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A mutation at the universally conserved position 529 in *Escherichia coli* 16S rRNA creates a functional but highly error prone ribosome

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ABSTRACT

A base substitution of G to U was constructed at position 529 in *Escherichia coli* 16S rRNA. The U529 mutant ribosomes were functional and present on polysomes but were highly error prone and caused a progressive loss of cell viability. They displayed elevated levels of readthrough of stop codons and frameshifting, and an increase in thermal sensitivity of β-galactosidase, suggestive of missense errors. These results demonstrate that the universally conserved G529 is involved in tRNA selection at the A site during protein synthesis.

Keywords: A site; rRNA mutant; 30S subunit; translational fidelity

INTRODUCTION

Many of the nucleotides in the 530 loop region of *Escherichia coli* 16S rRNA are universally conserved (Gutell et al., 1985; Noller, 1993). There is considerable evidence that this region is involved in functional interactions with elongation factor EF-Tu-tRNA and with mRNA (Moazed & Noller, 1990; Dontsova et al., 1992) and that it ensures the accuracy of translation (O'Connor et al., 1992). Positions 529, 530, and 531 are protected from chemical modification by tRNA placed in the A site on 30S ribosomes, whereas base 532 is protected by P site bound tRNA (Moazed & Noller, 1990; Noller, 1993) and has been crosslinked to mRNA (Dontsova et al., 1992) (see Fig. 1). Base changes at position 530 are lethal (Powers & Noller, 1990), and only low levels of the mutant ribosomes are found in the polysome fraction in vivo. In vitro studies showed an impaired interaction between mutant ribosomes and the EF-Tu-tRNA-GTP ternary complexes (Powers & Noller, 1993). Parallel studies with in vitro reconstituted U530 ribosomes demonstrated that although the mutant ribosomes were competent in polyphenylalanine synthesis in a polyU-directed translation system, they were unable to elongate on a natural mRNA (M. Santer et al., 1993). Mutations elsewhere on the 530 loop region caused less severe effects. These included base changes at position 517, which increased readthrough of nonsense codons and frameshifting (O'Connor et al., 1992); mutations at 523 (Gauthier et al., 1988) and 525 (Melancon et al., 1988), which conferred streptomycin resistance; and mutations at 527 and 528, which affected growth only when mutant rRNA was expressed at high levels (U.V. Santer et al., 1993).

The profound negative effect of mutations at position 530 on ribosome function and cell growth led us to investigate mutations at the adjacent base G529. In this paper we demonstrate that the G to U transversion at position 529 is a lethal mutation. The mutant ribosomes are functional in vivo and are found in polysomes and in 70S and 30S ribosomes at levels comparable to wild-type ribosomes. However, the mutant ribosomes are very error prone and display high levels of misreading. In addition, the mutant ribosomes have a reduced capacity to translate low abundance mRNAs. These results underline the importance of this region of 16S rRNA in the selection and binding of tRNA to the ribosome and in ensuring the accuracy of protein synthesis.

RESULTS

Construction and characterization of the lethal mutant U529

A G to U mutation at position 529 in 16S rRNA was introduced into plasmid pNO2680ΔH, which had the *rrnB* operon under control of the PL promoter. Expression

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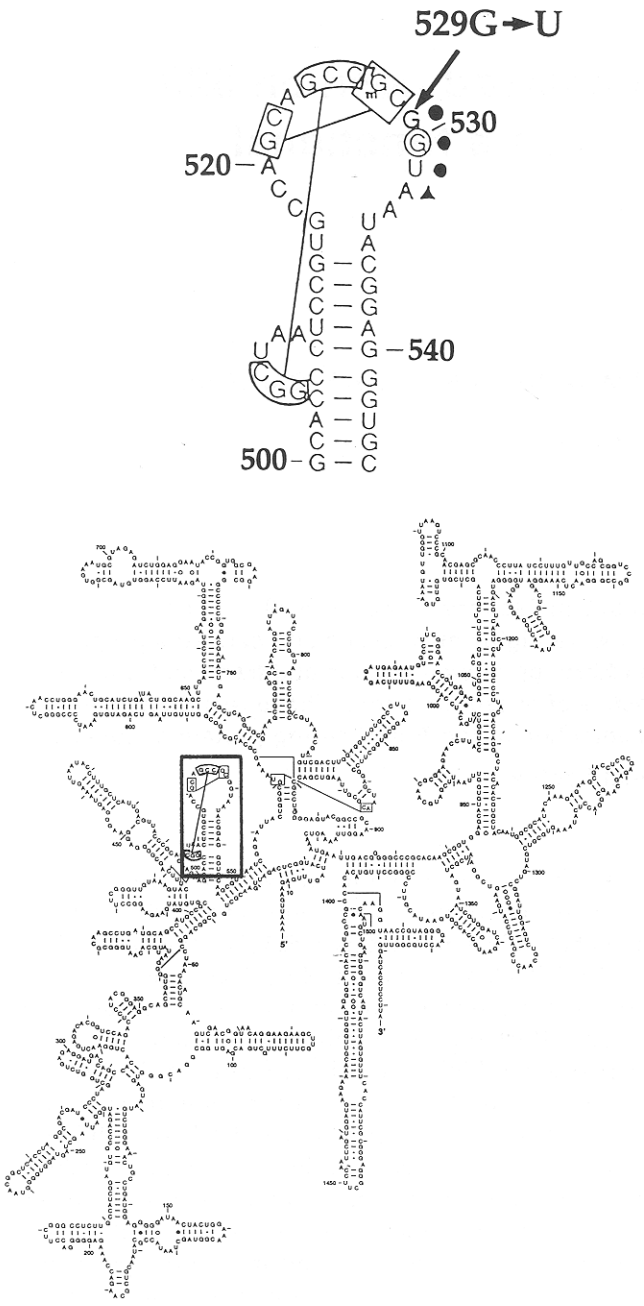

FIGURE 1. Sequence and secondary structure of the 530 region of *E. coli* 16S rRNA enlarged from the model of Gutell et al. (1994), with tRNA-dependent protections indicated: ●, A site; ▲, P site (Moazed & Noller, 1990).

of rRNA from the plasmid, pNOU529, was regulated by a temperature-sensitive repressor, cI857. Transcription was repressed at 30 °C, but at 42 °C the repressor was inactivated and expression of mutant rRNA occurred. The doubling time of *E. coli* DH1 with pcI857, carrying either mutant or wild-type plasmid, was 45 min at 30 °C. At 42 °C, the doubling time of the wild type was 36 min, whereas the mutant, after 1 h at 42 °C, had a doubling time of 230 min. This continued to increase with time at 42 °C until the cells eventually died.

To assess the effect of the U529 mutant 16S rRNA on viability, cells growing at 30 °C were shifted to 42 °C for 320 min, plated, and colonies counted. The results are shown in Table 1. The numbers of viable cells containing either mutant or wild-type plasmids at zero time were 6×10^8 colony forming units/O.D. After 320 min at 42 °C, there were 4.2×10^8 CFU/O.D. for cells carrying the wild-type plasmid and 4.7×10^5 CFU/O.D. for cells carrying the mutant plasmid. This represents almost a 1,000-fold decrease in viability for cells containing the U529 mutant ribosomes. The lethal character of U529 was confirmed by the inability to transform pNOU529 into strains lacking the cI repressor unless co-transformed with pcI857 (Jemiolo et al., 1991).

Expression and distribution of mutant 16S rRNA in subunits and polysomes

The level of expression and distribution of mutant 16S rRNA was measured by an adaptation of the reverse transcriptase primer extension method (Sigmund et al., 1988). Following induction of transcription by a shift to 42 °C, plasmid-encoded U529 16S rRNA rose to 26% (90–120 min) and then 48% (160 min) of the total 16S rRNA in ribosomes. At 120 min after induction, the distribution of mutant 16S rRNA in 30S subunits, 70S ribosomes, and polysomes was 29%, 23%, and 23%, respectively. Thus, the presence of U529 did not appear to affect assembly of 30S subunits or affect incorporation of subunits into polysomes. However, with longer times at 42 °C, the absolute amounts of polysomes and 70S ribosomes were reduced significantly in cells transcribing U529 rRNA, and there was a corresponding increase in the pool of free subunits. Cells with the mutant plasmid had 50% free 30S subunits compared to 20% with the wild-type plasmid after 150 min at 42 °C. The free 30S subunits had 30–40% mutant rRNA.

The severe effect of the U529 mutant on translational fidelity

Several reports have linked the 530 loop function to the selection of the correct ternary complex at the ribosomal A site (Moazed & Noller, 1990; Powers & Noller, 1990, 1993). Using a series of *lacZ* mutant constructs containing either a stop codon or a frameshift near the 5′ end of the *lacZ* gene, the effect of U529 on translational fidelity was tested. The data in Table 2 show that U529 ribosomes had the dramatic effect of severely reducing translational accuracy, causing a 2.4–6-fold increase in the level of readthrough of all six nonsense and four frameshift constructs tested. The extent of misreading produced by the U529 mutant was as significant as has been noted for any mutant in either 16S or 23S rRNA.

TABLE 1. Cell counts after expression of wild-type and mutant 16S rRNA.[a]

Plasmid	Growth conditions	A_{600nm} at plating	Viable cells per mL	Viable cells per A_{600nm} plated
pNO2680ΔH (WT)	30 °C	0.1	6×10^7	6×10^8
pNO2680ΔH (WT)	42 °C, 320 min	1.2	5×10^8	4.2×10^8
pNOU529	30 °C	0.1	6×10^7	6×10^8
pNOU529	42 °C, 320 min	0.64	3×10^5	4.7×10^5

[a] DH1with pc1857, carrying mutant or wild-type plasmid, was grown at 30 °C to an O.D. of 0.1 on LB broth supplemented with ampicillin (200 μg/mL) and neomycin (50 μg/mL). An aliquot was removed for a viable cell count at 0 min and the culture shifted to 42 °C. After 320 min, a second aliquot was removed and viable cells (colony forming units) were determined by serial dilution, plating on unsupplemented LB agar and growing at 30 °C for 48 h.

The levels of β-galactosidase synthesized from the wild-type lac plasmid (pSG25) were identical in cells with wild-type and mutant U529 ribosomes (Table 2). However, upon heating at 56 °C for 20 min, the β-galactosidase synthesized in cells containing mutant ribosomes was only 82% as active as the wild type (Table 3). Susceptibility of the enzyme to thermal denaturation suggested the possibility that the U529 ribosomes misread sense codons, inserting incorrect amino acids throughout the β-galactosidase chain, as well as causing frameshifting and readthrough events. Mutants of EF-Tu that cause increased misreading have analogous and quantitatively similar effects on the thermal lability of proteins (Hughes, 1991).

TABLE 2. Effects of the U529 mutation on stop codon readthrough and frameshifting.[a]

lacZ mutant	β-Galactosidase activity[b] In pNO2680ΔH (WT)	In pNOU529	Ratio of mutant/WT
Nonsense mutants			
pSG3/4 UGA	121.6 ± 8.4	417.1 ± 20.5	3.4
pSG34-11 UGA	39.0 ± 1.9	127.1 ± 10.0	3.3
pSG12-6 UAG	18.4 ± 0.8	44.9 ± 2.6	2.4
pSG163 UAG	36.0 ± 1.4	98.8 ± 2.9	2.7
pSG627 UAA	4.9 ± 0.3	16.2 ± 1.2	3.3
pSG853 UAA	12.0 ± 0.1	54.8 ± 4.0	4.6
Frameshift mutants			
pSGlac7 (+1)	45.8 ± 3.8	277.8 ± 16.1	6.1
pSG12DP (−1)	110.7 ± 3.5	376.2 ± 16.8	3.4
pSG375 (+1)	1.6 ± 0.2	9.2 ± 0.9	4.8
pSGCCCU (+1)	19.0 ± 1.4	59.5 ± 3.9	3.1
Wild-type *lacZ*			
pSG25 (P_{tac})	6,487.5 ± 645	7,593.0 ± 742.3	1.17
p90.91 (P_{lac} WT)	750.9 ± 28.4	441.5 ± 32.9	0.59
F'128	1,655.9 ± 54.9	910.2 ± 59.5	0.55

[a] Each culture was grown at 42 °C for 150 min (to induce transcription of mutant rRNA) before being assayed. The strains used are described in the Materials and methods section.

[b] Miller (1972) units. Each number is the mean activity of three to six independent cultures ± one standard error.

Differences in the level of synthesis of β-galactosidase by mutant and wild-type ribosomes: A reflection of the abundance of mRNA

In *E. coli* MC140, the constitutive level of wild-type β-galactosidase was unaffected by the U529 mutation when the *lacZ* mRNA was abundant (i.e., transcribed from the strong promoter in pSG 25; see Table 2). However, a decrease in β-galactosidase was observed when the *lacZ* gene was present on a single copy episome (F'128) or transcribed from a weaker promoter (p90.91). When the mRNA was transcribed from the chromosomal wild-type *lacZ* gene in *E. coli* DH1 (Table 4), a pronounced reduction in the level of β-galactosidase was correlated with induction of mutant ribosome synthesis. When the chromosomal *lacZ* gene in *E. coli* DH1/pcI857 was induced with IPTG immediately upon transfer to 42 °C and assayed for enzyme 15 min later, cells containing the U529 plasmid had essentially the same level of enzyme as cells containing the wild-type plasmid. Cells assayed for enzyme 60 min later expressed one-half the activity of cells assayed at 15 min. If induction of enzyme with IPTG was delayed to 30 min after growth at 42 °C, the level of β-galactosidase activity fell by 100-fold. Thus, the level of β-galactosidase expressed from the chromosomal *lacZ* gene declined dramatically as mutant ribosomes accumulated.

TABLE 3. Heat lability of β-galactosidase: a measure of missense errors by the U529 mutant ribosomes.[a]

Plasmid	% of initial activity	Ratio of activity relative to WT
pNO2680ΔH (WT)	80.8 ± 5.0	1.00
pNOU529	66.5 ± 1.9	0.82

[a] Measurements of percentage of initial activity were made after 20 min at 56 °C. All values are the mean of three independent measurements ± one standard error. Cell extracts were prepared as described in the Materials and methods section.

TABLE 4. β-Galactosidase activity in DH1 cells with U529 plasmid.[a]

Experiment	Length of induction of rRNA synthesis (at 42 °C) prior to adding IPTG (min)	Time of β-gal assay after IPTG addition (min)	β-Galactosidase activity[b]		
			pNOU529	pNO2680ΔH (WT)	Ratio of mutant/WT
I	0	15	100	90	1.1
		60	1,160	2,400	0.5
II	30	15	80	500	0.16
		70	60	1,100	0.05
		100	80	7,600	0.01

[a] DH1 pc 1857 cells with a chromosomal *lacZ* gene, containing WT or U529 pNO2680ΔH plasmid, were shifted from 30 °C to 42 °C at time 0 at an O.D. of 0.2 (Exp. I) or 0.05 (Exp. II). IPTG was added simultaneously (Exp. I) or after 30 min (Exp. II). β-Galactosidase was assayed at various times after IPTG addition, as indicated.
[b] Miller (1972) units.

DISCUSSION

We have produced a mutation in a functional region of 16S rRNA that is essential for maintaining translational fidelity (Moazed & Noller, 1990; Powers & Noller, 1990, 1993; Dontsova et al., 1992; O'Connor et al., 1992). The mutation, U529, has a drastic effect on cell growth, causing a greater than sixfold increase in doubling time within 90 min and ultimately leading to cell death. It is interesting that the 30S subunits containing mutant rRNA were found in polysomes in approximate proportion to their percentage in the total cellular pool. This is in contrast to a mutation at the adjacent position, 530, which is excluded from polysomes (Powers & Noller, 1990). Since the mutant U529 30S ribosomes are found in the polysomes, one can examine the effects of this mutation in vivo by regulating expression from a plasmid under the control of a repressable promoter. As the 529 mutant ribosomes accumulate in the cell, the growth rate decreases and the number of polysomes in the cell decreases, even in the presence of wild-type 30S subunits. This may reflect a progressive, generalized accumulation of inaccurately translated proteins. In fact, the demonstration of thermally sensitive β-galactosidase (from a high copy mRNA) suggests the presence of missense errors in all nascent cellular proteins. The synthesis of defective proteins, such as seen previously with mutants C to G at 726 (Prescott & Dahlberg, 1990) and G to C at 517 (O'Connor et al., 1992) in 16S rRNA or induced by low levels of streptomycin (Rosenberger, 1982), can have a profound effect on cell viability and could account for the observed lethality of the U529 mutation.

The U529 mutant ribosomes affected the level of translation of wild-type β-galactosidase. A 2–100-fold reduction in the steady state level of β-galactosidase was found when *lacZ* mRNA was present in amounts consistent with a single copy gene (Table 4). A similar reduction was also noted with weak promoters (p90.9/P_{lac} WT) or with the single copy gene F'128 P_{lac} WT (Table 2). Under ordinary conditions, transcription and translation are tightly coupled in *E. coli*, and ribosomal protein mutants have been found to influence premature termination of transcription (Jørgensen & Kurland, 1990). Consequently, the defects in the U529 mutant ribosomes may not be limited to translational events but might also affect transcriptional processes. This could account for the fact that the units of β-galactosidase did not increase with time when *lacZ* mRNA synthesis was induced in cells with U529 ribosomes (Experiment II, Table 4). When the *lacZ* mRNA was abundant (pSG25, P_{tac}), however, both mutant and wild-type strains produced equivalent amounts of β-galactosidase. If we generalize the differential effect of the mutant ribosomes on the translation of low abundance mRNA to the many mRNA species present at low levels in the cell, then clearly the mutant rRNA is expected to have disastrous consequences on cell physiology.

The severity of the effect of the 529 mutation on translational accuracy is clearly demonstrated by its effect on readthrough and frameshifting (Table 2). The lethal effect of the 530 mutation may be due to an inability to carry out proper elongation. A mutation at 531 (U to G) has no demonstrable effect on growth or protein synthesis (U.V. Santer et al., 1993). All three bases—529, 530, and 531—are part of the A site as judged by footprinting experiments (Moazed & Noller, 1990), but mutations at these three positions affect A site function in quantitatively different ways. The differential effects might reflect perturbations of different parts of the complex pathway leading to the proper placement of the aminoacyl-tRNA in the A site (Thompson, 1988; Ehrenberg et al., 1990; Weijland & Parmeggiani, 1994).

MATERIALS AND METHODS

The following strains of *E. coli* were used in these studies: *E. coli* DH1, HB101, and MC140 (O'Connor et al., 1992; M. Santer et al., 1993). Strains were transformed with plasmid pcI857, which encodes the temperature-sensitive λ repressor and the neomycin resistance marker. A derivative of plasmid

pNO2680 (Tapprich et al., 1990) lacking one *Hin*d III restriction site in the pBR322-derived portion of the plasmid was prepared and is referred to as pNO2680ΔH. This plasmid contains the *rrnB* operon under control of the λ P_L promoter (O'Connor et al., 1992).

The lac mutants used in this study to monitor readthrough and frameshifting carried the *lacZ* gene, transcribed from the tac promoter in the absence of any lac repressor, on a tetracycline-resistant plasmid derived from pACYC184 (O'Connor & Dahlberg, 1993). Strain MC140 (F^-*thi*$^-$Δ(lac-pro) recA$^-$), carrying the lambda temperature-sensitive repressor on a neomycin-resistant plasmid (pLG857) (O'Connor et al., 1992), was used as the host for all the *lacZ* mutants and plasmids carrying the *rrnB* operon under the control of the lambda P_L promoter (Gourse et al., 1985).

Site-specific mutagenesis

A 568-bp *Hin*d III fragment from positions 80 to 648 of 16S rRNA was inserted into M13 mp19. Oligonucleotide-directed, site-specific mutagenesis at base 529 was carried out using standard procedures (Kunkel et al., 1987). Screening for mutants was facilitated by the fact that a base change at position 529 abolished a *Sac* II site. The *Hin*d III fragment containing a G to U base change at position 529 was isolated from the RF of M13mp19 and cloned into plasmid pNO2680ΔH to give pNOU529. All base changes were verified by restriction enzyme analysis and DNA sequencing (Sanger et al., 1977) of the M13 insert and the reconstructed plasmid.

Growth experiments

Growth experiments were carried out with *E. coli* DH1 containing pNO2680ΔH or pNOU529, and plasmid pcI857. Cells were grown on LB agar with the appropriate antibiotic at 30 °C, suspended in broth, and grown at 30 °C in LB medium containing ampicillin and neomycin (200 mg/L and 50 mg/L) or only ampicillin. Unless otherwise indicated, the flasks were transferred to 42 °C when the cells reached A_{600nm} of 0.1. To establish whether cells were viable after growth at 42 °C, they were initially grown to an optical density of about 0.1 at 30 °C, transferred to 42 °C, and after 320 min, aliquots of each culture were serially diluted, plated on unsupplemented LB agar, and grown at 30 °C for 48 h.

Measurement of β-galactosidase and analysis of proteins

Cells were grown at 30 °C until A_{600nm} reached 0.15–0.2. An aliquot of cells was diluted fourfold into the appropriate medium prewarmed to 42 °C. *E. coli* DH1 cells were induced with IPTG (final concentration, 1 mM) immediately or 30 min later after transfer to 42 °C. At various times after addition of IPTG, β-galactosidase was assayed (Miller, 1972). To determine the degree of readthrough of nonsense codons and frameshifting, β-galactosidase assays were carried out on *E. coli* MC140 cultures grown overnight at 30 °C in LB medium supplemented with appropriate antibiotics (neomycin [50 mg/L], tetracycline [25 mg/L], and ampicillin [200 mg/L]). The cultures were diluted 1/50 and incubated with vigorous shaking at 42 °C for 2.5 h and then assayed as described (O'Connor et al., 1992).

Heat lability of β-galactosidase

Plasmid-encoded rRNA synthesis in MC140, pLG857, and pSG25 containing either pNO2680ΔH (wt) or pNOU529 was induced by growth at 42 °C for 3 h. Cell extracts were prepared by passage through a French press as described by Hughes (1991). Gel electrophoresis of β-galactosidase, purified by affinity immunochromatography (ProtoSorb, Promega, Madison, Wisconsin, USA), showed a single band of the enzyme and no evidence of enhanced proteolytic degradation in the mutant cells.

Isolation of rRNA from ribosome fractions and determination of levels of plasmid-encoded rRNA

E. coli cells containing mutant and wild-type plasmids were harvested at various times after being shifted to 42 °C. Total ribosomal RNA and ribosomal fractions were prepared. Polysomes and 70S and 30S ribosome fractions were separated by sucrose density gradient fractionation, and rRNA was isolated. The relative amounts of wild-type and mutant (plasmid-encoded) rRNA were determined by the primer extension method of Sigmund et al. (1988) using an oligonucleotide complementary to bases 547–532.

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