Functional Effects of a G to U Base Change at Position 530 in a Highly Conserved Loop of *Escherichia coli* 16S RNA†

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ABSTRACT: Any base change at position 530 introduced into *Escherichia coli* on a multicopy plasmid leads to cell death [Powers & Noller (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 1042–1046]. It was suggested that these mutants cannot carry out chain elongation. To define more precisely the function of base 530, we have studied ribosomes in which G530 was mutated to U530. *In vivo*, U530 16S rRNA was incorporated into 30S subunits and could combine with 50S to make 70S ribosomes. 16S rRNA *in vitro* transcripts containing U530 were assembled into 30S ribosomes, and their activity was tested in defined steps of protein synthesis. Mutant 30S ribosomes were as active as wild-type in poly(U)-dependent poly(Phe) synthesis, P- and A-site tRNA binding, and 30S initiation complex formation. 30S initiation complexes, in the presence of 50S, could react with puromycin like the wild-type. The rate, extent, and position of cross-linking of AcVal-tRNA in the P site to 16S RNA were identical in mutant and wild-type ribosomes. Although there appeared to be no defect in 70S initiation complex formation or in direct A-site binding of Phe-tRNA dependent on poly(U), U530 30S ribosomes were nevertheless defective in carrying out synthesis of fMet-Val dipeptide using natural mRNA. Mutant 30S ribosomes were also refractory to streptomycin-induced misreading although no misreading was observed in its absence. The defect in U530 ribosomes may be due to a block in correct binding of the first aminoacyl-tRNA, to infidelity in translation due to frameshifting, or to some other aspect of A-site interaction related to the use of natural mRNA instead of poly(U).

The 530 loop region of the 5′ domain of 16S rRNA (Figure 1) is highly conserved (Gutell et al., 1985), and contributes to a number of important ribosome functions (Moazed & Noller, 1987, 1990; Gauthier et al., 1988; Melançon et al., 1988; Shen & Fox, 1989; Woese & Gutell, 1989; Powers & Noller, 1990). The sequence GGU at positions 529–531 is universally conserved in small subunit rRNAs (Gutell et al., 1985) and appears to be part of the A site since tRNA in the presence of mRNA protects these bases from kethoxal addition (Moazed & Noller, 1990). Any base substitution for G530, introduced into *Escherichia coli* via a plasmid, leads to cell death despite the presence in the cell of an approximately equal quantity of chromosome-derived wild-type ribosomes (Powers & Noller, 1990). These mutations are, therefore, dominant lethal mutants, similar to mutations at position 1538 (Jacob et al., 1987), the 1400 region (Rottmann et al., 1988; Thomas et al., 1988), and positions 2060 and 2450 in 23S rRNA (Vester & Garrett, 1988). Thus, when any of the 530 mutants were cloned into the plasmid under control of the temperature-sensitive P_L repressor so that at 30 °C the cell remained viable, the cells ceased growing when the repressor was inactivated at 42 °C. Mutant 16S rRNA containing A530 was found in 70S ribosomes, but little in polysomes (Powers & Noller, 1990). These authors suggested that because the 530 mutant ribosomes were not present in polysomes the mutation interfered with chain elongation. We have attempted to define more precisely the functional defect of the mutant ribosomes. For this, we have used the U530 mutant (Figure 1) in a variety of procedures including *in vitro* protein synthesis assays done with 30S subunits reconstituted from *in vitro* synthesized mutant 16S rRNA.

Our data demonstrate that mutant 530 ribosomes are able to carry out elongation reactions of protein synthesis under standard assay conditions but are nevertheless deficient in making the first dipeptide from a natural mRNA.

MATERIALS AND METHODS

Bacteria, Plasmids, and Bacteriophage. *E. coli* HB101 and *E. coli* BL21/DE3 (Steen et al., 1986) have been previously described. *E. coli* BL21/DE3 contains a chromosomal gene for T7 RNA polymerase under control of the UV5 lac promoter. Plasmid pAR3056 contains the rrnB operon under control of the T7 promoter (Studier & Moffatt, 1986). Plasmid pKK3535 contains the rrnB operon (Brosius et al., 1981). pAR3056 with the *Sma* 1–6 deletion was constructed from pKK3535 with this deletion. The latter plasmid and a derivative of plasmid pNO2068 lacking one *Hin*dIII restriction site were obtained from A. E. Dahlberg. pWK1, a derivative of pUC19 capable of directing the *in vitro* synthesis of full-length 16S RNA by T7 RNA polymerase, has been described (Kryzosiak et al., 1988).

Chemicals and Reagents. Carrier-free [^{32}P]orthophosphate was purchased from New England Nuclear, Inc. Restriction enzymes and T4 DNA ligase were purchased from New England BioLabs. The Muta-Gene kit was obtained from BioRad, Sequenase was from U. S. Biochemical Corp., and Geneclean was from Bio101, La Jolla, CA. *E. coli* $tRNA^{Leu_5}$ (anticodon N*AA) and $tRNA^{Phe}$ were obtained from Subriden RNA, Rolling Bay, WA. They were aminoacylated with [^{3}H]leucine (237 200 dpm/pmol) and [^{14}C]phenylalanine (1028 dpm/pmol) to levels of 870 and 1200 pmol/A_{260} unit,

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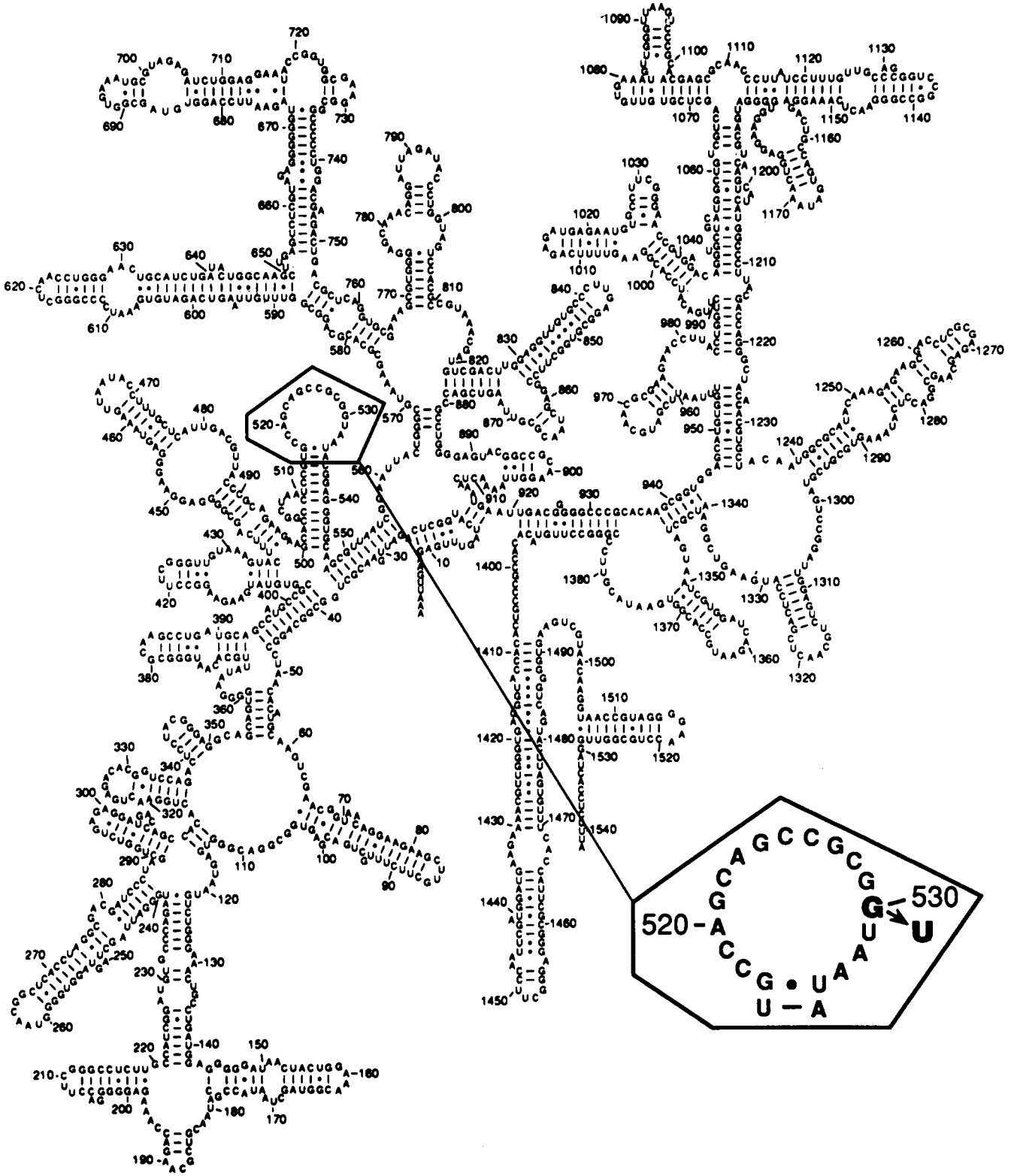

FIGURE 1: Location of the site of mutation. The secondary structure of *Escherichia coli* 16S RNA is according to Stern et al. (1989). The inset shows the site of the mutation described in this work in boldface type.

respectively. All other materials were obtained or prepared as in Denman et al. (1989a,b) or Cunningham et al. (1990, 1992a).

Site-Specific Mutagenesis. Site-specific mutagenesis at base 530 was carried out using standard procedures (Kunkel et al., 1987). Screening for mutants was facilitated by the fact that a G to T change abolished a *Sac*II site. A base change was verified by DNA sequencing (Sanger et al., 1977) of the M13 insert and the reconstructed plasmid.

Labeling of RNA and Ribosomes in Maxicells. E. coli BL21 (DE3) containing plasmid pAR3056 with the 530 mutant was utilized in maxicell experiments. pAR3056 both with the wild-type sequence and containing the *Sma* 1–6 deletion in the 16S rRNA gene were used as controls. The latter plasmid was used to measure the abolition of host chromosomal rRNA synthesis. Labeling of *E. coli* BL21 (DE3) carrying the various plasmids was carried out as described previously (Steen et al., 1986). Ten-milliliter

samples were normally labeled with 2 μCi/mL [^{32}P]orthophosphate for 20 min. Ribosomes were obtained from ^{32}P-labeled cells and separated on composite agarose–acrylamide gels (Stark et al., 1982).

In Vitro Preparation of Mutant Ribosomes. Plasmid pAR3056 containing the U530 mutation (see text) was cleaved at the single *Bst*EII site corresponding to G1504 of 16S RNA. After isolation of the linearized DNA from the reaction mixture by adsorption to and elution from a powdered glass suspension (Geneclean) as described by Cunningham et al. (1990), the DNA was cut with *Avr*II to generate a fragment corresponding to the segment C272–G1504 of 16S RNA. pWK1 was similarly cleaved at its single *Avr*II and *Bst*EII sites. The larger and smaller fragments of both cut plasmids were separated by gel electrophoresis. Ligation of the larger fragment from pWK1 with the smaller fragment from pAR3056 followed by transformation and plasmid purification as described by Cunningham et al. (1990) yielded pHIL98, a pWK1 with a G → U530 mutation. DNA sequence analysis (Cunningham et al., 1990) verified the U530 mutation as well as the correct sequence across both the *Avr*II and *Bst*EII ligation junctions. Transcription was performed according to Cunningham et al. (1991a) except that 5 mM NTP, 3000 units/mL T7 RNA polymerase, 30 μg/mL linearized plasmid, and a 6-h incubation were used. The RNA was isolated by the phenol-S200 method (Cunningham et al., 1991b) and was shown to be intact and full length by denaturing gel electrophoresis (Denman et al., 1989a). Ribosomes were reconstituted on a preparative scale as described by Cunningham et al. (1990) using a TP30/RNA ratio of 3. The presence of U530 in the mutant ribosomes was confirmed by sequencing 16S RNA from the mutant ribosomes. Phenol-extracted RNA was sequenced using reverse transcriptase and a primer complementary to residues 571–590 (Bakin & Ofengand, 1992).

Functional Assays. 70S formation, P-site binding of tRNA, A-site binding of tRNA, polyphenylalanine synthesis, 30S initiation complex formation (I site), fMet-puromycin formation, fMet-Val dipeptide synthesis, and misincorporation were all performed as described (Cunningham et al., 1992a).

Cross-Linking. Reaction and reverse transcription arrest analysis of the site of cross-linking were carried out as described by Cunningham et al. (1992a). Analysis of the extent of cross-linking was done as described previously (Denman et al., 1988).

RESULTS

In Vivo Assembly of 70S. To introduce a site-specific mutation from G to U at position 530, a 568 bp *Hin*dIII restriction endonuclease fragment from pKK3535 (Brosius et al., 1981) covering positions 80–648 of the 16S rRNA gene was cloned into M13 mp19, and mutagenesis was carried out as described (Kunkel et al., 1987). The *Hin*dIII fragment now containing the mutated base was cloned back into the plasmid pKK3535 which was used to transform *E. coli* HB101. No viable clones were recovered, which suggested that the base change at 530 is a lethal mutation. Powers and Noller (1990) have indeed demonstrated that base changes at 530 are dominant lethal mutations. To control the synthesis of mutant 16S rRNA and to determine if mutant rRNA was incorporated into 30S ribosomes, the *Hin*dIII fragment with the mutant 530 base was cloned into plasmid pAR3056. This plasmid contains the rrnB operon under control of the T7 promoter, and in *E. coli* BL21/DE3 which contains the gene for T7 RNA polymerase under control of the UV5 lac promoter, mutant rRNA synthesis can be induced with IPTG. *E. coli* was successfully transformed by plasmid pAR3056 coding for the mutation at position 530, and this cell–plasmid combination was used as a "maxicell" system (Steen et al., 1986) to study the production and assembly of ribosomes from the mutant gene. In this system, rRNA synthesis from chromosomal genes is turned off by rifampicin, while plasmid-coded rRNA synthesis is induced with IPTG, and is labeled with [^{32}P]orthophosphate.

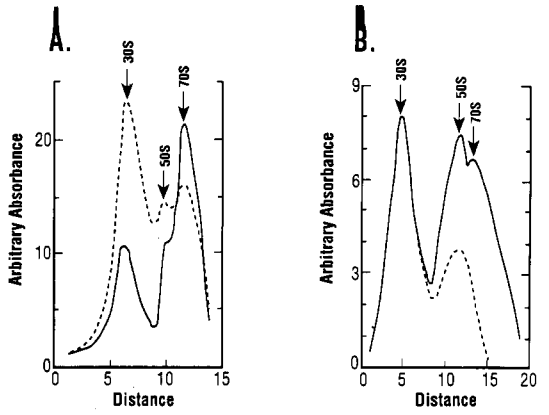

FIGURE 2: Relative *in vivo* 70S association ability of wild-type and mutant 30S ribosomes. Maxicells of *E. coli* BL21 (DE3) with plasmid pAR3056 containing wild-type or mutant base at position 530 were labeled with [^{32}P]orthophosphate after induction of T7 RNA polymerase with isopropyl β-D-thiogalactopyranoside. Lysates prepared in either 10 mM Mg^{2+} (panel A) or 6 mM Mg^{2+} (panel B) buffer were electrophoresed in acrylamide/agarose composite gels (Stark et al., 1982) containing 10 mM Mg^{2+} (panel A) or 6 mM Mg^{2+} (panel B) and autoradiographed. Densitometry of autoradiographs was measured with an LKB Ultrascan XL laser densitometer. G530 (—); U530, (- - -).

Extracts were prepared in 10 mM Mg^{2+} buffers, and the ribosomes were separated in 10 mM Mg^{2+} containing composite gels. In addition, in order to study the stability of 70S ribosomes containing mutant 16S rRNA under conditions where the Mg^{2+} level may more closely approximate *in vivo* conditions, extracts were prepared in 6 mM Mg^{2+} buffers and separated by electrophoresis at 6 mM Mg^{2+}. The densitometer trace of the autoradiographs of each set of separated ribosomes is shown in Figure 2A (10 mM Mg^{2+}) and Figure 2B (6 mM Mg^{2+}). At 10 mM Mg^{2+}, extracts of cells containing the mutant plasmid are depleted in labeled 70S ribosomes while the content of [^{32}P]30S ribosomes is increased compared to cells with the wild-type plasmid. The mutant 70S ribosomes that are present do contain mutant 16S rRNA, since no chromosomal coded 16S rRNA is produced under these experimental conditions (data not shown). At 6 mM Mg^{2+}, labeled 70S, 50S, and 30S ribosomes are present while extracts of cells containing the mutant plasmid have no labeled 70S ribosomes but only ^{32}P-labeled 50S and 30S subunits. These same extracts were analyzed in a two-dimensional gel system which separates ribosomes in the first dimension and rRNA in the second dimension (Stark et al., 1982). In the cells with the wild-type base at 530, there is a 70S ribosome component which contains ^{32}P-labeled 23S and 16S rRNA derived from 50S and 30S ribosomes. On the other hand, extracts obtained from cells containing plasmid coding for U530 have no labeled 70S ribosomes, but do have 50S and 30S ribosomes which contain labeled 23S and 16S rRNA, respectively (data not shown).

It appears that *in vivo* U530 containing 30S ribosomes can form 70S ribosomes, albeit at a reduced level at 10 mM Mg^{2+}. These data corroborate the results of Powers and Noller (1990). Moreover, 70S ribosomes containing mutant 30S subunits

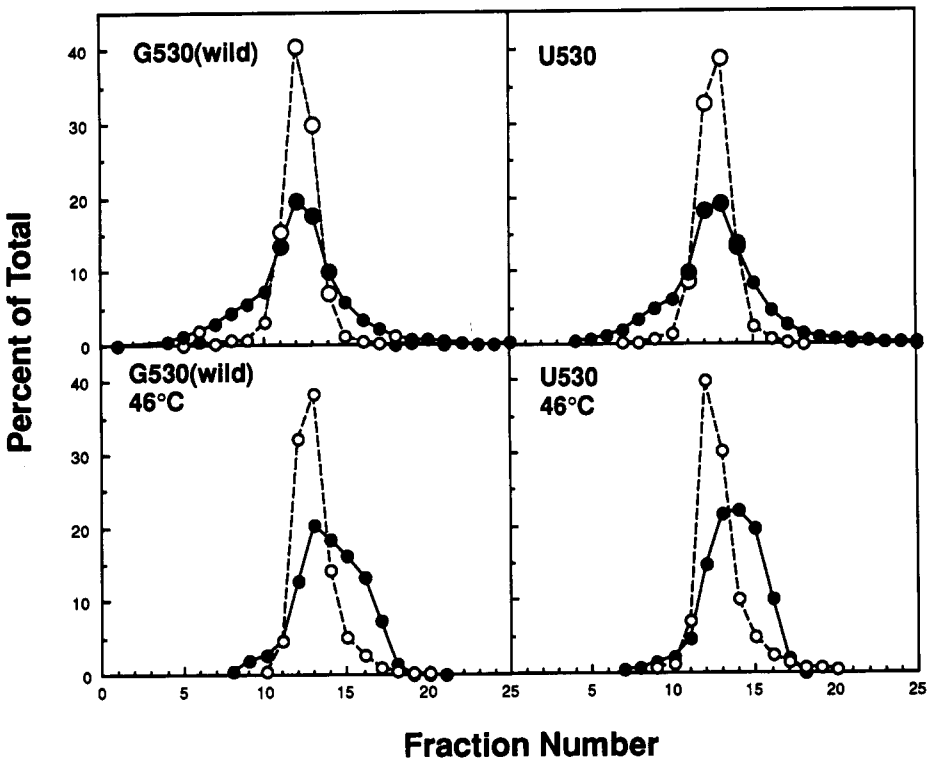

FIGURE 3: *In vitro* reconstitution of U530 and wild-type 30S ribosomes. Reconstitution of *in vitro* transcribed RNA with TP30 was done using 20 and 2 A_{260} units of RNA per sample for the upper and lower rows, respectively. Upper row, reconstitution conditions as described under Materials and Methods. Lower row, as above except that annealing was terminated after the 46 °C incubation. Analysis was by velocity gradient centrifugation. The direction of sedimentation is from right to left. Solid circles, A_{260} of fractions; open circles, marker ^{32}P-labeled 30S ribosomes. The two curves were normalized by expressing the value of each fraction as percent of the total recovered. The fractions shown in larger symbols were pooled for subsequent functional analysis as sample R215.

appear more sensitive to lower Mg^{2+} levels since they dissociate completely at 6 mM. The inability of mutant 30S ribosomes to form stable 70S particles *in vivo* may contribute to the absence of the mutant 30S ribosomes from the polysome fraction (Powers & Noller, 1990).

Protein Synthesis in Vitro. The results of Powers and Noller (1990) indicated that mutant 30S ribosomes were deficient in the polysome fraction but that they could participate in 70S ribosome formation. In order to determine the step in the process of protein synthesis blocked by the U530 mutation, the synthetic ribosome system described by Kryzosiak et al. (1987) was used. The U530 mutation was generated in plasmid pWK1 as described under Materials and Methods, the linearized plasmid was used to transcribe mutant 16S RNA *in vitro* (Kryzosiak et al., 1987; Denman et al., 1989a), and the RNA was reconstituted *in vitro* into mutant 30S particles (Figure 3). The top row shows the results for a preparative-scale reconstitution. Clearly, there was little or no difference in the ability of the U530 mutant to assemble in comparison to that of the wild-type sequence. However, if the annealing regime was terminated at 46 °C, rather than continuing to 50 °C as in the standard procedure (Denman et al., 1989a), there was a marked deficiency in assembly of both the G530 and U530 ribosomes (bottom row of Figure 3). The effect was not due to differences in the scale of the reconstitution as small (2 A_{260} units) reconstitutions under the standard conditions gave essentially the same results as the 20 A_{260} units scale preparation shown in the upper row of Figure 3 (data not shown). Although isolated and fully-modified 16S RNA can assemble correctly at 40 °C, as judged by cosedimentation with authentic 30S particles (Held et al., 1973; Denman et al., 1989a; Cunningham et al., 1990, 1991b, 1992b), the unmodified transcripts, although full-length, cannot do so. The annealing conditions used in our standard

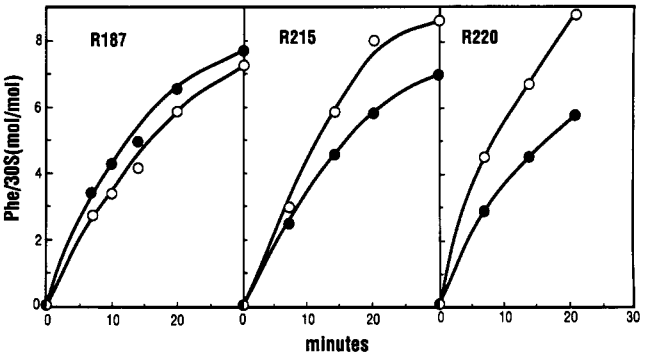

FIGURE 4: Kinetics of polyphenylalanine synthesis with wild-type and U530 ribosomes. Poly(U)-directed synthesis of polyphenylalanine was performed as described under Materials and Methods. Open circles, G530; solid circles, U530. Three independent ribosome reconstitutions are shown.

protocol were arrived at as a solution to the problem of assembling unmodified *in vitro* transcripts.

The mutant and control ribosomes were isolated from large-scale reconstitutions, activated, and used in a variety of *in vitro* assays of ribosome function. Figure 4 shows that the rate of polyphenylalanine synthesis directed by poly(U) was not inhibited by the U530 mutation, and therefore that the process of elongation *per se* is not affected. Three independent reconstitutions of the U530 mutant are shown to illustrate the degree of variability encountered. The rates are quantitated in Table I along with the values for the other assays. Both at 6 mM and at 15 mM Mg^{2+}, the U530 mutants were able to associate with 50S as well as the G530 control. This result is in contrast to the *in vivo* findings with U530. However, as it was necessary to use 15 mM Mg^{2+} in the gradient solutions in order to keep even the G530 30S from dissociating during the course of the analysis, it is possible that these conditions

Table I: Functional Activity of Synthetic 30S Ribosomes Bearing the U530 Mutation[a]

	subunit association		tRNA binding			peptide synthesis	
ribosome	6 mM Mg^{2+}	15 mM Mg^{2+}	P site	A site	I site	$(Phe)_n$	fMet-Val
U530 (R187)	94	81	103	94	117	118	31
U530 (R215)			88	105	90	79	18
U530 (R220)			91	112	91	66	20
	av 95	80	95	105	100	90	25

[a] All values are expressed as percent of control G530 from the same reconstitution. The average value has been rounded to the nearest 5%. The data for fMet-Val synthesis were taken from the plateau portion of Figure 6. The absolute values for G530 (R187) were as follows: for subunit association at 6 or 15 mM Mg^{2+}, 76 or 73%, respectively; for P-site, A-site, or I-site binding, 0.24, 0.29, or 0.30 mol of tRNA bound/mol of 30S, respectively; for $(Phe)_n$, 6.3 mol of Phe/mol of 30S per 20 min. The corresponding values for G530 (R215) for P-site, A-site, or I-site binding and for $(Phe)_n$ synthesis were 0.35, 0.30, 0.27, and 8.3, respectively. For G530 (R220), they were 0.28, 0.31, 0.27, and 8.9.

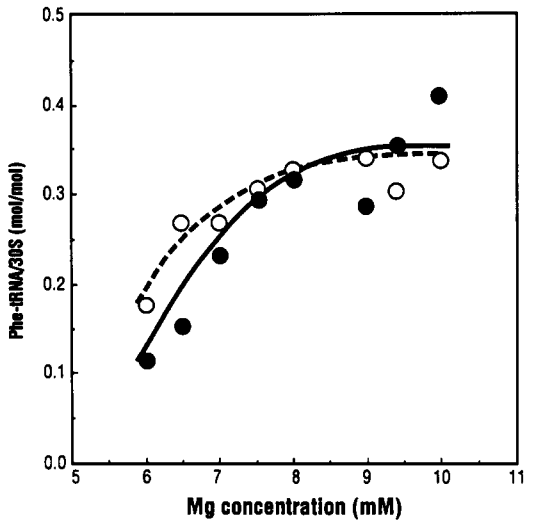

FIGURE 5: Magnesium concentration dependence of A-site binding with control and mutant 30S ribosomes. A-site binding was performed as described under Materials and Methods except that the step 1 reaction mixture was diluted with TC buffer before carrying out step 3. The dilution is plotted as the change in Mg^{2+} concentration. Open circles, G530; solid circles, U530.

failed to distinguish subtle differences between the G530 and U530 ribosomes which are manifested *in vivo*. The U530 30S was not different from the control G530 in message-dependent tRNA binding to the P site or in EFTu-dependent binding to the A site, when supplemented with 50S subunits. Factor-dependent binding to the I site of 30S in the absence of 50S was also not affected. In view of the suggestion by Powers and Noller (1990) that tRNA binding to the A site was affected by the U530 mutation *in vivo*, and our inability to detect any effect *in vitro* (Table I), A-site binding was further examined as a function of Mg^{2+} concentration (Figure 5). In this experiment, we were searching for any evidence that A- site binding was differentially affected by the mutation. The Mg^{2+} concentration in the *in vitro* assay was altered by the simple expedient of dilution of the step 1 reaction mixture with TC buffer before addition of the ternary complex formed in step 2. Thus, while only the Mg^{2+} concentration is plotted, in actuality all of the other components were diluted as well. Despite this more stringent test, dilution did not reveal any major distinction between the control and mutant ribosomes. The slight difference observed at the highest dilution (6 mM Mg^{2+}) is unlikely to be sufficient to explain the lethality observed *in vivo*.

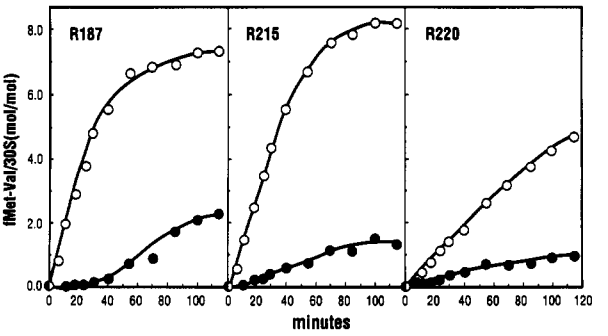

FIGURE 6: Rate of fMet-Val synthesis with control and mutant 30S ribosomes. Reaction was carried out as described under Materials and Methods. Open circles, G530; solid circles, U530. Three independent ribosome reconstitutions are shown.

Table II: Puromycin Reactivity of 70S–fMet-tRNA Complexes[a]

	fMet-PM/fMet-tRNA bound	
time (min)	G530	U530
5	0.92	0.97
10	0.79	0.95

[a] 30S fMet-tRNA initiation complexes formed with control and mutant 30S were, after addition of a 1.5-fold excess of 50S subunits and incubation for 5 min at 37 °C, reacted with 1 mM puromycin (PM) at 0 °C for the indicated times. The amount of fMet-tRNA bound was determined by filter-binding at 20 mM Mg^{2+}, and corrected for the blank value obtained in the absence of 30S subunits. The values were 0.24 and 0.29 mol of fMet-tRNA bound per mole of 30S added for the G530 and U530 ribosomes, respectively. 30S-dependent fMet-PM formation was determined by ethyl acetate extraction. There was no fMet-PM found in the absence of 50S subunits or in the absence of puromycin. The ratio of the amount of fMet-PM formed to the amount of fMet-tRNA bound is shown.

Only one assay, the ability to initiate and form the first peptide bond, was strongly affected by the mutation (Figure 6). Here also three independent reconstitutions are shown. Note that although the absolute magnitude of R220 is lower than R187 or R215, the percent activity of the mutant is about the same (Table I). Inability to form the first peptide bond could well explain the *in vivo* lethality. As fMet-Val formation encompasses many individual steps of initiation and elongation, it was necessary to further dissect the reaction to determine the inhibited step(s). Since the ability to form the 30S initiation complex (I site) was unaffected, the inhibition shown in Figure 6 must originate at a later stage. Several possible sites could be eliminated if the 30S initiation complex formed with the mutant could be shown to undergo the puromycin reaction since that should mean that the abilities to complex with the 50S subunit, to release IFs 1 and 3, to hydrolyze GTP and release IF2, and to form an amide bond were all intact (Hershey, 1987). This was the case (Table II). The puromycin reaction was complete in less than 5 min at 0 °C and resulted in the reaction of virtually all of the bound fMet-tRNA. This was also the case for the control ribosomes (Table II) and for isolated natural 30S subunits (Cunningham et al., 1992a). Therefore, the inhibited step(s) in the U530 mutant must be subsequent to 70S initiation complex formation. This issue will be considered further under Discussion.

Codon–Anticodon Interaction. Does the U530 mutation cause misreading? A way to test this possibility is to measure the ability of a ribosome to insert leucine into a peptide linkage in response to a poly(U) message. This approach was also used recently by Allen and Noller (1991), who found a modest increase in miscoding due to a C to U mutation at position 1469 in 16S RNA. The assay has been studied extensively by the Kurland group, who have found that different leucine

Table III: Misincorporation of Leucine in Response to a Poly(U) Message in the Presence and Absence of Streptomycin

ribosome	streptomycin	Leu × 100[a]	Leu + Phe[a]	E^b
30S	–	7.7	6.9	1.1
	+	125.0	6.5	19.4
G530	–	5.6	3.9	1.4
	+	61.8	4.7	13.2
U530	–	2.0	2.7	0.7
	+	5.3	3.6	1.5

[a] Moles of amino acid incorporated per mole of 30S subunit added per 10 min at 37 °C. The misincorporation assay was carried out as described under Materials and Methods. Values reported are averages of three time points. Blank values (30S omitted) were subtracted. [b] E is the ratio of (Leu × 100)/(Leu + Phe).

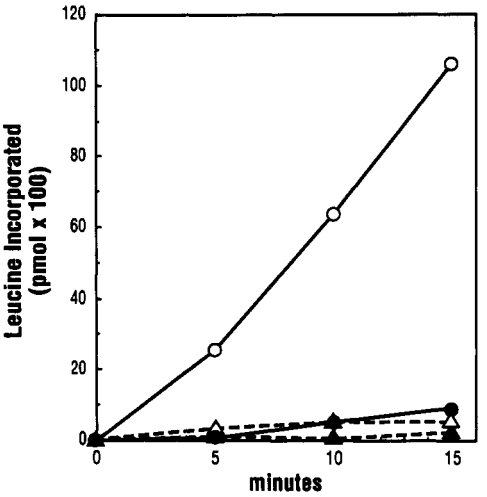

FIGURE 7: Rate of misincorporation of leucine using poly(U) as mRNA. The misincorporation assay was performed as described under Materials and Methods. Open symbols, G530; solid symbols, U530. Triangles, minus streptomycin; circles, plus streptomycin.

isoacceptor species have very different capacities for miscoding (Ruusala et al., 1982). We found that Leu-tRNA^{Leu} (anticodon N*AA) was effective in this assay. The results are shown in Table III. Although there was an apparent increase in stringency when the U530 ribosomes were used (compare the E value of 1.4 for the control with 0.7 for the mutant), all of the leucine incorporation values were very low when compared to those obtained upon streptomycin stimulation of misincorporation by the control G530 (Figure 7). We conclude that if fidelity is altered in the U530 mutant, it must be of a type which is not detectable by this *in vitro* assay. Note also that the total amount of Leu plus Phe synthesis was slightly lower in the mutant than in the control both in the absence (69%) and in the presence (77%) of streptomycin. In the poly(Phe) synthesis assay (Table I), the value was 66% for the same preparation.

Another important result of this experiment was the failure of the U530 ribosomes to miscode when streptomycin was added. Whereas streptomycin was able to stimulate the incorporation of leucine into a Leu-Phe copolypeptide with both synthetic and isolated 30S, there was virtually no effect when the U530 ribosomes were used. Clearly, streptomycin, if it can bind to the U530 mutant at all, is not able to induce miscoding. This resistance to the *in vitro* action of streptomycin correlates with the existence of two sites for *in vivo* streptomycin resistance on the opposite side of the RNA loop from U530 (see Figure 9).

As an alternative to the miscoding assay, cross-linking of tRNA to the P site (Ofengand et al., 1986, 1988) was used because it is a sensitive structural probe of the stereochemical

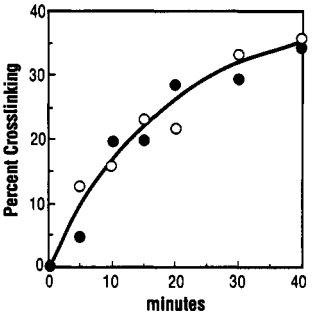

FIGURE 8: Rate and extent of cross-linking of P-site-bound AcVal-tRNA to control and mutant 70S ribosomes. Cross-linking was performed and assayed as described under Materials and Methods. Open symbols, G530; solid symbols, U530.

relationship between the anticodon of tRNA and the decoding-site nucleotides of 16S RNA [citations summarized in Cunningham et al. (1992a)]. Specifically, we asked if the U530 mutant could form the cmo^{5}U34–C1400 cross-link as well as the G530 control, and whether the site of cross-linking was altered. We found that the rate and yield of cross-linking did not change (Figure 8). Moreover, as shown by reverse transcription arrest analysis, the site of cross-linking also remained the same, that is, exclusively at C1400 (data not shown).

DISCUSSION

In Vivo Effects of the U530 Mutant. Introduction into *E. coli* of a plasmid containing a 16S RNA gene with any base substituted for G530 is a lethal event (Powers & Noller, 1990). We have confirmed these findings. *E. coli* cannot be transformed with a plasmid coding for 16S RNA with U530 when the normal P1 and P2 promoters are present on the plasmid, but can be transformed when the mutant 16S RNA gene is under control of the T7 promoter and T7 polymerase is absent from the cell. When the T7 polymerase gene is turned on, 16S RNA with U530 is made and incorporated into 30S ribosomes for a brief period before cell death occurs. The behavior of the mutant ribosomes was monitored by labeling the RNA of the plasmid-derived mutant, but not the chromosome-derived wild-type ribosomes. When the capability of the mutant 30S particles to form 70S was studied in this way, it became clear that the U530 mutation had affected the ability of the 30S to remain associated with 50S subunits in the cell. This was also observed by Powers and Noller (1990) for the A530 mutant but to a lesser extent and by a different method. These authors found a decreased amount of mutant plasmid-derived 16S RNA in the 70S fraction compared to wild-type.

A decreased ability to form 70S particles could conceivably explain the dominant lethal character of this mutation if it is supposed that stable mutant 30S initiation complexes form, thus sequestering mRNA, but that 70S initiation complexes, being less stable, do not allow elongation to occur. The approximately equal quantity of wild-type chromosomally-derived 30S particles would be expected to compete with the mutant 30S for mRNA, but since they would form 70S initiation complexes which would elongate, terminate, and dissociate, releasing the mRNA, over time all of the mRNA would become trapped in a nonproductive mutant 30S initiation complex. The success of this interpretation depends on the stability of the mutant 30S initiation complex, which is not known. Other explanations for dominant lethality are equally plausible, such as a defect in elongation, and more specifically in A-site recognition (Powers & Noller, 1990),

which could also result in sequestering essential components of the protein synthesis apparatus in nonfunctional complexes.

In Vitro Studies. The *in vitro* studies were initiated to try to localize the U530 effect. It was found that U530 ribosomes contain functional binding sites for initiator and elongator tRNAs and that they can participate in peptide bond formation and chain elongation as measured by poly(U)-directed polyphenylalanine synthesis. However, formation of an initiation-dependent peptide bond, fMet–Val, was blocked. Since reaction with puromycin was not affected, the 70S initiation complex could form. The next two steps are the binding of Val-tRNA to the A site and peptide bond formation. However, direct A-site binding was not affected (Table I), and peptidyl transferase activity also appeared intact since polyphenylalanine synthesis was not inhibited (Figure 4). What then is the defect in fMet-Val synthesis? We suggest three possible explanations.

First, consider the state of the A site when the first peptide bond is formed versus that during subsequent elongation. When the A site is initially occupied (A_i), the E site is empty but is filled during subsequent rounds of elongation (A_e) (Hausner et al., 1988; Nierhaus, 1990). A_i and A_e sites differ in several respects (Nierhaus, 1990). The effect of U530 could therefore be to block formation of an A_i site without affecting the A_e site.

Second, the fidelity of the decoding part of the A site on 30S may have been perturbed by the U530 mutation. This could occur by miscoding or frameshifting. Miscoding appears to be ruled out by the results shown in Table III. Frameshifting would not have been detected by the poly(U)-dependent polyphenylalanine synthesis assay, and thus remains a possibility.

Third, the U530 mutation may have perturbed the A site so that while it can still function with an artificial RNA such as poly(U), it no longer recognizes an mRNA containing the appropriate initiation signals. The inability of the mutant 30S to translate such an mRNA may thus be due to a failure of the mutant A site to respond in the way that a nonmutant site does to the various interactions between 16S RNA and regions of natural mRNA such as the Shine–Dalgarno region, the AUG codon with perhaps other surrounding primary structures (Gold et al., 1981), and enhancer regions (Olins & Rangwala, 1989; Thanaraj & Pandit, 1989).

All three models attempt to explain the inability of the mutant 30S ribosomes to participate in fMet-Val synthesis with natural mRNA by various alternative states of the A site. In physical terms, the A site has been partly defined by various bases in both 16S and 23S rRNAs which are protected by A-site-bound tRNA. These bases are, in 16S RNA, A1492, A1493, and G530, and to a lesser extent U531, A1408, and G1494 (Moazed & Noller, 1990). To understand how the protected G530 alters the A site when it is mutated to U, it is useful to consider whether all the bases protected by A-site-bound tRNA are topographically near each other on the surface of the ribosome.

Physical Juxtaposition of the 530, 1400, and 1500 Regions. There is contradictory data on the location of the 530 loop with respect to the 1400–1500 region. Both the 1400 region (Gornicki et al., 1984; Oakes et al., 1986) and the 1500 region (Oakes & Lake, 1990) have been shown by immunoelectron microscopy and like techniques to be located in the "cleft" area, or the "groove" region as it is called by Shatsky et al. (1991), of 30S ribosomes. Also, experimental evidence for the physical juxtaposition of these two regions has recently been obtained (Cunningham et al., 1992a). The 530 loop region, on the other hand, has been localized on the periphery of 30S ribosomes both by immunoelectron microscopy using antibody to m^7G527 (Trempe et al., 1982) and by electron microscopy (EM) localization of a biotinylated deoxyoligonucleotide complementary to the 530 loop region (Oakes & Lake, 1990). These experiments, carried out on isolated 30S subunits, place the 530 loop area some 70–100 nm away from the "cleft" region. This separation of the two regions, which perhaps not coincidentally also contains the three highly conserved sequence elements of 16S RNA, is also evident in the three-dimensional models of Stern et al. (1988a) and Brimacombe et al. (1988). In these models, the protection of G530 and U531 by A-site-bound tRNA (the anticodon arm of tRNA was equally effective) is viewed as an allosteric effect (Moazed & Noller, 1990).

An alternate model for the location of the 1400 and 530 regions, based on a reinterpretation of the EM data of Gornicki et al. (1984) and an alternative view of the extent of the "groove", places the two regions "close" to each other (Shatsky et al., 1991). This model, however, is not compatible with the interpretation Oakes and Lake (1990) give to their EM data. More recently, however, direct experimental evidence has been obtained which suggests that the 530 and 1400 regions may be juxtaposed, at least transiently. Dontsova et al. (1992) have shown that mRNA, bound to ribosomes via the Shine–Dalgarno sequence and initiator tRNA, which had thiouridine residues at positions +7 and +11 from the AUG codon, could be cross-linked via the thiouridines to 16S RNA. The +7 position was linked to A1395 and the +11 one to A532 of 16S rRNA. These data suggest, therefore, that A532 and A1395, which in previous models (Brimacombe et al., 1988; Stern et al., 1988a) were at a considerable distance from each other, may be close together. If that is the case, then G530 may be topographically part of the A site, just as are A1492 and A1493.

These differing points of view could be resolved if 30S ribosomes existed in two different conformations, one as the free subunit and the other when combined with mRNA and 50S ribosomes. In the former case, the 530 and 1400–1500 regions would be far apart (Trempe et al., 1982; Gornicki et al., 1984; Oakes et al., 1986; Oakes & Lake, 1990), while in the latter case the two regions would be close to each other (Dontsova et al., 1992). Since the U530 mutation only inhibited fMet-Val synthesis, a 70S reaction, this interpretation would suggest that the mutant base is physically at the A site and thus a change in G530 could directly interfere with functional occupancy of the site.

Functional Similarity to Mutants in the 1400 Region. Two other mutations in 16S rRNA produce the same pattern of functional behavior. The double mutant C1401/G1501 and the single mutant G1498 both are unaffected in A- and P-site binding, in poly(Phe) synthesis, in 30S initiation complex formation, and in 70S initiation complex formation as measured by the ability to react with puromycin, but like U530 cannot form the first peptide bond, fMet–Val (Cunningham et al., 1992a; Ofengand et al., 1993). These two mutants affect tertiary base pair formation in the region known to be physically close in the primary sequence to the decoding site, as marked by the cross-linking of C1400 to the P-site anticodon of tRNA (Ofengand et al., 1986, 1988). The functional similarity of all three mutants suggests that the 530 region may also be close to, and part of, the decoding site, in agreement with the cross-linking studies cited above (Dontsova et al., 1992).

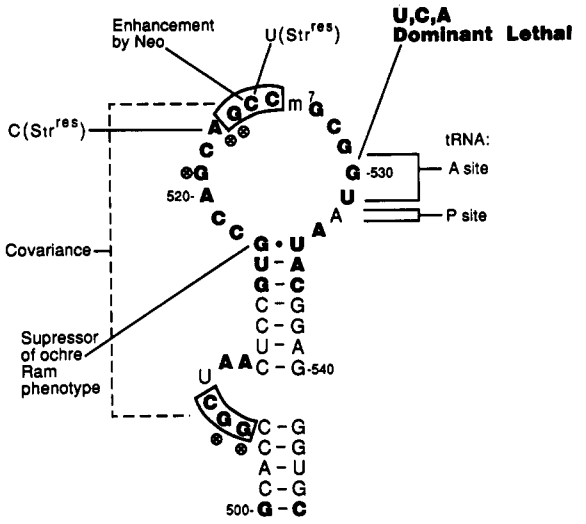

FIGURE 9: Molecular biology of the stem–loop at positions 500–545 of *E. coli* 16S rRNA. Bold letters: highly evolutionarily conserved (Gutell et al., 1985). tRNA: protection from chemical modification by tRNA bound to the A or P site (Moazed & Noller, 1990). U, C, A Dominant Lethal: changes at position 530 which are lethal (Powers & Noller, 1990). U(Str^{res}): a C to U change which results in streptomycin-resistant ribosomes (Powers & Noller, 1991). Enhancement by Neo: reactivity of C525 to dimethyl sulfate increased by neomycin and related amino glycoside antibiotics (Moazed & Noller, 1987). C(Str^{res}): a C to A change which results in streptomycin-resistant ribosomes (Gauthier et al., 1988; Melançon et al., 1988). Suppressor of ochre: transition at the corresponding site in yeast mitochondrial small-subunit rRNA results in suppression of ochre mutations (Shen & Fox, 1989). Ram phenotype: increases readthrough and frameshifting (O'Connor et al., 1992). Covariance: coordinated base changes in trinucleotide sequences which have maintained the potential for Watson–Crick pairing between them in 16S-like rRNAs from a variety of organisms (Woese & Gutell, 1989). X: sites interacting with protein S12 (Stern et al., 1988b).

Molecular Biology of the 530 Loop Region. This region has been shown by a variety of approaches to be at the translational core of ribosome function (Figure 9). G530 and also A1492 and A1493 are part of the A site (Moazed & Noller, 1990). G530 is adjacent to two ribosomal proteins, S12 and S4, which influence the accuracy of translation (Stern et al., 1989), and is close to the binding site on the 50S subunit of EFTu (Moazed et al., 1988) and EFG (Skold, 1983; Girshovich et al., 1981; Traut et al., 1986), proteins necessary for introducing a tRNA into the A site and translocation from the A site, respectively. Mutations in protein S12 (Okazaki et al., 1969) as well as mutation of A523 (Melancon et al., 1988) and C525 (Powers & Noller, 1991) lead to streptomycin resistance. Streptomycin-resistant ribosomes are more accurate in protein synthesis than wild-type ribosomes (Ruusala & Kurland, 1984). Both streptomycin sensitivity and resistance appear to be related to the maintenance of a pseudoknot structure involving bases 505–507/524–526 (Powers & Noller, 1991). Although streptomycin resistance *in vivo* cannot be tested with the U530 mutant since it is a dominant lethal, *in vitro* misincorporation studies have shown clearly that streptomycin causes little or no misreading with U530 ribosomes while there is considerable misreading with G530 ribosomes (Table III). Thus, U530 ribosomes appear to be resistant to streptomycin and may also have altered accuracy. Binding of streptomycin to U530 ribosomes has not yet been tested. Similarly, 30S ribosomes mutant at A523 were not induced by streptomycin to misread *in vivo* (Melancon et al., 1988) and a single base change at position 2661 in 23S rRNA also decreased misreading by streptomycin, as well as by neomycin and gentamicin (Melancon et al., 1992). It has been suggested that position 2661 may be involved in proofreading (Melancon et al., 1992) and this may also be the case for positions 523, 525, and 530. In the case of the 530 mutation, there is also the dominant lethal effect. Perhaps the 530 mutation increases proofreading and thus slows chain elongation so severely that it leads to cell death. Interestingly, none of the other 16S rRNA mutations or the 23S rRNA mutation which lead to streptomycin resistance are lethal to the cell (Melancon et al., 1988, 1992; Montandon et al., 1986; Powers & Noller, 1991).

Concluding Remarks. The mutation of G530 to U appears to abolish or interfere profoundly with dipeptide synthesis. In the cell, this is a dominant lethal event. Dominant lethality may be a consequence of the inability of U530 ribosomes to carry out the synthesis of the first peptide bond during translational initiation, but nevertheless bind to mRNA, fMet-tRNA, and 50S ribosomes, sequestering mRNA and preventing normal translation. Another possible consequence of cessation or slowing of the beginning of translational elongation is transcriptional termination. In *E. coli*, transcription and translation are closely linked, and translating ribosomes follow closely behind RNA polymerase. Stalled ribosomes may leave unprotected sections of mRNA which can trigger transcriptional termination (Adyha & Gottesman, 1978). This could result in the production of partial proteins which are lethal. Transcriptional termination may also be a consequence of the fact that U530 ribosomes may be subject to frameshifting. Frameshifting ribosomes could encounter out-of-phase termination codons which would lead to premature translational termination and lethality.

NOTE ADDED IN PROOF

After the revised version of this paper was submitted, the report by Powers and Noller appeared (Powers & Noller, 1993) in which the mutation of G530 to A was reported to interfere with EFTu-dependent A site binding of Phe-tRNA. The apparent contradiction between this report and our work, assuming A530 equivalent to U530, may be a result of the different ways in which A site binding was measured. Powers and Noller determined the decrease in protection of residues A1492 and A1493 which had been previously shown to be protected by A site bound Phe-tRNA as well as the anticodon stem–loop fragment of tRNA. Our studies, on the other hand, measured the physical affinity of Phe-tRNA for the ribosome in a more conventional cellulose nitrate filter binding assay. The A site perturbation detected by Powers and Noller may reflect the same event which resulted, in our work, in the inhibition of fMet-Val formation without seriously affecting poly(Phe) synthesis or A site binding of Phe-tRNA.

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