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Dominant lethal mutations in a conserved loop in 16S rRNA

(site-directed mutagenesis/rRNA mutations/λ P_L promoter/ribosomal A site)

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ABSTRACT **The 530 stem–loop region in 16S rRNA is among the most phylogenetically conserved structural elements in all rRNAs, yet its role in protein synthesis remains mysterious. G-530 is protected from kethoxal attack when tRNA, or its 15-nucleotide anticodon stem–loop fragment, is bound to the ribosomal A site. Based on presently available evidence, however, this region is believed to be too remote from the decoding site for this protection to be the result of direct contact. In this study, we use a conditional rRNA expression system to demonstrate that plasmid-encoded 16S rRNA genes carrying A, C, and T point mutations at position G-530 confer a dominant lethal phenotype when expressed in *Escherichia coli*. Analysis of the distribution of plasmid-encoded 16S rRNA in ribosomal particles, following induction of the A-530 mutation, shows that mutant rRNA is present both in 30S subunits and in 70S ribosomes. Little mutant rRNA is found in polyribosomes, however, indicating that the mutant ribosomes are severely impaired at the stage of polysome formation and/or stability. Detailed chemical probing of mutant ribosomal particles reveals no evidence of structural perturbation within the 16S rRNA. Taken together, these results argue for the direct participation of G-530 in ribosomal function and, furthermore, suggest that the dominant lethal phenotype caused by these mutations is due primarily to the mutant ribosomes blocking a crucial step in protein synthesis after translational initiation.**

Ribosomes are complex ribonucleoprotein particles that are responsible for the synthesis of proteins in all cells. An increasing body of evidence indicates that the essence of ribosomal function resides in its rRNA components (reviewed in refs. 1 and 2). Evidence for the direct involvement of 16S rRNA in the association of tRNA with ribosomes was first provided in early biochemical studies (3, 4). Rapid chemical footprinting methods (5, 6) have subsequently allowed for the identification of specific nucleotides in 16S rRNA that interact with tRNA (7). These residues are conserved in virtually all organisms and lie within phylogenetically constant structural elements (8, 9). More recently, these interacting residues have been unambiguously assigned to the ribosomal A or P sites, as conventionally defined (10).

Residues protected by A-site-bound tRNA form two clusters, within the 1400–1500 region and in the 530 stem–loop region, in relation to the phylogenetically derived secondary structure model for 16S rRNA (7, 10). Results of immunoelectron microscopy and model-building studies (11, 12) indicate that these regions are separated by a distance of 75–100 Å. The 1400–1500 region has been localized to the cleft of the 30S subunit (13), the site of codon–anticodon interaction (11, 14), whereas the 530 loop has been placed on the opposite side of the subunit, near ribosomal proteins S4, S5, and S12 (15). As all of the tRNA-dependent protections in 16S rRNA are obtained with the 15-nucleotide anticodon stem–loop fragment from $tRNA^{Phe}$, it seems unlikely that the protections in the 530 loop result from direct contact with tRNA. Rather, it has been argued that these protections are induced allosterically, in response to tRNA-dependent interactions at the decoding site (7).

It has long been realized that the base sequence of the 530 loop is one of the most highly conserved in nature (9, 16). The functional importance of this region was first suggested when G-530 was shown to be protected from kethoxal attack in tRNA-occupied ribosomes (4). More recently, it has been demonstrated that G-530, as well as the neighboring U-531, belong to the group of A-site-protected bases in 16S rRNA (10). To test directly the possibility that G-530 is important in ribosomal function, we systematically changed this residue to each of the other three possible bases. Here we demonstrate that 16S rRNA genes carrying these mutations at position 530 are lethal when expressed in *Escherichia coli*, despite the presence of seven wild-type rRNA operons in the host genome. We show that mutant rRNA is incorporated into 70S ribosomes and that these ribosomes are deficient at the stage of polysome formation and/or stability. In addition, we find no evidence for structural perturbation within ribosomes containing mutant rRNA. We conclude that residue G-530 plays a direct role in ribosomal function and, furthermore, that the mutant ribosomes are impaired primarily in elongation and, as a consequence, block protein synthesis after formation of a 70S initiation complex. These results are entirely consistent with evidence suggesting that this residue is involved in some aspect of the interaction of tRNA with the ribosomal A site.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Media. Strains DH1 (*RecA*) and GM2929 (*RecA*, *dam*$^-$, *Cam*^{r}) have been described (17). Strain CJ236 (*ung*$^-$, *dut*$^-$, *Cam*^{r}) and helper phage VCSM13 were provided by C. Craik (University of California, San Francisco). Strains C600 (λ*cI857*, *cro27*, S7) and NO3203 (λ*bio252*, *cI857*, ΔHI, *RecA*), used as hosts for experiments involving rRNA-encoding DNA mutations under control of the λ P_L promoter, were provided by M. Nomura (University of California, Irvine) and R. Gourse (University of Wisconsin). Plasmid pSTL102, a derivative of pKK3535 (18) containing the entire *E. coli rrnB* operon and selectable antibiotic resistance markers in the 16S and 23S rRNA genes, has been described (17). Plasmid pLK35, a derivative of pL *rrnB* (19), has been described (20). Physiological experiments were carried out in LB medium containing 10 g of tryptone, 5 g of yeast extract, 10 g of NaCl, and 100 mg of ampicillin per liter. Solid medium contained, in addition, 15 g of Bacto-agar per liter.

Quantitation of Plasmid-Encoded rRNA. Primer-extension analysis of plasmid-encoded rRNA, distinguishable by a C to U mutation at 1192 in 16S rRNA, was performed by the method of Morgan and coworkers (21) using a modified procedure (17). Relative band intensities of autoradiographs were determined by transmittance densitometry.

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RESULTS

Expression of G-530 Mutations Is Lethal. Site-directed mutagenesis (22, 23) was used to introduce A, C, and T point mutations at position G-530 in the 16S rRNA gene. We were unable to obtain expression of these mutant genes under control of the natural *rrnB* promoters. This result suggested that high-level, constitutive expression of these mutant genes is lethal to the host cell.

We tested this possibility further by the conditional rRNA expression system described by Gourse *et al.* (19). We placed the *rrnB* operon under transcriptional control of the bacteriophage λ P_L promoter/operator, by constructing plasmid pLK45 (Fig. 1*A*). Transcription is regulated by maintaining this plasmid in strain NO3203, which harbors the *cI857* temperature-sensitive allele of the *c*I λ repressor. Similar systems have been used to study deleterious mutations in the 1400–1500 region (24) and 3′ terminus (25) of 16S rRNA and in domain V of 23S rRNA (26).

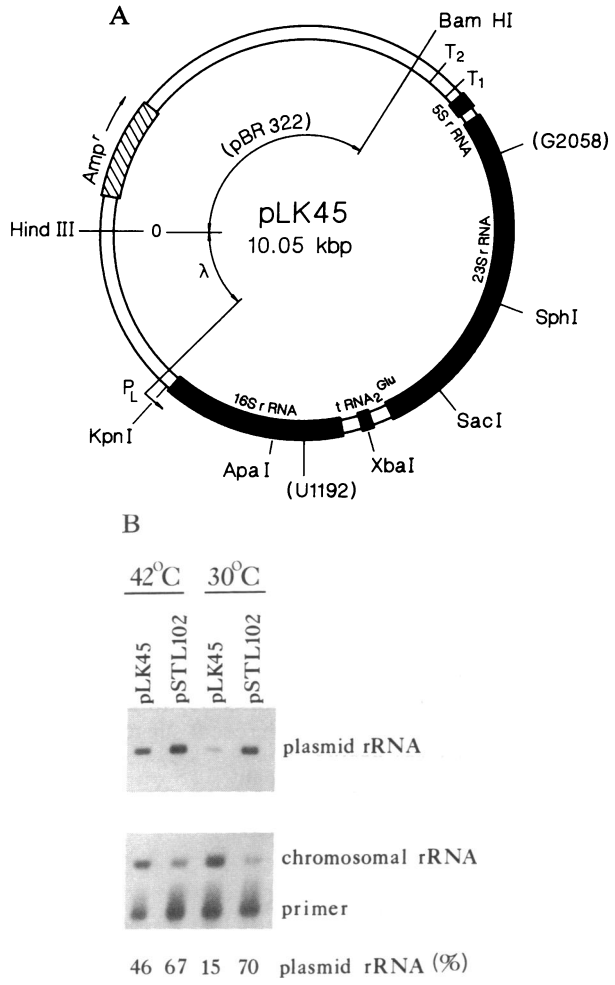

FIG. 1. rRNA expression system used in this study. (*A*) Plasmid pLK45 contains the *rrnB* operon from *E. coli*, under inducible control of the λ P_L promoter, and carries base changes in 16S and 23S rRNA genes allowing for quantitation of plasmid-encoded rRNA incorporated into ribosomes. pLK45 was constructed by introducing the antibiotic-resistance mutations present in plasmid pSTL102 (17) into plasmid pLK35 (20) (details will be published elsewhere). kbp, Kilobase pairs. (*B*) Primer-extension analysis of 16S rRNA extracted from ribosomes after isolation from strain NO3203, carrying plasmids pLK45 or pSTL102, and grown at either 30°C or 42°C. Cells were grown to an A_{650} of 0.8, harvested, and lysed, and rRNA was prepared as described (17). The proportion of plasmid-encoded rRNA in the various preparations is indicated.

Plasmid pLK45 also contains a C to U mutation at position 1192 of the 16S rRNA gene, which confers resistance to spectinomycin when expressed from the strong *rrn* promoters on multicopy plasmids (17, 27). While this mutation does not confer resistance to spectinomycin when expressed from the P_L promoter (unpublished observations), it may be used as a marker to discriminate between plasmid and chromosomally encoded rRNA, using a quantitative primer extension assay developed by Morgan and coworkers (21). We determined the relative amount of plasmid-encoded 16S rRNA present in ribosomes obtained from strain NO3203 carrying plasmid pLK45 (Fig. 1*B*). At 42°C (the "induced" temperature), ≈45% of the rRNA present is plasmid derived, whereas at 30°C plasmid-encoded rRNA accounts for only 15% of the total. This latter value is indicative of the "leakiness" in this system. As a control, we also prepared rRNA from NO3203 cells containing plasmid pSTL102 (17), which carries the 16S rRNA gene with the *spc*^{r} marker under the control of the natural, constitutive *rrnB* promoters (Fig. 1*B*). At both temperatures, the level of plasmid-derived rRNA is ≈70% of the total, in agreement with the results of Morgan and coworkers (21).

Mutant derivatives of pLK45, containing the G530 mutations, were introduced into host NO3203 for testing. Cell cultures carrying the mutant plasmids grew identically at 30°C to the culture carrying the control plasmid, whereas induction of mutant rRNA genes at 42°C resulted in an immediate decrease in growth rate and cessation of growth within several generations (Fig. 2*A*). This result was striking on selective plates as well; diluted cell cultures containing mutant plasmids grew identically to cells containing the control plasmid at 30°C but were completely inviable when grown at 42°C (Fig. 2*B*).

In a recent study, suppression of a deleterious mutation in the 16S rRNA gene was observed when the U-1192 mutation was carried in *cis* (28). To control for such pleiotropic effects, the G-530 mutations were placed into plasmid pLK40, identical to pLK45 but lacking the *spc*^{r} marker, and were introduced into NO3203. Upon induction, results identical to those described above were obtained (data not shown), demonstrating that the U-1192 mutation does not affect the phenotype of the G-530 mutants.

The A-530 Mutation Interferes with Polysome Formation. To examine the effect of these mutations on ribosomal assembly and function, one mutant, A-530, was studied in detail. Ribosomes and polyribosomes were prepared (29) from strain NO3203, carrying either plasmid pLK45 or pLK45/A-530, after induction of plasmid rRNA genes, and were separated on sucrose gradients (Fig. 3). The U-1192 marker was then used to monitor the presence of plasmid-encoded 16S rRNA in the various gradient peaks (Fig. 4). Analysis of gradient fractions containing control plasmid rRNA showed that ≈40% of the rRNA in free 30S subunits came from the plasmid. This ratio increased to 50% in the 70S ribosome and polysome fractions. In contrast, analysis of rRNA from the gradient prepared from cells containing the mutant plasmid revealed both a detectable increase in the level of plasmid-borne rRNA in the free 30S peak and a corresponding decrease in the 70S ribosome peak (Fig. 4). The most dramatic difference, however, was the scarcity of mutant rRNA present in the polysome peaks (10% or less).

We also determined the amount of plasmid-encoded 23S rRNA present in the various gradient fractions by using the erythromycin-resistance marker in pLK45 (data not shown). Equivalent amounts of plasmid-derived 23S rRNA were detected in the ribosome and polyribosome fractions for both the mutant and control plasmid. This indicates that the differences shown in Fig. 4 for cells carrying pLK45 versus pLK45/A-530 are indeed due to the presence of the mutant 16S rRNA.

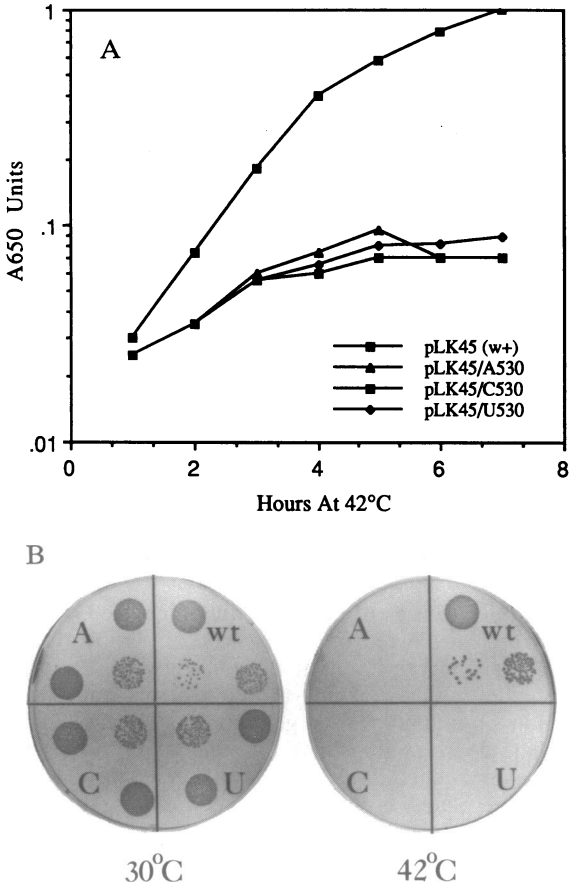

FIG. 2. Expression of rRNA genes carrying mutations at position G-530 in 16S rRNA is lethal. (*A*) Growth curves of strain NO3203 carrying control pLK45 or mutant plasmids grown at the nonpermissive temperature of 42°C. (*B*) Induction on solid medium. NO3203 containing pLK45 control (indicated as wt) or mutant plasmids were grown at 30°C (the permissive temperature) to an A_{650} of 0.8. Twenty-five microliters of 10^{-2}, 10^{-3}, and 10^{-4} dilutions was plated on solid medium and incubated at either 30°C or 42°C for 24 hr.

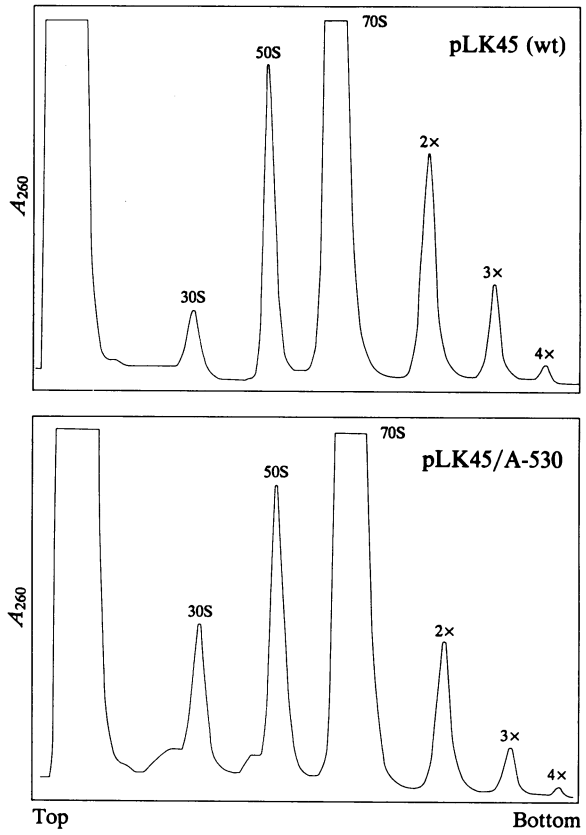

FIG. 3. Polysome profiles from NO3203, carrying either control pLK45 or mutant pLK45/A-530 plasmids. Freshly inoculated 80-ml cultures were grown at 30°C until an A_{650} of 0.1 and were shifted to 42°C for an additional 90 min (final A_{650}, ≈0.4). Cells were rapidly chilled and polysomes were prepared by the method of Ron *et al.* (29). Lysates were layered onto 10-ml 10–40% sucrose gradients, made in buffer containing 20 mM Tris·HCl (pH 7.8), 10 mM $MgCl_2$, 100 mM NH_4Cl, 2 mM dithiothreitol, and were centrifuged in an SW-41 rotor for 2.5 hr at 35,000 rpm and 4°C. Gradients were analyzed by using an ISCO gradient fractionator, and 0.375-ml fractions were collected.

We sought to determine the significance of the shoulder at the leading edge of the 30S subunit peak observed in the mutant gradient profile (Fig. 3); 5′-end mapping of the rRNA present in the 30S shoulder revealed the presence of rRNA species longer than mature-length 16S rRNA, suggesting that rRNA processing and/or assembly is blocked or otherwise perturbed (data not shown). The results of the rRNA analysis in Fig. 4, however, showed that the shoulder is not significantly enriched for mutant rRNA, which indicates that the mutant particles are not preferentially blocked at this point. Thus, the 30S shoulder most likely reflects a general decrease in subunit assembly and/or processing, resulting from expression of the lethal A-530 mutation (for example, due to a shortage of ribosomal proteins). Such a general effect may also account for the 50S subunit shoulder observed in the mutant profile (Fig. 3).

Higher-Order Structure of 16S rRNA in the A-530 Mutant. Detailed chemical probing, monitored by primer extension (5, 6), was used to examine the higher-order structure of the mutant 16S rRNA in 70S ribosomes. Here we sought to determine whether the deleterious nature of the G-530 mutations is accompanied by detectable structural perturbations, either within the 530 stem–loop itself or at other sites involved in tRNA binding.

A chemical probing analysis of A-site residues is presented in Fig. 5. Here we are necessarily probing a mixed population of ribosomes, containing both mutant and wild-type rRNA, where the relative proportion of mutant 16S rRNA is, as indicated above, approximately one-third of the total. Accordingly, we detect a decrease in the reactivity of position 530 to kethoxal, a guanine-specific probe, in the ribosome pool containing the A-530 mutant (Fig. 5*A*). Interestingly, we also detect decreased reactivity of G-529 to kethoxal in the mutant ribosomes, supporting the recent suggestion (10) that

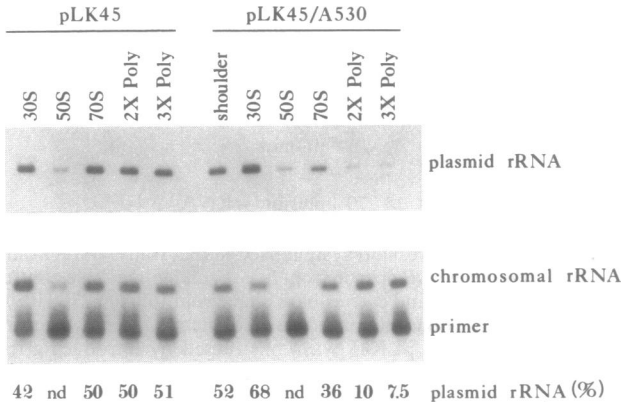

FIG. 4. Primer-extension analysis of rRNA extracted from the various peaks shown in Fig. 3. The relative proportion of plasmid-encoded rRNA is indicated. Poly, polysome; shoulder, the fraction of the mutant gradient containing the shoulder at the leading edge of the free 30S subunit peak; nd, not determined.

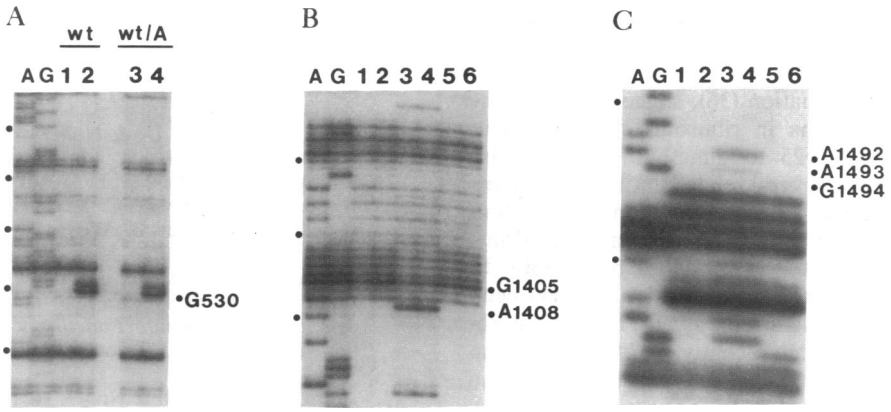

FIG. 5. Results of chemical probing of A-site residues in 70S ribosomes, prepared from NO3203 carrying either pLK45 or pLK45/A-530 plasmids, after induction of plasmid-encoded genes. (*A*) The 680 primer. Lanes: 1 and 3, unmodified control samples; 2 and 4, kethoxal-modified samples. wt, Samples obtained from cells carrying pLK45 control plasmid. (*B*) The 1490 primer. (*C*) The 1508 primer. Sources of template 16S rRNA in *B* and *C* were as follows: lanes 1, 3, and 5, pLK45 control rRNA; lanes 2, 4, and 6, mutant rRNA. Modification reagents used in *B* and *C* were as follows: lanes 1 and 2, unmodified controls; lanes 3 and 4, dimethyl sulfate; lanes 5 and 6, kethoxal. A and G refer to dideoxynucleotide sequencing lanes using rRNA obtained from strain MRE600 as template. Plasmid-encoded rRNA genes were induced and ribosomes were prepared as described in Fig. 3. Fractions containing 70S ribosomes were pooled and modified with dimethyl sulfate and kethoxal and sites of modification were identified by primer extension using reverse transcriptase (5, 6).

the stop prior to position 529 is, in fact, a "stutter" by reverse transcriptase normally pausing before G-530. We fail to detect any other differences in reactivities anywhere in the 16S rRNA chain, including other residues within the 530 loop and other A-site residues in the 1400 (Fig. 5*B*) and 1500 (Fig. 5*C*) regions. These results suggest that mutant and wild-type ribosomes are essentially isomorphic, although subtle differences may have been missed due to the wild-type background present in this assay.

DISCUSSION

We have used a conditional rRNA expression system, utilizing the λ P_L promoter/operator, to demonstrate that point mutations at position G-530 in 16S rRNA are lethal when expressed in *E. coli*. The presence of mutant rRNA in fully assembled ribosomal particles argues strongly that the lethal phenotypes we observe are expressed at the level of ribosomal function, despite the fact that over half of the ribosomes in the cell contain wild-type rRNA. A likely explanation for this dominant lethality is that there is a competition between ribosomes for components of the translational machinery that are present in relatively substoichiometric amounts within the cell. If the mutant ribosomes sequestered one or more of these components, thus rendering them unavailable to the wild-type ribosomes, translation would become arrested. Given this, we would expect the distribution of mutant rRNA within the various ribosomal fractions to reflect, at least to a first approximation, the step at which the mutant ribosomes are impaired (Fig. 4). The significant amount of A-530 mutant rRNA in the 70S ribosomal fraction, versus its relative paucity in the polysome fractions, suggests that the mutant ribosomes are impaired primarily in translation after the formation of the 70S initiation complex at some step in elongation. In addition, the increased proportion of mutant rRNA in the 30S subunit fraction indicates that the mutant ribosomes may be partially deficient in initiation and/or subunit association. Thus its universal conservation, the results of tRNA binding experiments (7, 10), and the results of this study lead us to the conclusion that residue G-530 participates directly in a critical ribosomal function involving the association of tRNA with the ribosomal A site.

Our results also indicate that the lethal nature of these mutations depends on gene dosage. Cells containing mutant plasmids display growth properties identical to those carrying the control plasmid pLK45 at 30°C, the repressed temperature (Fig. 2*B*). At this temperature, however, the proportion of mutant rRNA present in total ribosomal particles is nevertheless ≈15% (Fig. 1*B*) due to the leaky expression from the repressed P_L promoter. A minimum level of mutant rRNA (between 15% and 45%) must accumulate, therefore, before the lethal nature of the G-530 mutants is expressed. An attractive possibility is that this ratio correlates with the number of mutant ribosomes required to titrate the available initiation sites on mRNA in competition with wild-type ribosomes.

Fig. 6 summarizes our present understanding of several functional aspects of the 530 stem–loop. In addition to the relationship between residues G-530 and U-531 and A-site tRNA, there is evidence to suggest that the 530 stem–loop plays some role in maintaining translational accuracy. This structure is proximal to and interacts with ribosomal proteins

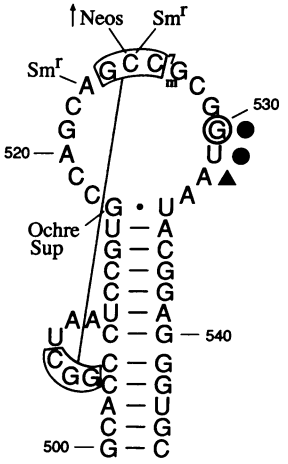

FIG. 6. Summary diagram indicating functionally important residues in the 530 loop. Residue G-530, the site where mutations confer a dominant lethal phenotype (this work), is circled. ●, A-site tRNA protections (10); ▲, P-site tRNA protections (10). C-525, which displays increased dimethyl sulfate reactivity in the presence of neomycin and related antibiotics (↑ Neos) (30), is indicated as are the sites of mutations resulting in ochre nonsense suppression in yeast mitochondria (31) and streptomycin resistance in chloroplasts and/or in *E. coli* (32, 33). Also indicated is the proposed tertiary interaction between residues 505–507 and 524–526 (34).

S4 and S12 (12), mutations in which affect the translational error frequency (35). Interestingly, several residues in the loop, including G-530, depend on S4 and/or S12 for their mature (and active) conformation (36), suggesting one possible role for these proteins in ribosomal function. The enhanced reactivity of C-525 to dimethyl sulfate in the presence of neomycin and several related antibiotics that are known to induce miscoding affirms a functional connection between the 530 loop and the decoding site, as these drugs also footprint residues located in the latter region (30). In addition, an A to C transversion at position 523 has been shown to confer resistance to streptomycin, both in chloroplasts and in *E. coli* (32). More recently, a C to U transition at position 525 (*E. coli* numbering) in chloroplasts has also been demonstrated to confer resistance to streptomycin (33). Lastly, an ochre nonsense suppressor has been identified in yeast mitochondrial 15S rRNA (31). This mutation is a G to A transition at position 517 (*E. coli* numbering), resulting in an A/C mismatch at the top of the 530 stem. This latter result provides direct evidence for a functional relationship between this structure and the process of codon recognition. All of the ribosomal protein and rRNA mutations that affect translational accuracy, as well as the antibiotics that induce miscoding, have an effect on the archetypal ribosomal event of A-site tRNA binding. Their effect, however, is peripheral in nature, in that it results in perturbation of the system but does not disable it. The bases involved in mutations conferring resistance to these drugs, as well as the sites of suppressor mutations, are accordingly, only moderately conserved phylogenetically. In contrast, the dominant lethal phenotypes resulting from mutation of G-530 are more in line with what would be expected if some crucial aspect of the mechanism itself were destroyed, as in altering the catalytic amino acid residue at the active site of a protein enzyme. It is interesting to note that the presumed reason for the lethality caused by the mutant ribosomes essentially amounts to the formation of aberrant initiation complexes, recalling the basis of the bactericidal action of streptomycin (reviewed in ref. 37).

The precise function of the 530 loop remains unknown. Its presumed distance from the site of codon–anticodon interaction suggests an involvement in A-site tRNA binding that is allosteric in nature. Elongation factor Tu has been mapped by immunoelectron microscopy to a region of the 30S subunit that is proximal to the location of the 530 loop (11); there is no direct evidence, however, for an interaction between them. Identification of the precise step during translation at which the G-530 mutants are blocked will provide an important link in our understanding of the intriguing role of the 530 loop in protein synthesis.

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