Mutations in the Intersubunit Bridge Regions of 23 S rRNA*

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The large and small subunits of the ribosome are joined by a series of bridges that are conserved among mitochondrial, bacterial, and eukaryal ribosomes. In addition to joining the subunits at the initiation of protein synthesis, a variety of other roles have been proposed for these bridges. These roles include transmission of signals between the functional centers of the two subunits, modulation of tRNA-ribosome and factor-ribosome interactions, and mediation of the relative movement of large and small ribosomal subunits during translocation. The majority of the bridges involve RNA-RNA interactions, and to gain insight into their function, we constructed mutations in the 23 S rRNA regions involved in forming 7 of the 12 intersubunit bridges in the Escherichia coli ribosome. The majority of the mutants were viable in strains expressing mutant rRNA exclusively but had distinct growth phenotypes, particularly at 30 °C, and the mutant ribosomes promoted a variety of miscoding errors. Analysis of subunit association activities both in vitro and in vivo indicated that, with the exception of the bridge B5 mutants, at least one mutation at each bridge site affected 70 S ribosome formation. These results confirm the structural data linking bridges with subunit-subunit interactions and, together with the effects on decoding fidelity, indicate that intersubunit bridges function at multiple stages of protein synthesis.

A fundamental feature of translation systems is the existence of two unequally sized ribosomal subunits that are joined together during the initiation phase of protein synthesis to form the functional ribosome, which in turn is resolved into the component subunits in a post-termination step (1). Analysis of bacterial 70 S ribosomes by cryoelectron microscopy showed that the 50 S and 30 S subunits are connected by a series of bridges (see Figs. 1 and 2 and Table 1) (2, 3). Subsequent analyses demonstrated the existence of the same bridges in yeast cytoplasmic ribosomes (4) and a subset of these bridge connections in mammalian mitochondrial ribosomes (5), suggesting that intersubunit bridges are conserved elements of ribosome structure. Besides fulfilling the obvious role of joining and holding the small and large ribosomal subunits together in an appropriate orientation during translation, structural analyses of ribosomes in different states of the translation cycle have shown that at least some of the intersubunit bridges are dynamic in nature and move during the translocation step (6). In addition, analyses of the interactions of initiation factor IF3 and ribosome recycling factor with the ribosome indicate that these factors may alter the orientation of several bridges, accounting for their effects on ribosome dissociation (7–10). Additional roles for bridges, including the transmission of signals between subunits and modulation of tRNA-ribosome interactions, have also been proposed (11).

The structures of Thermus thermophilus and Escherichia coli 70 S ribosomes have identified 12 distinct bridges (numbered B1a, B1b, B2a, B2b, B2c, B3, B4, B5, B6, B7a, B7b, and B8) (see Table 1), and the individual rRNA and ribosomal protein residues involved in bridge formation were also identified (3, 11, 12). Of the 12 intersubunit bridges, only a single bridge, B1b, involves a protein-protein interaction, whereas the majority involve wholly RNA-RNA interactions or a mixture of RNA-RNA and RNA-protein interactions (Fig. 1 and Table 1).

To investigate the potential roles of intersubunit bridges in the different steps of protein synthesis, we constructed mutations of the 23 S rRNA residues involved in the formation of bridges B1a, B2b, B2c, B3, B4, B5, and B7a and examined the mutants for their effects on viability, the accuracy of translation, and subunit association. The effects of mutations in bridges B2a and B6 have been examined separately.2 The majority of the mutants were viable in strains of E. coli expressing only mutant rRNA and had, at most, moderate effects on the levels of 70 S ribosomes in vivo. However, when 70 S ribosome formation was analyzed in vitro by mixing mutant 50 S subunits with wild-type 30 S subunits in the absence of any other ligands, the mutants exhibited a wide range of effects on subunit association. These results confirm the importance of bridge contacts in subunit association and suggest that the effects of rRNA mutations on 70 S ribosome formation may be masked in vivo by the binding of tRNAs, translation factors, and other ligands that help establish and maintain intersubunit contacts.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Plasmids**—The rRNA mutations were initially constructed in plasmid pLK35, in which the rnb operon is transcribed from the Ap1 promoter (13). Strain pop2136 carries the temperature-sensitive Acl repressor, and in this strain, transcription of plasmid-encoded rRNA is repressed at 30 °C but can be induced by a temperature shift to 42 °C (14). This strategy allowed the construction of potentially lethal mutations. Mutant rRNAs were subsequently expressed from

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2 M. O’Connor, unpublished observations.
the P1P2 promoters in plasmid pMO12 (15). Plasmid pMO12 is derived from pSC101 and is compatible with both the pACYC184-derived tRNA plasmid ptRNA67 and the lacZ and β-lactamase reporter plasmids used to monitor decoding fidelity. The Δlac ΔrecA Δ7 prn strain (16, 17) MC315, carrying deletions of all seven chromosomal rrr operons, was used to generate strains expressing only mutant rRNA.

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To facilitate subcloning, a KasI site was introduced into the region of the rnrB operon corresponding to h63 (see the legend to Table 1 for an explanation of the abbreviations used in this study) of 23 S rRNA. This variable region differs even between the seven rnr operons in E. coli (19). The nucleotide changes that create the KasI site (T1725C and C1726G) were combined with two additional base substitutions (G1734C and A1735G) designed to maintain base pairing in this stem loop, and no differences in growth, subunit association, or mis-coding were detected between this KasI mutant and wild-type rRNA. All PCR-generated fragments were initially cloned into a pLK35 derivative carrying the KasI site in h63, and the nucleotide sequences of the in vitro generated fragments were determined to ensure that no unanticipated mutations were present.

Generation of strains expressing only mutant rRNA involves replacement of a plasmid carrying a wild-type rnr operon with a plasmid encoding mutant rRNA. In strain MC315, the rnc operon is carried on a kanamycin-resistant, pSC101-derived plasmid, pCscB7 (20). This plasmid also carries the counters-selectable sacB gene, rendering MC315 sensitive to sucrose. Upon transformation of MC315 with pLK35-derived plasmids, the resident pCscB7 plasmid was eliminated by growth on sucrose-containing medium (20). This step was usually unnecessary for pMO12 transformants because the chloramphenicol-resistant pMO12 and kanamycin-resistant pCscB7 plasmids are both derived from pSC101 and cannot be stably maintained together in the same cell.

The growth rates of strain MC315 carrying pMO12 or its mutant derivatives were determined by diluting overnight cultures into fresh 2× YT medium (10 g/liter yeast extract, 16 g/liter Tryptone, and 5 g/liter NaCl) supplemented with chloramphenicol (50 mg/liter), incubating cultures with vigorous shaking at 37 °C, and monitoring subsequent growth with a Klett-Summernor colorimeter. The growth of mutant MC315 strains on solid medium was monitored at 30 and 37 °C by streaking cells on LB-agar plates supplemented with chloramphenicol and incubating for 24 and 72 h at 37 and 30 °C, respectively.

Sucrose Gradient Analysis of Ribosomes—MC315 strains expressing mutant rRNA were grown at 37 °C in 2× YT medium supplemented with chloramphenicol (50 mg/liter), Ribosomes were isolated from exponentially growing cells at a density of 1.0. Bacteria were collected by low-speed centrifugation and resuspended in 16% (w/v) sucrose in 6 mM MgCl₂, 60 mM NH₄Cl, 60 mM KCl, 50 mM Tris-HCl (pH 8.0), and 6 mM β-mercaptoethanol. After addition of lysozyme (final concentration of 50 units/ml), the suspension was lysed by five freeze-thaw cycles, and an S 30 lysate was prepared by centrifugation at 12,000 × g for 30 min. The volume of the lysate was increased 2-fold with TKNM-10 buffer (20 mM Tris-HCl (pH 8.0), 60 mM KCl, 60 mM NH₄Cl, 10 mM MgCl₂, and 6 mM β-mercaptoethanol) and loaded onto a 10–40% sucrose gradient in TKNM-10 buffer, followed by centrifugation at 17,000 rpm for 18 h in a Beckman SW 28 rotor. Gradient fractions were collected by displacement with 60% glycerol, and the absorbance of the fractions at 260 nm was monitored with an ISCO gradient fractionator.

Distribution of Mutant rRNA in 50 S Subunits, 70 S Ribosomes, and Polysomes—The rRNA mutants that were unable to support growth in the Δ7 prrn strain in the absence of wild-type rRNA were analyzed in strain DH1. This strain carries all seven intact chromosomal rnr operons and, when transformed with rrn plasmids, expresses a mixture of plasmid-encoded and chromosomally encoded rRNAs. Free 50 S and 30 S subunits, 70 S ribosomes, and polysomes were isolated by centrifugation of detergent lysates through 10–40% sucrose gradients (21). The distribution of mutant plasmid-encoded rRNAs in these fractions was analyzed by primer extensions as described (22), and the extension products were quantitated using a PhosphorImager.

Preparation of 50 S and 30 S Subunits—50 S and 30 S ribosomal subunits were dissociated from tight-couple 70 S ribosomes prepared as described above by sucrose gradient centrifugation under buffer conditions that promote dissociation (1 mM MgSO₄). First, cell lysates were diluted 2-fold with TKNM-10 buffer (10 mM MgSO₄) and loaded onto a 15–40% sucrose gradient in TKNM-10 buffer and centrifuged at ω₂t = 3.5 × 10¹¹ in a Beckman SW 28 rotor. The 70 S fraction from the gradients was collected, followed by concentration and buffer exchange using Amicon Ultra-15 centrifugal filter units (M, 100,000 cutoff). The Mg²⁺ concentration of the TKNM-10 buffer was lowered to 1 mM (TKNM-1), and concentrated and dissociated 70 S ribosomes were loaded onto a 10–25% sucrose gradient in TKNM-1 buffer and centrifuged at ω₂t = 2.7 × 10¹¹ in a Beckman SW 28 rotor. Fractions containing 50 S and 30 S ribosomal subunits were collected and concentrated, and the buffer was changed from TKNM-1 to TKNM-10.

In Vitro Reassociation Analysis of Ribosomes—70 S ribosomes were reassociated in TKNM buffer by adding 3 A₂₆₀ units of purified 30 S subunits to 3 A₂₆₀ units of purified 50 S subunits in a final volume of 1 ml. Parallel assays were performed for each mutant at six different MgSO₄ concentrations (TKNM buffer with 6, 7, 8, 10, 12, and 20 mM MgSO₄). Reassociation reactions were carried out for 30 min at 37 °C. 70 S ribosomes and 50 S and 30 S ribosomal subunits were fractionated by centrifugation through 10–25% sucrose gradients in TKNM buffer containing the same MgSO₄ concentration as was used for the reassociation reaction.

Stop Codon Readthrough and Frameshift Assays—The lacZ plasmids p400, p415, and pdnaXFSs carry UAG, UGA, and -1 frameshift mutations, respectively, in the 5′-end of the lacZ gene (23, 24). The Δlac AΔ7 prrn strain MC315 was used as a host for the ampicillin-resistant lacZ and pMO12-derived rRNA plasmids. β-Galactosidase activity was determined from logarithmic phase cells grown in minimal medium containing glucose (0.2%), thiamin, casamino acids, and ampicillin at 37 °C as described previously (15). Enzyme activities (calculated in Miller units) supported by the mutant ribosomes were expressed relative to the levels of activity obtained with wild-type ribosomes.

Missense Decoding Assays—Missense reading by the 23 S rRNA bridge mutants was analyzed using a β-lactamase missense mutant. In plasmid pKTS70G, a GGC glycine codon replaces the wild-type AGC serine codon at residue 70 of the β-lactamase gene (25).

Because a serine at position 70 is essen-
tial for β-lactamase activity, misreading of the GGC glycine codon by a serine tRNA is required to produce active enzyme. The influence of ribosomal mutants on miscoding in the β-lactamase mRNA was measured indirectly by assessing growth of ribosomal mutants carrying pKTS70G on increasing concentrations of ampicillin and determining the minimal inhibitory concentration of ampicillin for each rRNA mutant. For these experiments, cell growth was carried out in 96-well microtiter dishes using a 2-fold dilution series of ampicillin (25).

RESULTS

Construction, Viability, and Growth of 23 S rRNA Bridge Mutants—The 30 S and 50 S rRNA and protein constituents of the intersubunit bridges are listed in Table 1. Mutations at 23 S rRNA positions involved in intersubunit bridges were generated by PCR mutagenesis using oligonucleotides designed to change the wild-type base to all three mutant bases. Many of the bridge connections involve pairs of adjacent nucleotides, and in most of these instances, both positions were randomized in the mutagenic oligonucleotide. Bridge B4 involves interactions between the loop end of h34 in 23 S rRNA (nucleotides 713 and 717–718) and both h20 in 16 S rRNA and ribosomal protein S15. All four bases of the h34 loop (UAAC) were randomized in this case. pLK35 plasmids carrying the rRNA mutations were maintained at 30 °C in strain pop2136 to ensure that dominant deleterious mutations were not expressed during plasmid construction. Individual plasmids were sequenced to identify the nucleotide alterations at the site of mutagenesis. Sequenced mutants were then tested for their effects on growth and viability in strain MC315, which carries deletions of all seven chromosomal rrn operons. Construction of MC315-derived strains expressing mutant rRNA exclusively involved replacement of plasmid pCsacB7 with pLK35 derivatives carrying the selected mutations. The generation of ampicillin-resistant, kanamycin-sensitive isolates among the MC315 transformants was taken as evidence that the respective mutant 23 S rRNA retained sufficient function to support protein synthesis. Conversely, the inability to displace pCsacB7 with a mutant pLK35 plasmid indicated that the mutation had disrupted critical ribosomal functions to the extent that the mutant rRNA could no longer support protein synthesis.

Lethal Bridge Mutants—Upon transformation of MC315 with the mutant pLK35-derived plasmids, none appeared to have a dominant lethal effect on cell viability, and only a few mutants had recessive lethal phenotypes as judged by the inability of the mutant plasmids to displace pCsacB7. Derivatives of pLK35 in which the wild-type C1832–C1833 dinucleotide (bridge B2c) was changed to AA, AU, GA or UA were unable to eliminate pCsacB7 from MC315; only the GU and UU combinations were viable in this strain. In addition, whereas the C1836U and C1836A base changes (bridge B2b) were viable in MC315 when expressed from pLK35, the C1836G mutant was inviable. This result is consistent with the base pairing of C1836 to G1904. Only mutants supporting growth in Δ7 prrn strains were selected for further study.

Two lethal mutants at C1832–C1833 (bridge B2c) and the C1836A and C1836G (bridge B2b) mutants were subsequently analyzed in strain DH1, which carries intact chromosomal prrn operons and, when transformed with prrn plasmids, expresses a mixture of mutant and wild-type RNAs. The assembly and subunit association properties of these lethal rRNA mutants were analyzed by sucrose gradient centrifugation of lysates from the DH1 strains, and the distribution of the mutant RNAs among 50 S subunit, 70 S ribosome, and polysome fractions was subsequently determined by primer extension analysis (22). When analyzed by this approach, the RNAs of functional mutants that do not affect subunit association are evenly distributed among all fractions and account for ~50% of the rRNA (15). The ribosome profiles of the C1832A/C1833A mutant (B2c) resembled those shown in Fig. 3C, where the amount of free subunits was greatly increased, and the peak height of the 50 S fraction approached that of the 70 S fraction. Primer extensions showed that the mutant rRNA accounted for 67% of the rRNA in the 50 S fraction, whereas only 12 and 6% mutant rRNA were present in the 70 S and polysome fractions, respectively. The ribosome profiles of the C1832A/C1833U mutant differed only slightly from those of the wild type, showing only slightly elevated amounts of free subunits. However, the mutant RNAs were again selectively excluded from the 70 S and polysome fractions, and the mutant RNAs accounted for 44, 10, and 3% of the rRNAs in the 50 S, 70 S, and polysome pools, respectively. Thus, in both lethal C1832/C1833 bridge B2c mutants analyzed, the mutant rRNA is selectively excluded from the functional 70 S and polysome pools, consistent with a defect in subunit joining. The lack of substantial enrichment of the mutant RNAs in the 50 S pools and the absence of a greatly increased 50 S peak in the C1832A/C1833U mutant relative to the C1832A/C1833A mutant suggest that there may be additional defects in processing or assembly associated with this bridge B2c mutant.

The ribosome profiles of the C1836G mutant (B2b) resembled those in Fig. 3C, where the amount of free subunits was substantially elevated. Less severe effects were exhibited by the viable C1836A mutant; in this case, the amount of free 50 S subunits was only mildly increased. The mutant rRNA accounted for 51% of the rRNA in the 50 S fraction from the lethal C1836G mutant, whereas only 13 and 3% mutant rRNA were present in the 70 S and polysome pools, respectively. In the viable C1836A mutant, the mutant rRNA accounted for 42% of the 50 S fraction, whereas 13 and 14% were found in the 70 S and polysome pools, respectively. Although the mutant rRNA is excluded from the 70 S and polysome pools in both C1836 mutants in bridge B2b, consistent with an effect of the mutation on subunit association, the effect is more severe in the inviable mutant.

To facilitate subsequent analyses with lacZ and β-lactamase reporter gene constructs, the 23 S rRNA mutants supporting growth in strain MC315 were also constructed in the low copy number, chloramphenicol-resistant plasmid pMO12, which carries the rrrB operon under the control of the native Pp promoter. MC315 strains expressing only mutant 23 S rRNAs were generated by transforming MC315 with the pMO12 plasmids and isolating kanamycin-sensitive, chloramphenicol-resistant derivatives. Most of mutants that were viable when the rRNA was expressed from pLK35 were also viable when it was expressed from pMO12 plasmids. However, kanamycin-sensi-
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TABLE 2
Effects of 23 S rRNA mutations on doubling time and growth on solid medium

| 23 S rRNA position | Doubling time (min) | Growth on solid medium (37 °C) | Growth on solid medium (30 °C) |
|-------------------|---------------------|-------------------------------|-------------------------------|
| Wild-type         | 48 ± 1              | +++                           | +++                           |
| Bridge B1a        |                     |                               |                               |
| C1836A            | ND                  | ND                            | ND                            |
| C1836U            | 57 ± 6              | +++                           | –                             |
| A1932U            | 65 ± 1              | +++                           | –                             |
| Bridge B2b        |                     |                               |                               |
| C1832A            | ND                  | ND                            | ND                            |
| C1832U            | 53 ± 1              | +++                           | +                             |
| Bridge B2c        |                     |                               |                               |
| C1832G/C1833U     | ND                  | ND                            | ND                            |
| C1832G/C1833U/G1972A/G1973C | 54 ± 2 | +++                           | +                             |
| C1832U/C1833U/G1972A/G1973A | 59 ± 5 | +++                           | +                             |
| Bridge B3         |                     |                               |                               |
| C1947A            | 64 ± 3              | –                             | –                             |
| C1947G            | 58 ± 3              | +++                           | +                             |
| C1947U            | 63 ± 2              | –                             | –                             |
| A1960C            | 49 ± 1              | +++                           | +                             |
| A1960G            | 71 ± 1              | +++                           | +                             |
| A1960U            | 55 ± 1              | +++                           | +                             |
| C1947A/G1959U     | 75 ± 2              | –                             | –                             |
| A1960G/U1946C     | ND                  | ND                            | ND                            |
| Bridge B4         |                     |                               |                               |
| UAAC(loop 715)AUCU | 55 ± 1              | +++                           | +                             |
| UACC(loop 715)CCUC | 57 ± 2              | +++                           | +                             |
| UACG(loop 715)UGU | 54 ± 2              | +++                           | +                             |
| UACG(loop 715)GCCAC | 58 ± 1              | +++                           | +                             |
| Bridge B5         |                     |                               |                               |
| C1768U/G1769G     | 50 ± 2              | +++                           | +++                           |
| G1989A            | 51 ± 3              | +++                           | +++                           |
| G1989U            | 62 ± 3              | +++                           | +++                           |
| Bridge B7a        |                     |                               |                               |
| A1848C            | 48 ± 2              | +++                           | +                             |
| A1848G            | 51 ± 2              | –                             | –                             |
| A1848U            | 59 ± 1              | +++                           | +                             |
| G1894U            | 63 ± 2              | –                             | –                             |
| G1896C            | 50 ± 3              | +++                           | +                             |
| G1896U            | 65 ± 3              | +++                           | +                             |

Effects of 23 S rRNA mutations on doubling time and growth on solid medium. Growth on solid medium was determined after 24 or 72 h of incubation at 37 or 30 °C, respectively. +++, +++, +, and −, normal, moderate, slow, and no growth, respectively; ND, mutant not analyzed due to inviability or extremely slow growth in strain MC315.

The growth of MC315 strains expressing the C1836A (B2b) and C1832G/C1833U (B2c) mutant rRNAs from pMO12 plasmids were unstable during subculture, exhibited very slow erratic growth (~2-h doubling time), and were excluded from further analysis. It should be noted that these mutants did not exhibit remarkably slow growth in strain MC315 when expressed in plasmid pLK35. This discrepancy can perhaps be explained by the higher copy number of plasmid pLK35, which may allow increased expression of the mutant rRNAs to compensate for the suboptimal function of mutant ribosomes. A similar compensation of suboptimal function by increased ribosome concentration has been observed with mutations in ribosomal proteins S4 and S12 (26).

Viable Bridge Mutants—The doubling time of MC315 carrying the wild-type rrnB plasmid pMO12 was 48 min in rich liquid medium, and the doubling times of viable mutants varied between 50 and 71 min (Table 2). The most prominent effects on generation times were caused by the bridge B3 mutants C1947A, C1947U, and A1960G (doubling times of 64, 63, and 71 min, respectively); the bridge B2b mutant A1932U (doubling time of 65 min); the bridge B7a mutants G1849U and G1896U (doubling times of 63 and 65 min, respectively); and the bridge B2c mutant C1832U/C1833U (doubling time 65 min) (Table 2). Thus, mutations at each of the bridges had detectable effects on cell growth, indicating the importance of each of these regions for ribosome function.

Growth of the mutants was also analyzed on solid medium at both 30 and 37 °C (Table 2). Although all of the mutants had grown after overnight incubation at 37 °C, most mutants exhibited substantial growth defects at 30 °C. In general, mutants that had increased generation times in liquid medium at 37 °C grew poorly on solid medium at 30 °C. However, some rare mutants, including A1960C (B3) and A1848G (B7a), which had doubling times close to that of the wild type at 37 °C, failed to grow at the lower temperature. Conversely, the A1960G mutant (B3), which had one of the longest generation times at 37 °C in liquid medium, showed only modestly retarded growth at 30 °C. Incubation at 30 °C affected the growth of three of the four bridge B4 mutants only slightly, and no cold sensitivity was detected in any of the bridge B5 mutants. It should be noted that, although some mutants failed to grow on solid medium at 30 °C, these mutants did grow, albeit very slowly, in liquid medium at 30 °C.

Bridge B1a Mutants—h38 of 23 S rRNA is a prominent feature of the 50 S subunit and has been termed the “A-site finger,” given its proximity to the A-site tRNA (27). Bridge B1a involves interactions between A886, U887, and C888 at the loop end of h38 and protein S13 of the 30 S subunit (11, 12). All single-base mutations at these three residues were constructed; each of the nine mutations was viable in strain MC315 and had no apparent effect on growth rate. Analysis of cell lysates from these strains showed that the relative abundance of free subunits and 70 S ribosomes did not differ compared with the wild type (data not shown), suggesting that subunit interactions were not grossly distorted in these mutants. In light of these results, we constructed a more radical mutation by deleting residues 883–885 and 891–893, which shortened h38 by 3 bp but left loop 886–890 intact (referred to as Δ883–885/891–893). Our prediction was that shortening h38 in this manner should abolish its potential to contact protein S13. Surprisingly, this rRNA mutant was also viable in MC315, with only a modest effect on cell growth (doubling time of 53 min) (Table 2). During the course of these experiments, Sergiev et al. (28) reported the construction of an even more drastic mutant with the lower part of this helix (residues 872–905) completely deleted, but with only modest effects on cell growth and translation activities.

Mutations at C1832 and C1833 in Bridge B2c—Nucleotides C1832 and C1833 are part of a stable 4-bp stem in domain IV of 23 S rRNA (Fig. 2). Disruption of this helical element (h67) by mutations C1832G and C1833U most probably changes the conformation of the 50 S subunit and has been termed the “A-site finger,” given its proximity to the A-site tRNA (27). Bridge B1a involves interactions between A886, U887, and C888 at the loop end of h38 and protein S13 of the 30 S subunit (11, 12). All single-base mutations at these three residues were constructed; each of the nine mutations was viable in strain MC315 and had no apparent effect on growth rate. Analysis of cell lysates from these strains showed that the relative abundance of free subunits and 70 S ribosomes did not differ compared with the wild type (data not shown), suggesting that subunit interactions were not grossly distorted in these mutants. In light of these results, we constructed a more radical mutation by deleting residues 883–885 and 891–893, which shortened h38 by 3 bp but left loop 886–890 intact (referred to as Δ883–885/891–893). Our prediction was that shortening h38 in this manner should abolish its potential to contact protein S13. Surprisingly, this rRNA mutant was also viable in MC315, with only a modest effect on cell growth (doubling time of 53 min) (Table 2). During the course of these experiments, Sergiev et al. (28) reported the construction of an even more drastic mutant with the lower part of this helix (residues 872–905) completely deleted, but with only modest effects on cell growth and translation activities.
time of the strain containing the compensated mutant C1832G/C1833U combined with G1972A/G1973C was 54 min compared with a doubling time of at least 2 h for the uncompensated mutant C1832G/C1833U (Table 2). The same approach was used to rescue the severe growth defect caused by the mutation C1832U/C1833U with the compensatory base change G1972A/G1973A. However, in this instance, the effect of the compensatory mutations was less dramatic; the doubling time of the uncompensated C1832U/C1833U mutant was 65 min, and introduction of the compensatory G1972A/G1973A mutation resulted in only a partial recovery to a doubling time of 59 min. However, in the compensated mutant C1832U/C1833U/G1972A/G1973A, two of the four G-C pairs in the wild-type helix are replaced with two A-U pairs (Fig. 2), which most probably accounts for the incomplete recovery of the growth rate. This stable GC-rich stem was also disrupted by constructing mutations at G1972 and G1973 while leaving their base pairing partners C1832 and C1833 involved in bridge B2c formation unaltered. However, the G1972A/G1973A and G1972C/G1973A rRNAs were inviable in MC315. When the mutant rRNAs were expressed in strain DH1 in the context of mixed populations of wild-type and mutant ribosomes, the levels of free subunits were elevated in both mutants, and the ribosome profiles resembled those in Fig. 3B. Primer extension analyses showed that the mutant rRNA accounted for 62, 30, and 17% of the RNA in the 50 S, 70 S, and polysome fractions, respectively, from the G1972A/G1973A mutant. A similar distribution was observed with the G1972C/G1973A mutant, where the mutant rRNA accounted for 68, 39, and 18% of the rRNA in the 50 S, 70 S, and polysome fractions. Although the effects were less severe than those observed with the inviable C1832/C1833 mutants, these data show that the G1972/G1973 mutant rRNAs are clearly excluded from 70 S and polysome pools and slightly enriched in the 50 S fraction and are consistent with an effect of the mutations on subunit association. The inviability of G1972A/G1973A and G1972C/G1973A, together with the lethal effects of C1832/C1833 changes to AA, AU, GA, or UA noted above, is consistent with the requirement for helical stability in this region of 23 S rRNA. This in turn suggests that the effects of base changes at C1832 and C1833 on growth and ribosome function may be due to both the disruption of intersubunit contacts at bridge B2c and alterations to the structure of h67 of the 50 S subunit.

Bridge B3 Mutants in h70 of 23 S rRNA—The bridge B3 nucleotides C1947 and A1960 are base-paired to G1959 and U1946, respectively. Consequently, substitutions at these positions may have effects on the helical structure of region 1946–1960 in addition to any effects on intersubunit interactions. In an attempt to distinguish between these two possibilities, we investigated whether the slow growth of C1947A and A1960 are base-paired to G1959 and U1946, respectively. Consequently, substitutions at these positions may have effects on the helical structure of region 1946–1960 in addition to any effects on intersubunit interactions. In an attempt to distinguish between these two possibilities, we investigated whether the slow growth of C1947A and A1960 could be ameliorated by the introduction of compensatory base pairs at positions 1959 and 1946, respectively. However, the
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C1947A/G1959U mutant had an even slower growth rate (doubling time of 75 min) compared with the C1947A mutant alone (doubling time of 64 min), and the A1960G/U1946C mutant was inviable in MC315. These data indicate that, in addition to any effects of base pairing, the identities of the individual nucleotides in this helical region are critical for function. In bridge B3, G1482 and A1483 in 16 S rRNA form A-minor interactions with the consecutive G-C pairs C1947-G1959/G1948-C1958, and this interaction is likely to be eliminated by substitutions at these positions, as well as any obvious growth defect as judged by growth on solid rich medium at 37 °C. Although U714 is conserved by >98% in bacteria, and A715 and A716 are conserved by 90–98% in all three kingdoms (12), nonetheless, a wide range of four-base sequences functioned at these positions in E. coli under our experimental conditions, and there was no striking nucleotide preference at any of the four randomized positions. Given the apparent equivalence of all 12 loop 715 variants, only four mutants with sequences AUCU, CCUC, UUGU, and GCAC were studied further.

Effects of Bridge Mutations on Subunit Association in Vivo—One potential consequence of disrupting intersubunit bridge connections is a decreased ability to form or maintain 70 S ribosomes. We first addressed this by determining the distribution of 30 S and 50 S subunits and 70 S ribosomes in vivo using sucrose gradient analysis of cell lysates. None of the viable mutants gave rise to aberrant ribosomal particles on sucrose gradient profiles, suggesting that the 23 S RNA mutations did not affect 50 S subunit assembly. Based on the relative abundance of free subunits and 70 S ribosomes in the gradients, the mutants were divided into three groups. Gradient profiles of representative examples from each group are shown in Fig. 3. The gradient profiles of the first group of mutants (Fig. 3A) were identical to those of the wild type, and a majority (13 of 22) of the viable bridge mutants fall into this group. The second group (Fig. 3B) contains mostly bridge B7a mutants (A1848G, G1849U, and G1896C) and the bridge B2b mutant C1836U. The ribosome profiles of these strains showed a slight increase in the amount of free subunits relative to the 70 S ribosome fraction. The third group contains mutants that showed a substantial increase in the amount of free 30 S and 50 S subunits relative to the 70 S peak (Fig. 3C). 23 S RNA mutants A1932U (B2b); A1960G, C1947G, and C1947A (B3); and G1896U (B7a) all belong to this category. The substantial imbalance of the subunit/70 S ribosome ratio in the third group of mutants correlated well with the increased doubling times of these mutant strains (Table 2). However, not all of the slow growing mutants had altered ribosome profiles; the C1832U/C1833U (B2c) and C1947U (B3) mutants were among the slower growing mutants and yet had wild-type levels of subunits and 70 S ribosomes. Consequently, changes in subunit association patterns in vivo cannot alone explain all of the growth defects.

FIGURE 3. Sucrose gradient analysis of 50 S bridge mutants. Subunits and ribosomes were separated on sucrose gradients as described under “Experimental Procedures.” Mutants are grouped according to the relative abundance of free 50 S subunits versus 70 S fractions. Group A contains 23 S rRNA mutants with no visible effect on 70 S ribosome formation. The wild-type (Wt) profile is taken as a reference (A). Mutant G1896C represents a group with moderate effects on 70 S ribosome formation with a detectable increase in the fraction of free 50 S subunits (B). Mutant G1896U is representative of mutants with substantial effects characterized by a large amount of free subunits (C). Black arrows indicate 70 S, 50 S, and 30 S fractions. The direction of sedimentation is from left to right.

Mutations in Loop 715 of 23 S RNA (Bridge B4)—The loop end of h34 (nucleotides 713–718) in 23 S RNA interacts with both ribosomal protein S15 and residues 763–764 of 16 S RNA to form bridge B4 (11, 12). The importance of this interaction for subunit association is demonstrated by the observation that methylation of A715 disrupts subunit association in vitro (30). Randomization of nucleotides 714–717 yielded 12 different mutants with sequences differing from the wild-type sequence: 5′-UAAC-3′. These sequences were CCUC, CCUG, GCUU, AUCU, CGCC, UUGU, GCCC, GCAC, CAUC, UACG, GAUC, and CAGC. All 12 mutants were viable in MC315, and none conferred any obvious growth defect as judged by growth on solid rich medium at 37 °C. Although U714 is conserved by >98% in bacteria, and A715 and A716 are conserved by 90–98% in all three kingdoms (12), nonetheless, a wide range of four-base sequences functioned at these positions in E. coli under our experimental conditions, and there was no striking nucleotide preference at any of the four randomized positions. Given the apparent equivalence of all 12 loop 715 variants, only four mutants with sequences AUCU, CCUC, UUGU, and GCAC were studied further.
The pronounced temperature sensitivity of many of the mutants prompted us to investigate whether subunit association in vivo was compromised during growth at low temperature. MC315 strains expressing wild-type and C1836U (B2b), A1848G (B7a), A1932U (B2b), and C1947U and A1960C (B3) mutant tRNAs were grown at 30 °C, and the distributions of subunits and 70 S ribosomes were investigated by sucrose gradient analysis of cell lysates. These mutants were among those showing the most pronounced growth defects at 30 °C. In all cases, the gradient profiles of strains grown at 30 °C did not differ from the profiles of strains grown at 37 °C; and consequently, the reduced growth of these mutants at 30 °C cannot be ascribed to a decreased efficiency of subunit association at low temperatures.

Analysis of 70 S Ribosome Formation in Vitro—Formation of 70 S ribosomes in vivo is influenced not just by the relative affinities of the 30 S and 50 S subunits for one another but also by the binding of ligands such as tRNAs and translation factors. To examine the effects of disrupting bridge contacts on subunit joining in the absence of any of the ligands found in vivo, we analyzed 70 S ribosome formation in vitro using purified subunits and defined buffers.

Mutant 50 S subunits were purified from tight-couple 70 S ribosomes by sucrose gradient centrifugation under conditions that promote dissociation (1 mM Mg²⁺). Each assay contained 3 A₂₆₀ units of 50 S ribosomal subunits and 3 A₂₆₀ units of 30 S ribosomal subunits (~2-fold molar excess of 30 S over 50 S subunits). The in vitro reassociation efficiency of mutant 50 S subunits was tested at six different Mg²⁺ concentrations (6, 7, 8, 10, 12, and 20 mM MgSO₄) in TKNM buffer. The reassociation efficiency at different Mg²⁺ concentrations reflects the ability of 50 S subunits to form 70 S ribosomes. The concentration of Mg²⁺ ions in vivo is ~2–4 mM; however, subunit association and ribosome functions are also influenced by the polyamines present in the cell. Both Mg²⁺ ions and polyamines are known to stabilize RNA structures and are crucial for functioning of the translational apparatus. To keep the experimental system simple and easily interpreted, we used the previously described assay conditions in which reassociation was carried out in Mg²⁺-containing TKNM buffers from which polyamines were excluded (31, 32). The optimal Mg²⁺ concentration required for functioning of E. coli ribosomes in vitro is normally 10–12 mM. At 6 mM MgSO₄, wild-type 50 S and 30 S subunits reassociate with 50–60% efficiency (31), as was also observed in this study (Fig. 4, first panel). The efficiency of 70 S ribosome formation at 20 mM MgSO₄ was >95% and was used as a control. Reaction mixtures were incubated at 37 °C for 30 min, followed by sucrose gradient centrifugation at 4 °C in TKNM buffer containing 6, 7, 8, 10, 12, or 20 mM MgSO₄ as appropriate. Analysis of the ribosome profiles allowed the bridge mutants to be divided into four classes (A, B, C, and D) according to the efficiency of 70 S ribosome formation (Fig. 4).

The profiles of class A mutants are similar to those observed in the control assay utilizing wild-type 50 S subunits. The gradient profiles of the G1989U mutant were taken as being representative of class A mutants (Fig. 4). 50 S subunits containing 23 S rRNA mutations A1932U (B2b); C1832U/C1833U/G1972A/G1973A (B2c); C1947U, A1960U, and A1960C (B3); C1768U/U1769G, G1989A, and G1989U (B5); and G1986C (B7a) all belong to this group (Fig. 4).

Mutants of class B are characterized by an altered sedimentation coefficient of the reassociated ribosomes at low Mg²⁺ concentrations (6–10 mM) (Fig. 4). Mutant A1848G (B7a) is representative of class B mutants. The association efficiency of these mutants is very high even at low Mg²⁺ concentrations (6 and 8 mM) (Fig. 4). However, the 50 S and 70 S peaks are not resolved, and the sedimentation of mutant 70 S ribosomes is visibly slower (~50–60 S) than observed for wild-type ribosomes at the same Mg²⁺ concentrations. At 10 mM Mg²⁺, the broad 70 S peak is still poorly resolved from the 50 S peak and sediments at a position slower than seen with wild-type 70 S ribosomes. At 12 mM Mg²⁺, the reassociated ribosome fraction sediments like wild-type 70 S ribosomes, and the 50 S and 70 S peaks are well resolved. Interestingly, all loop 715 mutants (bridge B4) belong to this group. Despite these defects, the generation times of loop 715 mutants were only slightly decreased (Table 2). Class B also contains mutants Δ883–885/891–893 (B1a), C1836U (B2b), and G1849U (B7a).

Class C mutants include bridge B2c mutants C1832U/C1833U and C1832G/C1833U/G1972A/G1973C and bridge B7a mutants A1848C, A1848U, and G1896U (Fig. 4). A typical example of this group is A1848C. This group of mutants is characterized by a reduced reassociation efficiency compared with wild-type ribosomes analyzed under the same conditions. However, in contrast to class B, the sedimentation coefficient of reassociated ribosomes is not different from that of wild-type (70 S) ribosomes. All of these mutants reduce 50 S subunit reassociation efficiency by 20–40% (Fig. 4, fourth panel).

The remaining mutants, bridge B3 C1947A and A1960G, constitute class D and exhibit the most severe effect on 70 S ribosome formation in vitro. The reassociation profiles show some of the characteristic features of both class B and C mutants, and A1960G is representative of class D (Fig. 4). There is a low efficiency of 70 S ribosome formation at all Mg²⁺ concentrations, and particularly at low Mg²⁺ concentrations, the reassociated ribosomes have a sedimentation coefficient of <70 S. The normal 70 S sedimentation position is achieved only at 20 mM Mg²⁺. At low Mg²⁺ concentrations, the 50 S and 70 S fractions sediment as one peak, and only a small shoulder of reassociated ribosomes can be detected at 6 mM Mg²⁺. Even at 8 mM Mg²⁺, the 50 S and 70 S fractions are still part of two partially overlapping peaks. Because the 50 S subunits used in reassociation experiments were purified from 70 S ribosomes, any 50 S subunit assembly defects can be excluded as a potential cause of this association defect. The C1947A and A1960G mutants were among the slowest growing and were also among the mutants that showed the greatest defects in 70 S ribosome formation in vivo (Fig. 3). In summary, these bridge B3 mutations have a severe effect on the reassociation efficiency, and high Mg²⁺ concentrations are required to compensate for the absence or misformation of bridging interactions.

Effects of Mutations on Readthrough of Stop Codons, Frameshifting, and Miscoding—In a final set of experiments, the effects of bridge mutations on the fidelity of translation were addressed using a series of lacZ nonsense and frameshift constructs. Assays of β-galactosidase levels in wild-type and
mutant tRNA strains harboring the lacZ UAG and UGA constructs p400 and p450, respectively, showed that, with the exception of bridge B4 mutants, each bridge region contained at least one mutant affecting stop codon readthrough (Fig. 5). However, none of the bridge mutants analyzed in this study affected −1 frameshifting (data not shown). In addition, the β-galactosidase levels obtained with an in-frame lacZ construct, pGLZ23 (33), did not differ between mutants.

Little or no readthrough of either UAG or UGA stop codons was observed with the Δ883–885/891–913 (B1a); C1832G/FIGURE 4. Analysis of 70 S association in vitro. 3 A260 units of mutant 50 S subunits were mixed with 3 A260 units of wild-type 30 S subunits and incubated at 37 °C for 30 min before being loaded onto 10–25% sucrose gradients with appropriate MgSO4 concentrations, followed by ultracentrifugation. Six different MgSO4 concentrations (6, 7, 8, 10, 12, and 20 mM) were used for association analysis. Ribosomal mutants were classified into four different groups (classes A, B, C, and D) according to the pattern of 70 S ribosome formation. The association of wild-type 50 S and 30 S subunits under these assay conditions is shown in the upper panel. Mutants used as references for each class are shown in black boxes. The direction of sedimentation is from left to right.
C1833U/G1972A/G1973C and C1832U/C1833U/G1972A/G1973A (B2c); and A1960C and A1960U (B3) mutants. However, unlike the loop 715 mutants in bridge B4, where none of the rRNA alterations affected readthrough, other base changes at C1832-C1833 (B2c) and A1960 (B3) affected decoding of stop codons. The remaining bridge mutants are divided between those that supported detectable readthrough of both UGA and UAG and those that showed some preference for one or other stop codon. With the possible exception of G1989, where both base changes at this position preferentially stimulated UAG readthrough, different base substitutions at other rRNA positions affected both termination codons or had differential effects on UAG or UGA readthrough. An example is the three base changes at C1947 in bridge B3: C1947U stimulated readthrough of both codons, whereas C1947A and C1947G exhibited preferences for UGA and UAG, respectively.

Missense suppression was assessed indirectly by measuring growth of the ribosomal mutants carrying the Ser70 → Gly β-lactamase missense mutant in medium containing increasing concentrations of ampicillin. Although most of the mutants did not differ from the wild type in their response to ampicillin, mutants C1947U (B3), G1989U (B5), and A1848U/G1849U (B7a) showed a 2-fold increase in resistance to the antibiotic, indicating that these ribosomal mutants promote miscoding at sense codons in addition to their effects on stop codon reading.

**DISCUSSION**

Intersubunit bridges are prominent and evolutionarily conserved features of ribosomes and have been linked to a variety of functions in translation through structural, genetic, and biochemical investigations. Given the inferred importance of intersubunit bridges, it is surprising that few inviable mutants were recovered in our systematic mutagenesis of bridge residues and that most of the rRNA base changes had relatively modest effects on subunit association in vivo (Fig. 3 and Table 2). However, in vivo, subunits are joined to form a 70 S ribosome with the aid of initiation factors and ligands such as tRNAs, which span both subunits and may also aid in maintaining the association between subunits. During the elongation phase of protein synthesis, A- and P-site or P- and E-site tRNAs may also help maintain subunit association. Moreover, many bridges involve multiple interactions. Consequently, defects in bridge contacts may be masked through the action of ligands that contact both subunits and the multiplicity of intersubunit contacts at each individual bridge.

The in vitro assay for 70 S ribosome formation addresses subunit association in the absence of any other ligands, and subunit joining under these conditions should be more dependent on bridging contacts than is the case in vivo. Assays of subunit association in vitro (Fig. 4) showed that several of the mutants, including Δ883–885/891–913 (B1a), A1848C and A1848U (B7a), the four loop 715 variants (B4), and C1832U/C1833U (B2c), which were unaffected in 70 S ribosome forma-
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...in vitro, displayed detectable defects in subunit association in vitro. Moreover, whereas C1947U, A1960C and A1960U (B3), G1896C (B7a), and C1832U/C1833U/G1972A/G1972A (B2c) showed no effects on subunit association either in vivo or in vitro, the other base substitutions that we constructed at these positions displayed altered subunit association properties, confirming the involvement of these regions in bridging interactions. Only the G1989U, G1989A, and C1768U/U1769G mutants (B5) were unaffected in subunit association under both in vivo and in vitro conditions. The A1932U mutant in bridge B2b is unique; A1932U mutant ribosomes showed little or no defects in subunit association in vitro but were among the mutants with the most prominent defects in 70 S ribosome formation in vivo (Fig. 3C). Because subunit joining in vivo is influenced not just by subunit affinities but also by the binding of tRNAs, mRNA, and translation factors, the in vivo defect in 70 S ribosome formation in the A1932U mutant potentially derives from defects in ligand-ribosome interactions. Alternatively, because bridges participate in domain movement within and between ribosomal subunits, the increased levels of free subunits in the A1932U mutant in vivo could reflect destabilization of 70 S ribosomes caused by defects in bridge function after subunit joining. In summary, the majority of the rRNA mutations we created are viable, and with the exception of the bridge B5 mutants, at least one base change at each bridge produces detectable effects on subunit association in vivo and/or in vitro. The results of the mutagenesis experiments thus confirm the structural data linking these rRNA residues with subunit-subunit interactions and the formation of 70 S ribosomes.

Based on the in vitro reassociation behavior, mutants were grouped into four classes, with classes B, C, and D representing varying defects in 70 S ribosome formation and class A being unaffected in subunit association (Fig. 4). Class C mutant 50 S subunits displayed a decreased ability to associate, as if the loss of intersubunit bridge contacts simply decreased the affinity of the mutant 50 S subunits for the 30 S particles or altered the stability of the 70 S ribosomes. Class B mutants are clearly distinct from class C mutants, as the subunits associated readily at all MgCl₂ concentrations, but the 70 S ribosomes had an aberrant sedimentation, particularly at low MgCl₂ concentrations. 70 S ribosomes from class D mutants also displayed an aberrant sedimentation but, in addition, showed a reduced efficiency of 70 S ribosome formation. The altered sedimentation of 70 S ribosomes in class B and D mutants could reflect a novel conformation of the 70 S ribosomes caused by the loss of critical bridging interactions or, possibly, the formation of alternative, aberrant intersubunit bridges. Alternatively, the anomalously slow sedimentation patterns may reflect dissociation of the 70 S ribosome during centrifugation, as described over 30 years ago by Infante and co-workers (34–36). Hydrostatic pressure during centrifugation dissociates the 70 S ribosomes, and the dissociated but unresolved subunits sediment at an anomalous rate of ~61 S for E. coli ribosomes (35). This anomalous behavior is also influenced by MgCl₂ concentrations (37), as shown in Fig. 4. The latter interpretation would posit that the 70 S ribosomes of bridge mutants are more sensitive to pressure-induced dissociation than wild-type ribosomes. The differing association patterns of class B and D mutants may thus represent the effects of altered bridges on both subunit association and the stability of 70 S ribosomes.

The effects of the bridge mutations on stop codon readthrough and missense decoding indicate that intersubunit bridge functions are not limited to the initiation and recycling phases of protein synthesis. In the high resolution structures of the ribosome, bridges B1a and B2a are close to tRNA-binding sites (3, 11), and we reported previously the stimulation of stop codon readthrough and frameshifting by various bridge B2a mutations (15). However, we did not detect any increases in error frequency in the bridge B1a mutant examined here (Fig. 5), although some modest increases in translational errors were observed in a more radical B1a mutant (28). Unexpectedly, the results of the misreading assays presented in Fig. 5 show that, even though many of the bridges are distant from the tRNA-binding sites on the ribosome, alterations to bridges B2b, B2c, B3, B5, and B7a all affect the accuracy of decoding. Conceivably, decreased fidelity might be due to indirect effects on tRNA interactions with the large subunit. Alternatively, because there are substantial conformational changes in the small ribosomal subunit during decoding that precede peptide bond formation on the large subunit, the effects of altered bridge connections on decoding may derive from the inappropriate propagation of signals between subunits (12, 38, 39).

Bridge B1a is formed though contacts between ribosomal protein S13 and region 880 of h38 in 23 S rRNA. Protein S13 also contacts the large subunit protein L5, forming bridge B1b. Bridges B1a and B1b are the only contacts between the head and central protuberance regions of the 30 S and 50 S subunits, respectively. Region 880 of 23 S rRNA also contacts the elbow region of A-site tRNA, whereas L5 residues contact the P-site tRNA. During translocation, both bridges B1a and B1b undergo substantial movement; B1a intersubunit contacts are broken, and B1b engages in alternative S13-L5 interactions (3). All of these observations indicate that region 880 of 23 S rRNA is functionally important, and one would predict that ribosome function should be strongly affected by mutations in this 23 S rRNA region. Surprisingly, our data and those of Sergiev et al. (28) show that truncations of h38 (deletions of 3 bp and of nucleotides 872–905, respectively) are well tolerated in vivo, with relatively modest effects on cell growth, fidelity, subunit association, and elongation factor G-dependent GTPase activity. Bridge B1a has also been disrupted via mutagenesis of the 30 S protein S13 (40). Deletion of the rpsM gene encoding protein S13 results in disruption of both bridges B1a and B1b, and although this strain is viable, it is extremely slow growing, and the S13-deficient ribosomes are severely compromised in subunit association. Knowledge of the S13 residues involved in formation of bridges B1a and B1b and contacts with the P-site tRNA has allowed the design of S13 variants defective in specific bridging or tRNA contacts. These experiments showed that loss of the S13 residues involved in tRNA contacts or formation of bridge B1a has little phenotypic consequences, whereas residues involved in bridge B1b formation severely compromise growth. These S13 data are consistent with our results indicating that bridge B1a is largely dispensable for ribosome function, and the S13 data further suggest that bridge B1b is the more critical bridge connecting the head of the 30 S sub-
unit to the central protuberance of the 50 S subunit. It is noteworthy that, although h38 is conserved among bacterial, archaeal, and eukaryal ribosomes, it is considerably shortened in mitochondrial ribosomes, where bridge B1a is apparently absent (5).

Bridge B2b involves interaction (i) between loops 780 and 790 of 16 S rRNA and C1836, C1837, and G1922 in 23 S rRNA and (ii) between loop 1516–1519 capping h45 of 16 S rRNA and A1919, C1920, and A1932 in 23 S rRNA. Mutagenesis and biochemical studies have implicated region 790 of 16 S rRNA in initiation factor IF3 binding and subunit association (41). In 23 S rRNA, the C1836G mutant analyzed here is one of the few recessive lethal mutants we recovered, and sequestration of the C1836G mutant rRNA in the subunit pools of mixed ribosome populations, together with the subunit association defects of the viable C1836U mutant, indicates that bridge B2b is important for association. The anomalous behavior of the A1932U mutant discussed above suggests that bridge B2b also functions in steps after subunit joining.

Bridge B2c is formed through the interaction of the backbone of C770-G771 and G1514-G1515 in 16 S rRNA with the backbone of C1832-C1833 in 23 S rRNA via ion-mediated phosphate interactions (12). Phosphorothioate substitutions at C770 in 16 S rRNA have been shown to inhibit 70 S ribosome formation, confirming the importance of this interaction (42). Our analysis of the bridge B2c mutants indicated that, provided Watson-Crick pairing with positions 1972 and 1973 is maintained, C1832/C1833 mutations have relatively minor effects on subunit association, consistent with the bridge contact being made via backbone interactions. The 16 S rRNA nucleotides involved in bridges have been analyzed via combinatorial mutagenesis, and in these experiments, the wild-type nucleotides C770, G1514, and G1515 are enriched in the functional ribosomes (43), again suggesting that bridge B2c is important for subunit association.

In bridge B3, two A-G pairs in h44 of 16 S rRNA (G1417-A1483 and A1418-G1482) form A-minor interactions with two consecutive G-C pairs in h71 of 23 S rRNA (C1832-C1833 in 23 S rRNA via ion-mediated phosphate interactions (12). Phosphorothioate substitutions at C770 in 16 S rRNA have been shown to inhibit 70 S ribosome formation, confirming the importance of this interaction (42). Our analysis of the bridge B2c mutants indicated that, provided Watson-Crick pairing with positions 1972 and 1973 is maintained, C1832/C1833 mutations have relatively minor effects on subunit association, consistent with the bridge contact being made via backbone interactions. The 16 S rRNA nucleotides involved in bridges have been analyzed via combinatorial mutagenesis, and in these experiments, the wild-type nucleotides C770, G1514, and G1515 are enriched in the functional ribosomes (43), again suggesting that bridge B2c is important for subunit association.

In bridge B4, the loop 715 residues in 23 S rRNA interact with the small subunit protein S15. In the wild-type E. coli ribosome, loop 715 forms a U-turn and interacts with a hydrophobic area of protein S15 (12). However, our randomization of this loop showed that a number of four-base sequences were tolerated at this region of 23 S rRNA. Using modification interference experiments, Maival and Remme (30) showed that bridge B4 is essential for 70 S ribosome formation. Dimethyl sulfate modification of A715 inhibits subunit association at 6 mM Mg2+ but does not affect association at high Mg2+ concentrations. However, it has been argued that, because the modified N-1 position of A715 is not in direct contact with the 30 S subunit, the effect of the dimethyl sulfate methylation may derive from charge-charge repulsion with Arg52 of protein S15 (12). Although largely lacking a distinct phenotype in vivo, our selected loop 715 mutants showed some effects on subunit association in vitro, confirming the involvement of bridge B4 in 70 S ribosome formation.

Bridge B5 is one of four bridges (B2a, B3, B5, and B6) that involve interactions between h44 of 16 S rRNA and 23 S rRNA. Unique among our bridge mutants, none of the bridge B5 mutants we analyzed affected subunit association either in vivo or in vitro, possibly suggesting that the other 23 S rRNA interactions with h44 in the adjacent bridges B3 and B6 may render bridge B5 dispensable for subunit association functions. However, both substitutions at G1989 in bridge B5 affected the fidelity of decoding, suggesting that this bridge may play a role in subunit communication.

In bridge B7a, A702 in 16 S rRNA stacks on A1848 in 23 S rRNA (12). Consistent with the importance of this interaction, all three substitutions at A1848 affect subunit association in vitro and/or in vivo. In addition, A702 and A1848 interact with the C-G pairs G1846-C1894 and G1845-C1895 in 23 S rRNA (12). Mutants at the adjacent nucleotides G1849 and G1896 show substantial defects in subunit association, and G1896U promotes high levels of UAG readthrough. During ratcheting of the subunits, bridge B7 is broken by the lateral movement of the A702 region in the 30 S subunit (3, 12). The mutagenesis data indicate that bridge B7a plays a critical role in establishing and maintaining subunit contacts and also influences the decoding activities in the 30 S subunit.

Bridges have been linked to ribosomal functions other than subunit joining and modulation of tRNA-ribosome interactions. These activities include the relative displacement of 30 S and 50 S subunits during translocation (3) and initiation factor IF3-ribosome and ribosome recycling factor-ribosome interactions and the transmission of signals between the functional centers of both subunits (8, 9–10). The rRNA mutants we have constructed should now allow us to examine the contribution of each intersubunit bridge to these ribosomal functions.

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