exert a detrimental effect in a wild-type EF-G background. This is consistent with the increase in doubling times observed in all strains containing the C1400U/A2058G combination (Table 2). Similar effects, however, do not appear to result from the C1192U mutation (Fig. 1A), which also resides in the decoding region of the ribosome (Dontsova et al., 1992; Moine & Dahlberg, 1994), as both PEM100 pSTL102 and JM83 pSTL102 display approximately equal distribution of mutant 16S rRNA across all fractions.

One model to explain the mechanism by which these particular rRNA mutations suppress the ts defect of

EF-G G502D is that they restore the binding of EF-G G502D to the ribosome, at least to an extent allowing limited elongation to occur. In the context of the proposed role of EF-G in modulating communication between the peptidyltransferase and decoding regions, restored binding of the protein would effectively restore the link between these two functional centers on the ribosome. Previous genetic experiments identified frameshift suppressor mutations in domain V of 23S rRNA that provides support to the concept of a functional association between the decoding center and the peptidyltransferase region (O'Connor & Dahlberg, 1993). Although the existence of such a cooperative link has been suspected, there has been a lack of conclusive evidence demonstrating how the domains communicate (Bogdanov et al., 1995). The communication that is modulated by EF-G may be conveyed via structural perturbations associated with the hydrolysis of GTP; indeed, it is by this "molecular-switch" mechanism that other GTPases mediate their effects (Bourne et al., 1990; Bourne, 1995). In effect, the binding of EF-G to the ribosome and the subsequent hydrolysis of GTP would occur upon receiving the "all-clear" signal from the peptidyltransferase center. Hydrolysis of GTP may allow the proper association of EF-G domain IV with the decoding region of the ribosome, consequently promoting translocation (Rodnina et al., 1997). The transition of the ribosome from the pretranslocational to posttranslocational state would result in the dissociation of the EF-G-GDP (Inoue-Yokosawa et al., 1974).

Results from the western blot analysis (Fig. 2) support the model. The rRNA mutants protect EF-G G502D from degradation, presumably by restoring function as they effect proper EF-G-ribosome interactions during the elongation cycle. The requirement for both a 16S and a 23S mutation further supports the concept that the mechanism of suppression involves the restoration of a link between the two ribosomal subunits at the decoding region and the peptidyltransferase domain.

MATERIALS AND METHODS

Bacterial strains and plasmids

Escherichia coli ts strain PEM100 harbors a missense mutation in *fusA* (Hou et al., 1994b). The mutated gene product (EF-G G502D) contains an aspartic acid residue in place of a glycine residue at position 502 in the amino acid sequence. As a result the strain displays slower elongation rates at 30 °C in vivo, and is completely restrictive for growth at 42 °C. *E. coli* strain JM83 (*ara* Δ*(lac-proAB) ϕ80 lacZ*Δ*M15*) (Hou et al., 1994b) is the parent strain of PEM100 and was routinely used as a control strain. *E. coli* K-12 strain SB221 (*lpp hsdR* Δ*trpE5/leuB6 recA1/F' laqI^q lac$^+$ pro$^+$*) (March & Inouye, 1985) and strain DH1 (*sup*E44 *hsd*R17 *rec*A1 *end*A1 *gyr*A96 *thi*-1 *rel*A1) (Hanahan, 1983) were employed for plasmid maintenance and plasmid isolation procedures.

The plasmid pECEG contains the wild-type *fusA* gene under the control of the tandem promoter *lpp^{P5}lac^{PO}* (Hou et al., 1994a). Hence the production of EF-G from this plasmid is induced in the presence of IPTG. All plasmids employed for suppressor analysis were derivatives of pKK3535, which contains the entire *E. coli rrn*B (rRNA) operon (Brosius et al., 1981). The derivatives pKK1192U and pKK1400U encode for 16S rRNAs containing the C1192U and C1400U substitutions, respectively; pKK2058G encodes for 23S rRNA containing the A2058G substitution. The plasmid pSTL102 encodes both the C1192U substitution in 16S rRNA (conferring resistance to spectinomycin) and the A2058G substitution in 23S rRNA (conferring resistance to erythromycin) (Triman et al., 1989). A derivative of pSTL102, pKK1400U/2058G, was constructed from *Bgl*II-*Xba*I restriction fragments of pKK1400U and pSTL102. It encodes both C1400U in 16S rRNA and A2058G in 23S rRNA, conferring spectinomycin sensitivity and erythromycin resistance.

Hydroxylamine mutagenesis

Hydroxylamine mutagenesis was used to isolate rRNA mutations that suppress the ts phenotype of PEM100 (Humphreys et al., 1976). Mutant plasmids were transformed into PEM100 and incubated at 42 °C. Plasmid DNA was isolated from colonies and retransformed to confirm suppression of the ts phenotype of PEM100.

Ribosomal RNA analysis

Cultures were grown in LB medium (5 g NaCl/L), 200 μg mL ampicillin, at either 30 °C or 42 °C to an A_{430} of 0.8 (*N.B.*: for cultures of PEM100 pSTL102 and PEM100 pKK1400U/2058G grown at 42 °C a large inoculum was used to reach an A_{430} of 0.8 within approximately 3 h). Cells were collected by centrifugation and ribosomes and total cellular RNA extracted (Godson & Sinsheimer, 1967).

Polysomes, 70S, 50S, and 30S ribosomal fractions were separated by sucrose density gradient centrifugation (De Stasio & Dahlberg, 1990), and the mutant and wild-type rRNAs were extracted and analyzed by primer extension (Sigmund et al., 1988).

Determination of cellular EF-G content

Western blot analysis was used to determine the cellular EF-G content after shifting cultures to 42 °C. Cultures were grown in LB medium at 30 °C to an A_{430} of 0.4. One half of each culture volume was then shifted to 42 °C and incubations were continued for 3 h, after which 1-mL samples were removed from all cultures and cells were collected by centrifugation. Cells were lysed in SDS-sample buffer and equal amounts of total protein were separated by SDS-PAGE (March & Inouye, 1985) and then transferred to nitrocellulose by semidry electroblotting. The EF-G content was determined by immunoblotting using anti-EF-G antiserum, an Amersham ECL Plus western blotting detection system and a BioRad FlourS MultiImager.

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