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Base changes at position 792 of *Escherichia coli* 16S rRNA affect assembly of 70S ribosomes

(subunit association/site-directed mutagenesis/maxicell analysis/initiation factors)

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ABSTRACT **To investigate the function of base 792 of 16S rRNA in 30S ribosomes of *Escherichia coli*, the wild-type (adenine) residue was changed to guanine, cytosine, or uracil by oligonucleotide-directed mutagenesis. Each base change conferred a unique phenotype on the cells. Cells containing plasmid pKK3535 with G^{792} or T^{792} showed no difference in generation time in LB broth containing ampicillin, whereas cells with C^{792} exhibited a 20% increase in generation time in this medium. To study the effect on cell growth of a homogeneous population of mutant ribosomes, the mutations were cloned into the 16S rRNA gene on pKK3535 carrying a spectinomycin-resistance marker (thymine at position 1192), and the cells were grown with spectinomycin. Cells containing G^{792} or C^{792} showed 16% and 56% increases in generation time, respectively, and a concomitant decrease in ^{35}S assimilation into proteins. Cells with T^{792} did not grow in spectinomycin-containing medium. Maxicell analyses indicated decreasing ability to form 70S ribosomes from 30S subunits containing guanine, cytosine, or uracil at position 792 in 16S rRNA. It appeared that C^{792}-containing 30S ribosomes had lost the ability to bind initiation factor 3.**

To study the relationship between the primary structure and function of 16S rRNA, single base changes have been introduced at position 792 by site-directed mutagenesis. This approach has been used to establish the crucial role in translational initiation of base 1538 (1) and the surrounding region at the 3′ end of 16S rRNA (2). Position 792 is an adenine residue, is highly conserved in small-subunit rRNAs (3), and is in a conserved, single-stranded region (positions 787–795) that was suggested to be at the interface between the 30S and 50S ribosomal subunits (4–8). A base change at position 791 decreases subunit association (8). There is evidence that at least four bases in the loop region covering positions 787–795 (Fig. 1)—namely, bases 790, 791, 794, and 795—also interact with tRNA (9). To find out whether position 792 has an effect on ribosome function, this base has been altered to guanine, cytosine, or uracil. Each base change at position 792 differentially affects ribosome function. Both 70S ribosome formation and protein synthesis are progressively decreased by changes to guanine, cytosine, and uracil. An A → C change leads to a loss of initiation factor 3 (IF3) binding. These results are similar to those obtained by Tapprich *et al.* (8), which demonstrate that a G → A base change at position 791 decreases 70S ribosome formation, IF3 binding, and protein synthesis.

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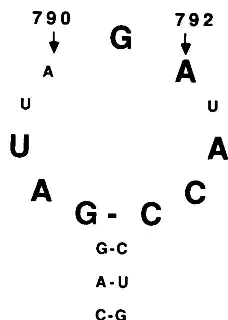

FIG. 1. Primary and secondary structure of 792 region of 16S rRNA. Large letters indicate highly conserved bases in eubacteria, archaebacteria, eukaryotes, and chloroplasts (3).

MATERIALS AND METHODS

Bacteria, Plasmids, and Bacteriophage. *Escherichia coli* HB101 (10), XL1 Blue (11), and BL21(DE3) (12) have been described. *E. coli* BL21(DE3) contains a chromosomal gene for phage T7 RNA polymerase under control of the UV5 *lac* promoter. Plasmid pAR3056 contains the *rrnB* operon under control of the T7 promoter (13). Plasmid pKK3535 contains the *rrnB* operon (14, 15). Plasmid pKK3535 with a thymine at position 1192 (T^{1192}) of the 16S rRNA gene, which confers spectinomycin resistance on host cells (16, 17), pAR3056 with the Sma 1–6 deletion, and pKK3535 with the mutation at position 791 were gifts from A. E. Dahlberg (Brown University, Providence, RI). Bacterial strains carrying plasmids were grown in LB broth with either ampicillin (200 μg/ml) or ampicillin (200 μg/ml) plus spectinomycin (250–300 μg/ml).

Radiochemicals and Enzymes. Carrier-free [^{32}P]orthophosphate was purchased from New England Nuclear. Trans^{35}S-label ([^{35}S]methionine/[^{35}S]cysteine mixture) was purchased from ICN. Restriction enzymes, T4 DNA ligase, and DNA polymerase and Klenow fragment were purchased from New England Biolabs. The Muta-Gene kit was obtained from Bio-Rad.

Site-Specific Mutagenesis. Mutagenesis at base 792 was carried out by standard procedures (18, 19). Synthetic oligodeoxynucleotides were generously provided by A. E. Dahlberg. Screening for mutants was facilitated by the fact that an A → G change created a *Kpn* I site. From this mutant the C and T changes were made. To screen for the C and T mutants we tested for the loss of a *Kpn* I site. In each case

Abbreviation: IF3, initiation factor 3.
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the base change was verified by DNA sequencing (20, 21) of the M13 insert and the reconstructed plasmid.

Labeling of RNA and Ribosomes in Maxicells. *E. coli* BL21(DE3) cells containing plasmid pAR3056 with A^{792}, G^{792}, C^{792}, or T^{792} were used in maxicell experiments. pAR3056 containing the Sma 1–6 deletion in the 16S rRNA gene was used as a control to measure the abolition of host chromosomal rRNA synthesis. pAR3056 with A^{791} was constructed from a clone provided by W. Tapprich and A. E. Dahlberg. The normal base at 791 is a guanine. This construction was included as a control for 70S assembly since Tapprich *et al.* (8) had shown that the G → A mutation at 791 interferes with 30S–50S association.

Labeling of *E. coli* BL21(DE3) carrying the various plasmids was carried out as described (17). Cells (10 ml) were generally incubated with [^{32}P]orthophosphate (2 μCi/ml; 1 μCi = 37 kBq) for 20 min. Ribosomes and rRNA were obtained from ^{32}P-labeled cells by procedures described previously (22, 23). Densitometer traces of autoradiographs of ribosomes or rRNA were done with an LKB Ultroscan XL laser densitometer.

Gel Electrophoresis of Labeled Proteins. Cells carrying pKK3535 plasmid constructions with T^{1192} and either A^{792}, G^{792}, or C^{792} were grown on modified ZPM medium (15) containing 0.1% Casamino acids, 0.5% LB broth, and ampicillin (200 μg/ml) until the culture reached an OD of 0.3 at 600 nm. Spectinomycin was then added to a final concentration of 300–500 μg/ml. One-half to 1 hr after the addition of spectinomycin, Trans^{35}S-label (6.25 μCi/ml) was added and incorporation was allowed to proceed for 60 min. Cells were harvested and lysed, and proteins were separated by SDS/PAGE (24). Gels were stained with Coomassie blue and autoradiographed.

Growth Experiments with Single and Double Mutants. *E. coli* HB101 cells containing plasmid pKK3535 with various base alterations were grown from isolated colonies in 10 ml of LB ampicillin (200 μg/ml) broth or LB ampicillin (200 μg/ml)/spectinomycin (250 μg/ml) broth at 37°C. Generation times were measured during logarithmic growth. At least three determinations of generation time were carried out for each cell/plasmid combination.

IF3–Ribosome Binding Studies. Ribosomal 30S subunits containing either A^{792} (wild type) or C^{792} in 16S RNA were prepared from plasmid-transformed HB101 as described (25). IF3 was prepared from an overproducing strain (26), purified, and reductively methylated with [^{14}C]formaldehyde and sodium cyanoborohydride as described (27). The resultant [^{14}C]methyl-IF3 had a specific activity of 25,000–35,000 cpm/μg. IF3 binding reactions were carried out essentially as described (25) in a total volume of 100 μl containing 20–24 pmol of 30S subunits and various concentrations of [^{14}C]-methyl-IF3 (0.5–9.0 mol of IF3 per mol of 30S subunits), and the subunit-bound IF3 was quantitated by sucrose density gradient centrifugation essentially as described (25).

RESULTS

Growth Rates in the Absence and Presence of Spectinomycin. To study the ability of a homogeneous population of mutant ribosomes to sustain cell growth, cells containing pKK3535 with T^{1192} and one of the four bases at 792 were grown in two types of media (Fig. 2). Cells with A^{792} or G^{792} had similar generation times in ampicillin broth. Cells with C^{792} had a generation time of 72 min. In ampicillin/spectinomycin broth, G^{792}- and C^{792}-containing cells grew 16% and 56% more slowly than wild-type cells, whereas cells with the T^{792} plasmid did not grow. It is clear, therefore, that ribosomes constructed from 16S rRNA with U^{792} are incapable of participating in a level of protein synthesis that will support cell growth.

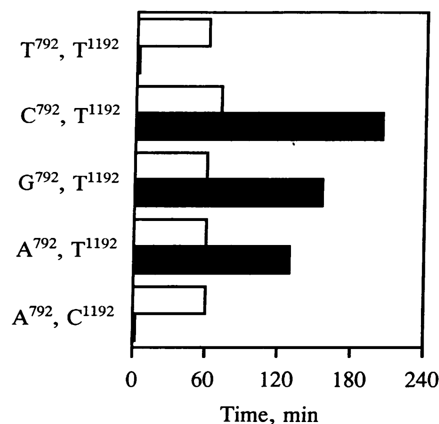

FIG. 2. Generation times of *E. coli* HB101 containing mutant plasmids. Cells were grown as described in *Materials and Methods* with ampicillin (open bars) or ampicillin and spectinomycin (filled bars). Generation times represent the average of three determinations and varied by 5–10% in spectinomycin broth. Generation time of cells with wild-type (A^{792}, C^{1192}) plasmid is also shown. Note inability of cells with T^{792}, T^{1192} plasmid to grow in spectinomycin broth.

Protein Synthesis by Plasmid-Coded Ribosomes. To determine to what extent ribosomes containing mutations at position 792 synthesize protein, ^{35}S-incorporation experiments were carried out. Cells with the various mutant plasmids and cells without plasmid were grown to mid-logarithmic phase, and spectinomycin was added, followed 30–60 min later by the addition of Trans^{35}S-label. Total cellular proteins were separated in an acrylamide gel. Coomassie blue staining (Fig. 3*A*) showed that almost identical quantities of protein were separated in each lane. Autoradiography (Fig. 3*B*) showed that *E. coli* HB101 cells that did not contain plasmid (and thus were spectinomycin-sensitive) incorporated very little ^{35}S (lane 1). Cells that were spectinomycin-resistant because they carried plasmid

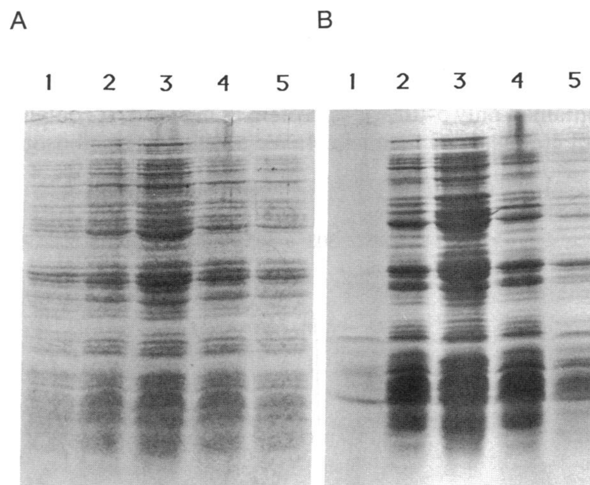

FIG. 3. Protein synthesis by plasmid-coded ribosomes. Cells containing pKK3535 plasmids with T^{1192} (except lane 1) were grown to mid-logarithmic phase (0.3 OD at 600 nm), and spectinomycin was added, followed 60 min later by the addition of Trans^{35}S-label for 60 min. Equal aliquots of each lysate were analyzed by SDS/PAGE (24) followed by Coomassie blue staining (*A*) and autoradiography (*B*). Lane 1, *E. coli* lacking plasmid; lane 2, cells containing pKK3535 with T^{1192}; lane 3, cells carrying plasmid as in lane 2, but labeled in the absence of spectinomycin; lanes 4 and 5, cells containing pKK3535 with T^{1192} and G^{792} or C^{792}, respectively, mutations of the 16S rRNA gene.

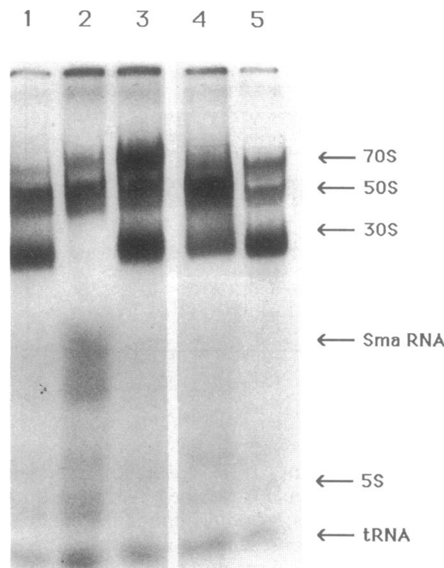

FIG. 4. Plasmid-coded ribosomes in maxicells of *E. coli* BL21(DE3) with plasmid pAR3056 containing wild-type or mutant base at position 792. Maxicells were labeled with [^{32}P]orthophosphate after induction of T7 RNA polymerase with isopropyl β-D-thiogalactopyranoside. Lysates prepared in 10 mM Mg^{2+} buffer were electrophoresed in acrylamide/agarose composite gels (22, 23) and autoradiographed. Lane 1: cells carrying the A^{791} mutant provided by W. E. Tapprich and A. E. Dahlberg [30S ribosomes containing this mutation are deficient in 70S formation (8)]. Lane 2: cells containing the Sma 1–6 deletion in 16S rRNA; no 30S ribosomes were produced, although labeled 50S and 70S ribosomes are apparent. Lane 3: cells containing the wild-type (A^{792}) plasmid. Lanes 4 and 5: cells containing plasmid with C^{792} or G^{792}, respectively.

pKK3535 with T^{1192} gave similar labeled protein profiles whether they were incubated in the presence of spectinomycin (lane 2) or in its absence (lane 3), although more ^{35}S was incorporated in the absence of the drug, because both host- and plasmid-coded ribosomes were functioning during the labeling period. When cells carrying the G^{792} (lane 4) or C^{792} (lane 5) mutation in addition to T^{1192} were analyzed, densitometer scanning of the Coomassie blue-stained gel (Fig. 3*A*) and the autoradiograph (Fig. 3*B*) indicated that although lane 5 contained only 5–10% less protein than lane 4, it contained about 40% less ^{35}S than lane 4. We conclude that ribosomes containing C^{792} are less able to support protein synthesis than ribosomes containing G^{792}.

Production of Mutant rRNA and Ribosomes. To examine transcription, processing, and assembly into ribosomes of mutant rRNA, we carried out maxicell experiments using a host/plasmid system composed of *E. coli* BL21(DE3) and pAR3056. Cells were transformed with pAR3056 containing A^{792}, G^{792}, C^{792}, or T^{792} or with plasmid containing the Sma 1–6 deletion to measure the extent of host chromosome-coded 16S rRNA synthesis. In this plasmid rRNA synthesis is under the control of the T7 promoter. Plasmid-coded rRNA was labeled with [^{32}P]orthophosphate. Aliquots of labeled cells were lysed and deproteinized, and rRNAs were separated in acrylamide/agarose composite gels. Cells carrying the various plasmids made mature forms of 23S and 16S rRNA, except for the cells carrying plasmid with the Sma 1–6 deletion, which did not produce 16S rRNA (data not shown).

To determine whether ribosomes could be assembled from mutant rRNA molecules, aliquots of maxicell lysates prepared in buffer with 10 mM Mg^{2+} to preserve 70S ribosomes were analyzed by one-dimensional gel electrophoresis. This procedure separates 70S, 50S, and 30S ribosomes (Fig. 4; densitometer traces of the autoradiographs are shown in Fig. 5). For comparative purposes we included ribosomes from cells containing plasmid with A^{791} instead of G^{791}. Tapprich *et al.* (8) showed that this mutant 16S RNA was assembled into 30S ribosomes but that these ribosomes were defective in 70S assembly. Their data are corroborated by our finding that maxicells with the A^{791} plasmid contained few 70S ribosomes (Fig. 4, lane 1; Fig. 5*A*) compared to the level of 70S ribosomes in maxicells where 30S ribosomes were produced from plasmid containing the normal 16S rRNA gene (Fig. 4, lane 3; Fig. 5*D*). When ribosomes produced from plasmid encoding C^{792} or G^{792} 16S rRNA were analyzed, the autoradiographs (Fig. 4) indicated a progressive decrease in 70S ribosome formation as the base at position 792 was changed from adenine (lane 3) to guanine (lane 5) to cytosine (lane 4). Densitometry (Fig. 5) clearly showed that G^{792}- and C^{792}-containing 30S ribosomes have drastically altered ability to make 70S ribosomes when compared to 30S ribosomes containing A^{792}. The traces also suggested a lower level of 30S ribosomes containing C^{792}. A decreased ability to form 70S ribosomes was also observed with the T^{792} mutation (data not shown). To quantitate the decreased ability of U^{792}-containing 30S ribosomes to form 70S ribosomes, two-dimensional separation of ribosomes and rRNAs was carried

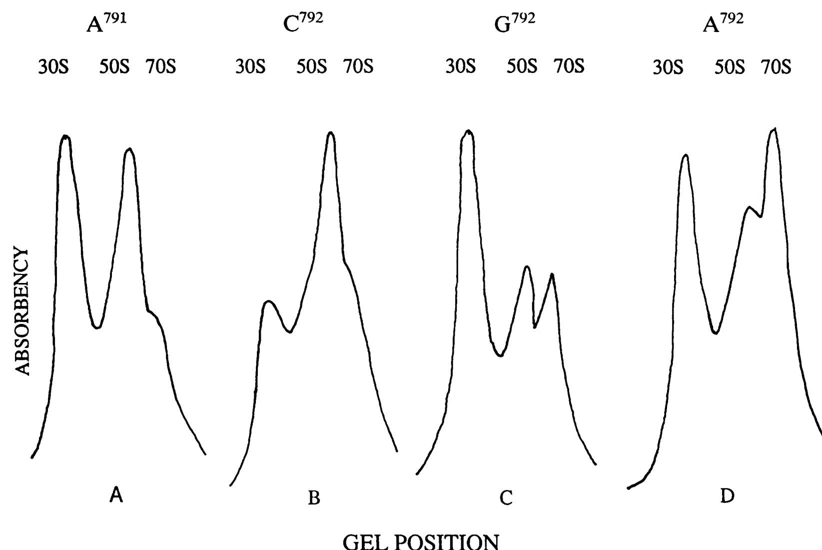

FIG. 5. Densitometer traces of autoradiograph in Fig. 4. (*A*) Lane 1, A^{791}. (*B*) Lane 4, C^{792}. (*C*) Lane 5, G^{792}. (*D*) Lane 3, A^{792}.

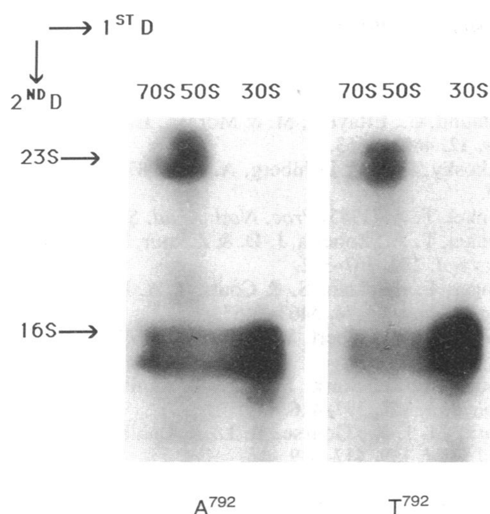

FIG. 6. RNA composition of ribosomes produced in maxicells containing A^{792} or T^{792} plasmid. Extracts of cells were prepared in 6 mM Mg^{2+} buffers, and ribosomes were separated in agarose/acrylamide composite gels with the same level of Mg^{2+}. The 70S, 50S, and 30S ribosomes separated in the first dimension (1st D) were dissociated into free RNAs, which were separated in a second dimension (2nd D) (22) and detected by autoradiography. The positions of 23S and 16S rRNAs are indicated.

out (Fig. 6). In this experiment cell extracts were prepared in buffers with 6 mM Mg^{2+} and the ribosomes were separated in gels with 6 mM Mg^{2+}. Electrophoresis in the first dimension separated 70S, 50S, and 30S ribosomes, whereas rRNA were separated in the second dimension. The autoradiograph (Fig. 6) indicated that less "mutant" 16S rRNA was present in 70S ribosomes than "wild-type" 16S rRNA. Densitometry (not shown) of these autoradiographs demonstrated that the level of 16S rRNA in 70S ribosomes in cells containing the T^{792} mutation was about 30% of the level of 16S rRNA in 70S ribosomes derived from cells carrying the A^{792} plasmid.

IF3 Binding to 30S Ribosomes. Tapprich *et al.* (8) presented evidence that 30S ribosomes mutant at position 791 showed

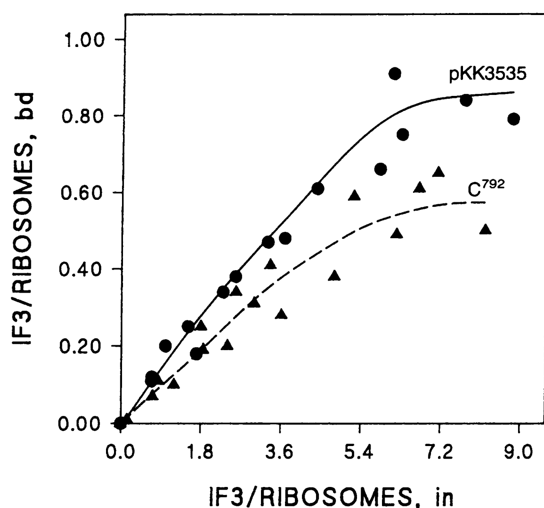

FIG. 7. Saturation binding of [^{14}C]methyl-IF3 to wild-type and mutant 30S subunits. Twenty to twenty-four picomoles of wild-type (pKK3535, ●) or mutant (C^{792}, ▲) 30S subunits were incubated with various concentrations of [^{14}C]methyl-IF3 and the reaction mixtures were centrifuged in 15–30% (wt/vol) sucrose density gradients. Gradients were fractionated and the amount of 30S subunit-bound IF3 was determined by liquid scintillation counting. Abscissa indicates molar ratio of added IF3 (in) to ribosomes; ordinate indicates IF3 bound (bd).

a 10-fold loss in affinity for IF3. To test whether the A → C mutation at 792 would have a similar effect, we examined the ability of IF3 to interact with the mutant subunits. As shown in Fig. 7, binding of IF3 to mutant subunits was reduced by ≈40%. It should be pointed out that the 30S subunits employed in these studies consisted of both wild-type subunits containing chromosomally encoded 16S rRNA and mutant subunits containing C^{792} 16S rRNA. If 30S subunits containing C^{792} were present at <50% (1, 8), then almost all of the IF3 binding to subunits can be ascribed to interaction with the wild-type subpopulation. Thus, it appears that the A → C mutation at position 792 in 16S rRNA results in abolition of the IF3 binding capacity of 30S subunits containing this mutation.

DISCUSSION

Comparative primary structure analyses on small-subunit rRNAs from a wide variety of sources have established that there are conserved primary and secondary structures (3). Included in these conserved regions are positions 787–795 in the *E. coli* numbering system. Base number 792 is an adenine in all small-subunit RNAs except for two mitochondrial RNAs. This strong conservation of primary structure indicates a role for this base and this region in 30S ribosome function. Two roles have been suggested for this portion of 16S rRNA, namely, subunit association (4–9) and tRNA interaction (9). Damage to either one of these functions would affect translation to varying degrees. Replacing the adenine with a guanine at position 792 increased the generation time by about 16%, while a change to a cytosine increased it by 56%. The decrease in growth rate was correlated with a decrease in ^{35}S assimilation into proteins by as much as 30–40% in cells translating with C^{792}-containing 30S ribosomes. Ribosomal 30S subunits containing C^{792} or G^{792} participated in 70S assembly at greatly reduced levels (Figs. 4 and 5); this decrease in ability to form 70S ribosomes may have accounted for the decrease in protein synthesis and the large increase in generation time that were observed when cells were required to grow in the presence of spectinomycin, using only mutant ribosomes. At the same time the ability of these mutant 30S subunits to combine with 50S subunits had only a small effect on the generation time of cells when a heterogeneous population of 30S subunits was used, simply because the mutant 30S subunits competed, however modestly, with normal 30S subunits for binding to 50S subunits. On the other hand, 30S ribosomes with U^{792} appeared to have no effect on growth rate in a mixed population of ribosomes, suggesting that U^{792} mutant 30S ribosomes do not interfere with normal 30S ribosomes during translation. However, when cells were required to grow using only U^{792}-containing 30S ribosome, they could not do so.

The inability of U^{792}-containing 30S ribosomes to support growth may be due to one or more of three effects. (*i*) The level of functional 70S ribosomes is so low that cell growth is not sustained. For example, pKK3535-coded ribosomes normally account for 50–60% of the cells' population of ribosomes (1, 8). In the presence of spectinomycin only these plasmid-coded ribosomes are functioning. If mutant 30S ribosomes have the same *in vivo* level of association with 50S ribosomes as they do *in vitro* at 6 mM Mg^{2+}, then the total population of 30S ribosomes associated with 50S ribosomes is about 17% of the total cell population of ribosomes. (*ii*) IF3 binding is impaired by the base change at position 792. (*iii*) Ribosomal 30S subunits containing U^{792} may be less stable than those containing G^{792} or C^{792}.

The decreasing growth rate, and finally the inability to grow, is correlated with a change of A^{792} to guanine, cytosine, or uracil, which progressively decreases 70S ribosome assembly. Position 791, like 792, also has a profound effect on

subunit assembly (8), which suggests that a number of bases in this region of 16S rRNA are all essential for 30S–50S interaction. Positions 791 and 792 appear to be on the platform area of the 30S subunit, which places them at the interface between the 30S and 50S subunits (28–31). This region also appears to be an IF3 binding region (32). The presence of two adjacent bases in 16S rRNA that are important for subunit association and IF3 binding provides strong support for the role of this region of 16S rRNA in protein synthesis.

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