G1401: A Keystone Nucleotide at the Decoding Site of *Escherichia coli* 30S Ribosomes

Philip R. Cunningham,‡ Kelvin Nurse, Carl J. Weitzmann,§ Didier Nègre,‖ and James Ofengand*

Roche Institute of Molecular Biology, Roche Research Center, Nutley, New Jersey 07110

Received April 6, 1992

ABSTRACT: 16S ribosomal RNA contains three highly conserved single-stranded regions. Centrally located in one of these regions is the C1400 residue. Zero-length cross-linking of this residue to the anticodon of ribosome-bound tRNA showed that it was at or near the ribosomal decoding site [Ehresmann, C., Ehresmann, B., Millon, R., Ebel, J-P., Nurse, K., & Ofengand, J. (1984) *Biochemistry 23*, 429–437]. To assess the functional significance of sequence conservation of rRNA in the vicinity of this functionally important site, a series of site-directed mutations in this region were constructed and the effects of these mutations on the partial reactions of protein synthesis determined. Mutation of C1400 or C1402 to any other base only moderately affected a set of in vitro protein synthesis partial reactions. However, any base change from the normal G1401 residue blocked all of the tested ribosomal functions. This was also true for the deletion of G1401. Deletion of C1400 or C1402 had more complex effects. Whereas subunit association was hardly affected, 30S initiation complex formation was blocked by deletion of C1400 but much less so by deletion of C1402. Alternatively, tRNA binding to the ribosomal A site was more strongly affected by deletion of C1402 than by deletion of C1400. P site binding was inhibited by either deletion. HPLC analysis of the in vitro reconstituted mutant ribosomes showed that none of the functional effects were due to the absence or gross reduction in amount of any ribosomal protein. It is concluded that G1401 in the 16S RNA of the *Escherichia coli* ribosome is a nucleotide critical for ribosome function, whereas the adjacent C1400 and C1402 are not. A model is proposed to account for these effects.

The ribosome, a ribonucleoprotein complex of over 2×10^6 daltons, is perhaps the most complex of all cellular organelles. It is nevertheless surprisingly consistent in structure throughout phylogeny. All ribosomes are constructed from 2 high molecular weight RNAs, up to 2 small molecular weight RNAs, and approximately 50–80 proteins distributed between two unequally sized subunits. Though some notable variations in structure exist, the basic architecture and function of the ribosome are conserved in all organisms.

Considerable progress has been made recently in determining how the ribosomal proteins interact with the high molecular weight RNA components in *Escherichia coli* (Stern et al., 1989), and models for how the RNA and protein components are arranged within the small (30S) subunit of the *E. coli* ribosome have been proposed (Brimacombe et al., 1988; Stern et al., 1988). The two main reaction centers of the ribosome, namely the peptidyl transferase region of the large (50S) subunit (Moazed & Noller, 1989, and references cited therein) and the decoding site on the 30S subunit (Ofengand et al., 1986; Moazed & Noller, 1990), have also been localized on the *E. coli* ribosome. Despite this recent burst of structural information, the way in which the ribosome functions at the molecular level is still not understood, although the accumulated evidence from a large number of experiments indicates that the RNA of the ribosome plays a much more important functional role than heretofore believed (Dahlberg, 1989; Raué et al., 1990; Noller, 1991).

Although the secondary structure of the small RNA is highly conserved phylogenetically, extensive sequence conservation is found only at three single-stranded regions (Raue et al., 1988, 1990). One of these sequences (518–533) is in the 5′-domain, while the remaining two (1394–1408; 1492–1505) are in the 3′-minor domain (Figure 1). All three regions have been implicated in tRNA binding (Moazed & Noller, 1990; Krzyzosiak et al., 1987; Cunningham et al., 1988; Denman et al., 1989a) and other protein synthesis functions (Cunningham et al., 1988; Denman et al., 1989a). In particular, C1400, which is located in the center of the 1394–1408 sequence, has previously been shown by us to be at the decoding site of the ribosome (Ofengand et al., 1979; Ehresmann et al., 1984; Ehresmann & Ofengand, 1984).

A way to obtain a better understanding of the role of such conserved ribosomal RNA sequences in the mechanism of protein synthesis is to make ribosomes that contain site-specific mutations in the conserved sequences of the RNA moiety. However, the genes for ribosomal RNA are found in seven copies in *E. coli*, each in a separate operon distributed throughout the chromosome, and as such have proven refractory to study by in vivo genetic techniques. One approach to circumventing this problem, pioneered by Dahlberg (Stark et al., 1982), has been to place a single copy of an rRNA gene on a multicopy plasmid and to generate mutants in the plasmid-borne gene. Typically, such transformed cells make half of their rRNA from the mutant gene. Our approach has been to develop a totally in vitro system for the synthesis of mutant 16S ribosomal RNA and for the reconstitution of this RNA into functional 30S ribosomal subunits (Krzyzosiak et al., 1987, 1988). We term this the "synthetic" ribosome system. The main virtues of this system over the plasmid-borne in vivo system are that all of the ribosomes generated are mutant with no wild-type background, the processes of ribosome assembly and ribosome function are separately controlled, and quantities of purely mutant ribosomes can be made and tested for function in the individual steps of protein synthesis.

* Author to whom correspondence should be addressed.
‡ Present address: Department of Biological Sciences, Wayne State University, Detroit, MI 48202.
§ Present address: Pall Corp., Glen Cove, NY 11542.
‖ Present address: Laboratoire de Biologie Moleculaire, Universite Claude Bernard, 69622 Villeurbanne Cedex, France.

0006-2960/92/0431-7629$03.00/0

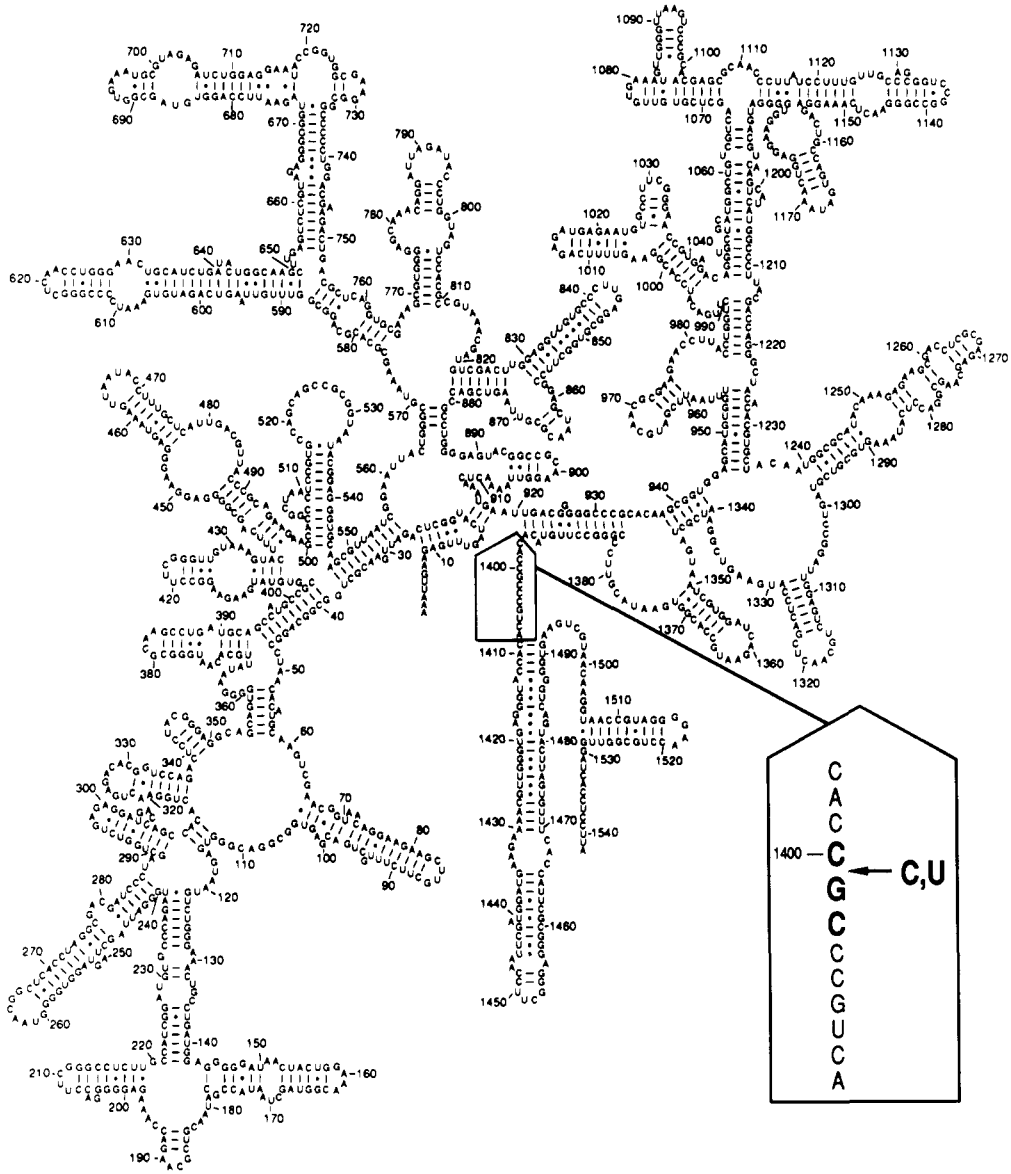

FIGURE 1: Position of the mutated nucleotides. The secondary structure of *E. coli* 16S RNA is according to that of Stern et al. (1989). The inset shows the mutated positions described in this work in boldface type. The mutants with U or C inserted between C1400 and G1401, U1400.1 and C1400.1, respectively, were described previously (Denman et al., 1989a,b).

In our initial studies, this approach was used to effect base changes at C1400, the residue cross-linked to the anticodon of tRNA. Despite the virtually complete conservation of this sequence throughout nature (Table I) and the unique cross-linking activity of this residue or its equivalent in ribosomes from a variety of species (Ehresmann et al., 1984; Ehresmann & Ofengand, 1984; Ciesiolka et al., 1985; Nurse et al., 1987), mutation of C1400 to other bases had little effect on ribosome function (Denman et al., 1989a). However, it was noted that deletion of residue G1401 completely inactivated the ribosome, although deletion of the adjacent residues, C1400 or C1402, did not. To explore a possibly unique functional role for G1401, ribosomes containing all possible base changes at positions 1401 and 1402 were made and compared with the C1400 mutants in a set of assays which probed the known partial reactions of protein synthesis.

EXPERIMENTAL PROCEDURES

General Techniques. Oligonucleotide synthesis, annealing of deoxyoligomers, preparation of plasmid DNA for sequencing, for retransformation, and for transcription, preparation of the double-digested vector DNA, HPLC analysis of ribosomal proteins, and 30S subunit reconstitution were all as described by Cunningham et al. (1990).

Construction of Mutants by Cassette Mutagenesis. The two complementary oligodeoxynucleotides described by Krzyzosiak et al. (1987) which terminated in *Bsm*I and *Nco*I sites were synthesized, except that random nucleotide substitutions were programmed at position 1401 in one oligomer and at position 1402 in the other oligomer. The heteroduplex (0.5 pmol), formed by annealing the two oligomers, was added to 0.1 pmol of vector DNA which had been double-digested with *Bsm*I and *Nco*I, and the mixture ligated as described previously (Cunningham et al., 1990). The resulting mixture was used to transform competent DH5 cells. Ampicillin-resistant transformants were selected on LB agar containing 100 μg/mL ampicillin. Twenty-four colonies were chosen. Plasmid DNA was prepared from each and used to retransform DH5 cells. The retransformants were selected on LB agar containing 100 μg/mL ampicillin. Plasmid DNA for sequencing was prepared from each of the chosen colonies, and the mutations were identified by sequencing through both ligation junctions.

Table I: Conservation of Residues 1397–1407 of *E. coli* 16S RNA among Other Organisms

	residue[a]										
	1397	1398	1399	1400	1401	1402	1403	1404	1405	1406	1407
archetype (433 sequences)	C	A	C	C	G	C	C	C	G	U	C
chloroplasts											
Chlamydomonas moewusii *Chlamydomas eugametos*	A	●	●	●	●	●	●	●	●	●	●
mitochondria											
Coturnix coturnix *Gallus gallus*	U	●	●	●	●	●	●	●	●	●	●
Aedes albopictus	U	●	U	●	●	●	●	●	A	●	●
Drosophila yakuba *Drosophila virilis*	●	●	U	●	●	●	●	●	●	●	●
Caenorhabditis elegans *Ascaris suum*	A	●	U	●	A	U	●	●	A	●	●
Strongylocentrotus purpuratus *Paracentrotus lividus*	●	●	U	●	●	●	●	●	●	●	●
Aspergillus nidulans *Podospora anserina*	A	●	●	●	A	●	U	●	●	●	●
Saccharomyces cerevisiae	A	●	U	●	A	●	U	●	A	●	●
Schizosaccharomyces pombe	A	●	●	U	A	●	U	●	●	●	●
Prototheca wickerhamii	●	C	●	●	●	●	●	●	●	●	●
Chlamydomonas reinhardtii	●	●	U	U	●	●	●	●	●	●	●
Tetrahymena pyriformis	●	●	●	●	●	●	●	●	A	●	●
Paramecium primaurelia *Paramecium tetraurelia*	●	●	●	U	●	●	●	●	A	●	●
Trypanosoma brucei *Crithida fasciculata* *Leishmania tarentolae* *Leptomonas* sp.	U	G	U	U	●	●	●	●	A	C	●

[a] Numbered according to Stern et al. (1989). Sequences were from the compilation by Neefs et al. (1991), Dubin and Hsuchen (1983), Delaney and Cattolico (1991), Desjardins and Morais (1991), and Okimoto et al. (1992).

Plasmid sequencing was performed as described by Cunningham et al. (1990) using a primer which corresponded to residues 1430–1450 of the RNA sequence. Using this procedure six mutations at two loci were generated with a single heteroduplex.

Transcription. Plasmid DNA, linearized by digestion with *Bsu*36I, was transcribed and the RNA purified as described by Cunningham et al. (1990). RNA transcripts were routinely checked for sequence in the mutated region and for integrity by glyoxal–Me_2SO denaturation (Denman et al., 1989b). In addition, the identity of mutants A1401, C1401, A1402, G1402, and U1402 was confirmed by sequencing the RNA extracted from the isolated mutant 30S ribosomes.

Materials. The following materials were prepared as described previously (Cunningham et al., 1990): mRNA for the I site and fMet-Val dipeptide assays, ribosomal high-salt wash (HSW) used as the source of initiation factors, 30S and 50S subunits, and total 30S ribosomal proteins (TP30) using the described LiCl–urea procedure.

Functional Assays. Assays for 70S formation were performed by incubating 100 nM 50S subunits and 67 nM 30S subunits in 50 mM Hepes (pH 7.5), 50 mM NH_4Cl, 15 mM $Mg(OAc)_2$, 5 mM DTT, and 20 μg/mL poly(U_2,G) at 37 °C for 10 min, followed by addition of 100 nM deacylated $tRNA^{Val}$ and incubation for 20 min longer. The samples, 0.2–0.3 mL, were analyzed as described by Denman et al. (1989b). The recovery of added 30S subunits as the sum of 30S and 70S particles ranged from 35 to 40%. The analogous recovery of 50S subunits was 60–65%. P site binding of tRNA, A site binding of tRNA, Phe, Val copeptide synthesis, 30S initiation complex formation (I site), and fMet-Val dipeptide synthesis were performed as described by Cunningham et al. (1990). All assays were adjusted so that the amount of product measured was proportional to the amount of 30S subunits added. A site binding in the absence of EFTu was <11% of that in its presence for all mutants studied. I site binding in the absence of HSW was <6% of that in its presence over the entire range of Mg^{2+} concentrations used for all of the mutants studied.

RESULTS

Sequence Conservation around the Sites of Mutation. The single-stranded sequence within which mutations were made (Figure 1) is very highly conserved throughout phylogeny. As indicated in Table I, 433 of 457 complete sequences currently available, or 95%, were identical to the *E. coli* sequence. Moreover, if organellar ribosomes are excluded, the identity is complete, there being no known cytoplasmic or bacterial sequences which differ from the archetype in this region. The degree of conservation among the organellar, mainly mitochondrial, ribosomes varies, both in terms of frequency and in the nature of the replacement, when analyzed position by position. Position 1397 is the most variable with 15 replacements of C by either A or U. A1398 is more conserved with

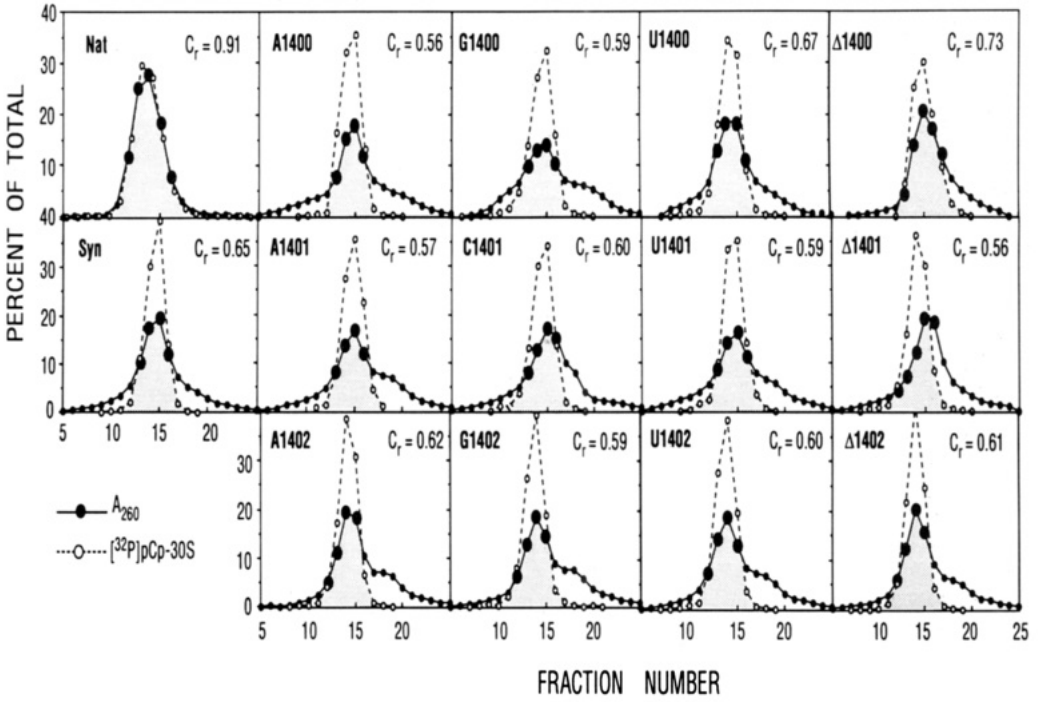

FIGURE 2: Reconstitution of natural and mutant ribosomes. Samples were prepared and analyzed as described under Experimental Procedures. One-milliliter fractions were collected. (Solid circles) A_{260}; (open circles) [^{32}P]pCp-30S. *The large solid ovals indicate the fractions pooled for functional analyses.* Each point was calculated by dividing the actual value for A_{260} or ^{32}P by the total recovered from each gradient. Thus, the areas under all of the peaks are equal. The coefficient of reconstitution (C_r) shown in each panel was obtained by taking the ratio of the area represented by the large solid ovals to the total A_{260} area. Integration was performed numerically by summing the lesser of the A_{260} or ^{32}P percentages for each fraction. The results for A1400, G1400, and U1400 are replotted from Denman et al (1989b).

only one replacement by C and, in the trypanosomes, four replacements of A by G. Positions 1399 and 1400 are variable with 13 and 8 examples each, respectively, of the replacement of C by U. G1401 is changed to A in six instances. C1402 and C1403 are highly conserved, being replaced by U in only two and four examples, respectively. C1404 is completely conserved. G1405 is replaced by A in 11 cases. U1406 is also highly conserved, while C1407 is so far invariant. It is worth noting that except for replacement of C1397 by A and the single occurrence of C1398, all replacements are conservative, that is, purines for purines and pyrimidines for pyrimidines.

This high degree of conservation implies a functional requirement for these particular residues. When coupled with the knowledge that C1400 is geographically close to the decoding site of the ribosome, it was a reasonable deduction that mutation of this region should produce interesting effects on ribosome function.

Preparation and Characterization of Mutant Ribosomes. 30S ribosomes mutant at positions 1400–1402 were prepared following procedures previously described (Cunningham et al., 1990) with appropriate modifications. This involved mutation of a suitable 16S RNA gene by the cassette method, in vitro transcription with T7 RNA polymerase, and reconstitution with a mixture of 30S ribosomal proteins. Sucrose gradient analysis of the ribosomes produced is shown in Figure 2. The [^{32}P]30S position (dashed line) served as an internal marker of the position and shape of the peak for authentic 30S subunits, it previously having been shown that [^{32}P]30S and isolated 30S subunits migrate identically (Krzyzosiak et al., 1987). The amount of such marker 30S material was always much less than 1% of the total, so that its activity would not interfere with the measurement of even poorly active mutant ribosomes.

Ribosomes reconstituted with 16S RNA isolated from 30S ribosomes (Nat) were able to reconstitute a particle which sedimented exactly like the marker, whereas the 30S subunits reconstituted from 16S RNA produced in vitro (Syn) assembled less well. Although the sedimentation peaks of the marker and Syn particles coincided, the Syn particles were clearly heterogeneous. Since the same protein preparation and reconstitution conditions were used in both samples, the difference must lie in the RNA. Recent experiments have shown that the method of isolation of the RNA has no effect on this result and that the minor difference in sequence at the 5′-end between "natural", i.e., isolated, 16S RNA and the in vitro product is also not responsible (Cunningham et al., 1991). By inference, it was concluded that the increased heterogeneity of reconstitution is a result of the lack of base modification(s) in the in vitro product.

The degree of heterogeneity was quantitated by calculating a coefficient of reconstitution, C_r, as described in the legend of Figure 2. Both by comparing C_r values and by visual inspection, all of the mutants appear similar and only slightly more heterogeneous than the Syn sample. The A_{260} absorption peaks of all of the mutant samples except for Δ1401 coincided with the marker 30S, although in some cases a slower sedimenting shoulder was clearly discernible. In particular, note that the 1401 mutants did not differ in any particular respect from the 1400 or 1402 mutants despite their greatly decreased functional ability (see below). The sucrose gradient analysis was also used as a preparative procedure. Only the fractions that lay within the authentic 30S peak, indicated by the enlarged ovals, were pooled for subsequent functional analysis. When some of the side fractions were compared to the pooled peaks in terms of ribosomal function, they proved to be <10% as active. Moreover, even the pooled peak fractions are more heterogeneous than Nat ribosomes, since Syn particles are only about half as active as Nat 30S (Denman et al., 1989a; Cunningham et al., 1991).

The ribosomal protein content of each of the mutant 30S subunits was determined (Table II). Except for a few cases, all of the proteins from S2 to S21 were present in unit stoi-

Table II: Protein Content of Mutant Reconstituted 30S Subunits[a]

protein	A1400	G1400	U1400	Δ1400	A1401	C1401	U1401	Δ1401	A1402	G1402	U1402	Δ1402
S2	1.1 (<0.1)	1.0 (0.1)	1.4 (0.4)	0.7 (0.3)	1.0 (0.1)	1.1 (<0.1)	0.9	1.1 (0.2)	0.9 (0.1)	0.8 (0.1)	1.1 (<0.1)	0.7 (0.2)
S3	1.0 (<0.1)	1.0 (<0.1)	1.0 (<0.1)	0.9 (0.2)	0.8 (0.1)	0.9 (0.1)	0.7	1.0 (0.2)	1.0 (<0.1)	1.0 (0.1)	1.1 (<0.1)	1.0 (0.1)
S4	0.9 (<0.1)	1.0 (0.1)	0.8 (0.1)	0.8 (0.3)	0.9 (<0.1)	1.0 (0.1)	0.9	1.0 (0.1)	0.9 (<0.1)	0.9 (<0.1)	0.9 (0.1)	0.9 (0.1)
S5	1.0 (<0.1)[b]	0.9 (<0.1)[b]	1.3 (0.4)	0.8 (0.1)[b]	0.9 (0.1)	1.0 (0.1)	0.9[b]	1.1 (0.2)	0.9 (<0.1)	0.9 (0.1)	1.0 (<0.1)	0.9 (<0.1)
S6	1.1 (0.1)	1.0 (<0.1)	1.1 (0.2)	0.8 (0.1)	1.1 (<0.1)	1.2 (0.1)	1.0	1.2 (0.1)	1.0 (0.1)	0.9 (0.1)	0.9 (<0.1)	0.9 (<0.1)
S7	0.9 (<0.1)	0.9 (0.2)	1.1 (0.1)	0.9 (<0.1)	0.9 (0.1)	0.9 (0.1)	0.8	1.1 (0.1)	0.9 (<0.1)	0.9 (0.1)	1.0 (<0.1)	1.1 (<0.1)
S8	1.0 (<0.1)[c]	1.1 (<0.1)[c]	1.2 (0.2)	0.9 (0.3)[c]	0.9 (0.2)	1.0 (0.1)	1.0	1.1 (0.1)	1.0 (<0.1)	1.0 (<0.1)	1.1 (0.1)	1.0 (0.1)
S9	1.0 (<0.1)[b]	0.9 (<0.1)[b]	1.0 (0.1)	0.8 (0.1)[b]	1.0 (<0.1)	1.0 (0.1)	0.9[b]	1.1 (0.1)	0.9 (<0.1)	0.9 (0.1)	1.0 (<0.1)	0.9 (0.1)
S10	1.1 (0.1)	1.1 (0.1)	1.2 (0.1)	0.8 (0.1)	1.0 (0.1)	1.2 (0.2)	1.0	1.1 (0.1)	1.0 (<0.1)	0.8 (<0.1)	1.0 (<0.1)	1.0 (<0.1)
S11	1.1 (0.1)	1.2 (0.2)	1.1 (0.2)	0.9 (0.4)	1.0 (0.1)	1.1 (0.1)	0.9	1.0 (0.1)	0.9 (<0.1)	0.8 (0.1)	0.8 (<0.1)	0.9 (<0.1)
S12	1.1 (0.2)	1.1 (0.1)	1.0 (<0.1)	0.8 (0.2)	1.0 (0.1)	1.1 (0.3)	0.8	1.0 (0.1)	1.0 (0.1)	1.1 (0.1)	1.0 (<0.1)	1.0 (0.1)
S13	1.0 (<0.1)[c]	1.1 (<0.1)[c]	1.0 (0.1)	0.9 (0.3)[c]	1.0 (<0.1)	1.0 (0.1)	1.0	0.9 (0.1)	0.9 (0.2)	1.1 (0.1)	1.1 (0.1)	1.0 (0.1)
S14	1.0 (<0.1)	1.1 (<0.1)	0.9 (0.1)	0.9 (0.3)	0.9 (<0.1)	0.9 (<0.1)	0.9	0.9 (0.2)	1.0 (0.1)	0.9 (0.1)	1.0 (<0.1)	1.0 (<0.1)
S15	0.9 (0.1)	1.1 (0.1)	0.9 (<0.1)	1.0 (0.3)	0.9 (<0.1)	1.0 (<0.1)	0.9	1.0 (0.2)	1.0 (0.1)	1.0 (<0.1)	1.0 (<0.1)	1.0 (<0.1)
S16	0.9 (<0.1)	1.0 (<0.1)	1.0 (0.2)	0.8 (0.2)	0.9 (<0.1)	1.0 (0.1)	1.0	1.1 (0.1)	1.1 (0.1)	1.0 (0.1)	1.0 (<0.1)	1.0 (0.1)
S17	0.8 (<0.1)	1.1 (0.1)	1.0 (0.2)	0.8 (0.3)	1.0 (<0.1)	1.1 (0.1)	1.0	1.1 (0.1)	1.0 (0.1)	0.8 (<0.1)	0.9 (0.1)	0.9 (<0.1)
S18	1.0 (<0.1)	1.2 (<0.1)	1.0 (<0.1)	0.9 (0.3)	1.0 (<0.1)	1.1 (<0.1)	1.0	1.0 (0.1)	0.9 (0.1)	0.9 (<0.1)	0.9 (<0.1)	0.9 (0.1)
S19	1.0 (0.2)	1.1 (0.2)	0.9 (0.1)	0.8 (0.3)	0.9 (<0.1)	1.0 (0.1)	0.9	0.9 (0.1)	0.9 (<0.1)	0.9 (0.1)	1.0 (0.1)	1.0 (<0.1)
S20	0.8 (0.1)	0.9 (<0.1)	0.9 (0.1)	0.7 (0.2)	0.9 (<0.1)	1.0 (<0.1)	0.9	1.0 (<0.1)	0.9 (0.1)	0.9 (<0.1)	0.8 (0.1)	1.0 (0.1)
S21	1.0 (0.1)	1.1 (0.1)	0.9 (0.1)	0.8 (0.2)	0.8 (<0.1)	0.9 (0.1)	0.6	1.0 (0.2)	0.9 (0.1)	0.9 (<0.1)	0.9 (0.1)	1.1 (0.1)
n	2	2	2	3	2	2	1	4	2	2	2	2

[a] Analyses were as described under Experimental Procedures. Values are expressed as protein/RNA mole ratios, normalized (Denman et al., 1989b) to reconstituted synthetic 30S. *n* is the number of analyses with average deviation ($n = 2$) or standard deviation ($n = 3$ or 4) in parentheses. Values <0.8 or >1.2 are underlined. [b,c] Pairs of proteins eluting together.

chiometry (±20%) in each mutant. The few larger deviations from stoichiometry, underlined in Table II, are not considered significant in view of the fact that in the cases where previous measurements were made, i.e., for A1400, G1400, U1400, Δ1400, Δ1401, and Δ1402 (Denman et al., 1989b), values closer to unit stoichiometry were obtained. For example, S2 in U1400 was 1.4 in Table II but 1.0 in the previous paper. Similarly, S20 in Δ1400 is 0.7 here but previously was 1.2. A complete comparison with the previously reported results shows that of the 240 protein values in Table II, only 2 appeared consistently low in both sets of measurements. Both cases involved S2, which was, in Δ1400, 0.7 here and 0.5 previously, and in Δ1402, 0.7 in Table II and 0.6 previously. An average value of 0.6 and 0.65 for S2 in these two deletion mutants is somewhat surprising in view of the fact that no proteins are known to interact with this region of the RNA (Stern et al., 1989). In any event, the magnitude of the decrease is insufficient to account for the large activity changes described below. The conclusion from Table II is that none of the functional effects described below can be ascribed to the absence of a given ribosomal protein from the mutant 30S subunit. This is an important point and one which is only possible to test experimentally in the synthetic ribosome system.

Function of Mutant Ribosomes in Protein Synthesis. Six in vitro assays of ribosome function (Table III) were developed to measure ribosome activity at various stages of the protein synthesis cycle. The subunit association assay measured the ability of 30S and 50S subunits to form 70S particles under conditions approximating those used for the P site and Phe,Val assays. In those cases where subunit association was low, measurements were also made at 20 mM Mg^{2+}, corresponding to system B of Denman et al. (1989b), but no difference from the values given in Table III was found (data not shown). Comparison of the standard conditions used in this work with system A of Denman et al. (1989b) for both synthetic and A1401 ribosomes also showed no difference between these two conditions. The assay is independent of the binding of tRNA to the P site. When deacylated tRNA was omitted, either in the presence or absence of mRNA, the percent association of Syn particles only decreased from 86 to 79%.

Table III: Functional Effects of Site-Specific Nucleotide Substitutions and Deletions at Positions 1400–1402[a]

mutant	subunit assoc	tRNA binding			peptide synthesis	
		P site	A site	I site	fMet-Val	Phe, Val
substitutions						
A1400[b]	110	140	80	70–110	140	115
G1400[b]	100	115	65	65–100	55	145
U1400[b]	90	135	125	70–110	115	135
A1401	35	30	45	20	15	20
C1401	25	15	20	5	5	15
U1401	30	20	20	5	10	15
A1402	70	65	75	55	95	85
G1402	80	70	65	70	115	75
U1402	95	100	80	110	100	100
deletions						
Δ1400[b]	80	20	60	10–20	<5	220
Δ1400	80	5	50	10	5	90
Δ1401[b]	35	<5	<5	<5	<5	15
Δ1401	15	5	10	5	5	15
Δ1402[b]	100	5	30	65–105	<5	200
Δ1402	95	10	25	65	5	125

[a] Assays were performed as described under Experimental Procedures. The values are expressed as percent of a synthetic wild-type sequence reconstituted at the same time with the same TP30 preparation. Each mutant ribosome was reconstituted two or more times with a separate preparation of RNA, and the average value was rounded to the nearest 5%, which is judged to be the level of accuracy of the measurements. [b] Data are from Denman et al. (1989a). fMet-Ser was measured instead of fMet-Val, and the coupled transcription–translation assay was used. The subunit association values are the average values for systems A and B, series I, as described in Denman et al. (1989b). The I site assay of these mutants are not reported previously.

The tRNA binding and peptide synthesis assays have been described in detail previously with respect to kinetics, Mg^{2+} optima, dependence on other components, and proportionality to 30S ribosomes added (Denman et al., 1989a; Cunningham et al., 1990). The P site assay was mRNA- and 30S-dependent, and the A site assay was additionally dependent on the presence of EFTu, which stimulated all binding values by at least 10-fold. Formation of the 30S initiation complex consisting of 30S subunits, mRNA, and fMet-tRNA (I site assay) likewise was stimulated 20-fold by a crude initiation

Table IV: Effect of Increased mRNA, Mg^{2+}, and Initiation Factor Concentration on fMet-tRNA Binding to 30S Subunits

				mol of fMet-tRNA bound/mol of 30S[a]					
expt	HSW (μg)	Mg^{2+} (mM)	mRNA (μM)	i30S	G1401	A1401	C1401	U1401	Δ1401
I	45	12	0.23	0.53	0.30	0.06	0.01	0.03	0.01
	90	18	0.23	0.51	0.26	0.07	<0.01	0.03	0.01
	90	18	1.59	0.52		0.05	0.01		<0.01
II	90	15	0.23	0.57	0.31	0.02	<0.01		
	90	20	0.23	0.56	0.30	0.04	<0.01		
	90	25	0.23	0.58	0.33	0.05	<0.01		

[a] f[^{3}H]Met-tRNA binding to 30S ribosomes was analyzed as described under Experimental Procedures. i30S denotes 30S subunits isolated from 70S ribosomes; HSW, ribosomal high-salt wash containing initiation factors; mRNA, synthetic mRNA prepared as described under Experimental Procedures. G1401 is the wild-type sequence.

factor preparation and was dependent on mRNA. The Phe,Val assay measured mRNA-dependent peptide bond formation in the absence of initiation, while the fMet-Val assay measured the ability to initiate and form the first peptide bond. This latter activity was also dependent on mRNA and initiation factors. All assays were adjusted so that the amount of product formed was directly proportional to the amount of 30S subunits added. Thus, the values represent the average values of the probably heterogeneous population of 30S particles rather than the activity of the most active subpopulation which would be the result if ribosomes were used in excess. The I site assay was particularly important for assessing the function of mutant ribosomes because it is the only one which is independent of prior association with the 50S subunit. Thus, effects on function and on subunit association could be independently monitored by this assay.

All possible substitutions at positions 1400–1402 were made, as well as deletions at each of the three positions (Table III). The effects of deletions are more complex to interpret since they change the spatial arrangement in the mutated region in addition to removing the naturally occurring nucleotide. The substitution mutants of C1400 and all three deletion mutants were reported previously (Denman et al., 1989a). They are included here for comparison with the newly generated mutants at positions 1401 and 1402. In addition, a second series of deletion mutants, prepared 1.5 years after the first series, was included as a separate entry to illustrate the degree of reproducibility of the results. The values are all expressed relative to the synthetic wild-type sequence (Syn) reconstituted with the same ribosomal protein mixture. Syn 30S was 40–60% as active as isolated 30S in the tRNA binding and peptide synthesis assays and 90–100% as active in the subunit association assay. Nat 30S was virtually as active as isolated 30S subunits in all of the assays (Denman et al, 1989a; Cunningham et al., 1990, 1991).

Replacement of C1400 by A, G, or U had little in vitro effect (Denman et al., 1989a). Only G1400 showed a lower activity in A site binding and in fMet-Val activity. Since the latter assay requires prior A site binding of Val-tRNA, both effects could be due to a small effect of G substitution on A site binding. Note the lack of any appreciable effect on I site binding. These latter results were not previously reported. Substitution of C1402 by the other bases had no strong effect on any of the assays. In marked contrast to these results, replacement of G1401 by any other base was strongly inhibitory in all of the assays with the possible exception of A1401 in A site binding. Not only was subunit association strongly affected along with those assays depending on association, but I site binding, which is independent of 50S association, was severely blocked. An unusual feature was noted in this assay in the case of A1401. Whereas the low activity of C1401 and U1401 was present from the first assay and in each reconstituted preparation, A1401 was transiently quite active (40–55%) in the I site assay in three of the four reconstituted preparations before decaying to the final stable value shown in the table. This behavior was not the result of repeated freeze–thaw cycles because a separate sample was taken for each assay. It was not observed with the other assays, and the most recent reconstitution did not show this behavior at all.

The newly prepared set of deletions behaved like those reported previously with two exceptions. The Phe,Val assays for Δ1400 and Δ1402, which were overstimulated previously, now were not more active than the Syn control, and the subunit association ability of Δ1401 was now reduced even further. In general, the behavior of Δ1400 can be understood as primarily a block in I site binding to 30S subunits (and also in P site binding to 70S ribosomes), which also therefore blocks fMet-Val synthesis but does not much affect subunit association or A site binding. The ability to make peptide (Phe,Val assay) with an apparently inhibited P site has been discussed previously (Denman et al., 1989a). The effect of deleting G1401 is consistent in all respects with the substitution results. The same inhibitions were found, only to a greater extent. Deletion of C1402 caused more complex effects, possibly because it can be considered equivalent to deleting C1403 or C1404. Like Δ1400, subunit association was not inhibited, but in contrast to Δ1400, A site binding was reduced while I site binding was largely unaffected. P site binding was also inhibited. This mutant appears to be defective in both P and A site binding as well as in some initiation step subsequent to formation of the initial 30S complex since it does not seem likely that all of the fMet-Val inhibition could be due only to the decreased A site activity. The retention of Phe,Val activity in the face of inhibited P and A site binding may be explained in the same way as for Δ1400. It is worthy of note that this is the only mutant described here or elsewhere (Cunningham et al., 1990), where P site and I site activity have differed.

A relatively trivial explanation for the lack of activity of the G1401 mutants might be that the affinity constants for tRNA, mRNA, and/or factors have been decreased as a result of the base changes such that under the standard assay conditions little reaction occurs. To test this possibility,, all of the mutants at position 1401 were tested for I site binding under varied conditions (Table IV). This assay was chosen because it is not complicated by the necessity to first associate with the 50S subunit. Experiment I shows that doubling the amount of crude initiation factors (HSW), increasing the Mg^{2+} concentration from 12 to 18 mM, and increasing the mRNA concentration 7-fold had no effect. In experiment II, still higher concentrations of Mg^{2+} were tried, but again there was no effect. The apparent increase from 0.02 to 0.05 in the case of A1401 is probably an artifact, as even 12 mM Mg^{2+}

gave 0.06 in experiment I. In another experiment, a 10-fold increase in concentration of fMet-tRNA had no effect on the amount of fMet-Val synthesis by A1401, being 17 and 18%, respectively, of the Syn control. We conclude from these results that the severe functional inhibition due to the mutation of G1401 is not simply a result of moderate changes in binding affinities of the ligands but rather must reflect either drastic quantitative changes or a qualitative effect.

DISCUSSION

The Synthetic Ribosome System. The method of making and analyzing ribosomal RNA mutants used in this work has a number of advantages over the in vivo methods used by other workers (Tapprich et al., 1990; Zimmerman et al., 1990; Leclerc & Brakier-Gingras, 1990). Mutations that interrupt protein synthesis can be as readily studied as those which are inert, as illustrated by the examples of both classes of such mutants described above. In vivo, inactivating mutants like the 1401 substitutions are either dominant lethals which kill the cell or recessive lethals which are silent or slow the growth rate. This dual phenotype is due to the unavoidable presence of a more or less equal amount of wild-type ribosomes derived from the host cell genome which either compete with or substitute for the mutant ribosomes derived from the genome of the introduced plasmid. In addition, the presence of host cell ribosomes gives a high background when in vitro assays of ribosome function are performed. The synthetic ribosome system, by contrast, has no background value of wild-type ribosomes because all of the rRNA is mutant.

Three approaches have been used to overcome the contribution from host cell ribosomes, but each has its own limitations. Introduction of an antibiotic resistance mutation into the plasmid ribosomal RNA gene to be mutated and use of the antibiotic to suppress host cell ribosome function (Hui & de Boer, 1987; Triman et al., 1989) suffer from the danger that the mutation (Hui et al., 1988) and/or the presence of the antibiotic can have unsuspected consequences. The specialized ribosome system (de Boer et al., 1985; Hui & de Boer, 1987; Jacob et al., 1987), in which both the anti-Shine–Dalgarno sequence of the ribosomal RNA to be mutated and the Shine–Dalgarno region of the mRNA for a protein which can be readily assayed are coordinately altered, has been successfully used for measuring protein synthesis in vivo (Hui et al., 1988). It has not yet been tested in the partial reactions of protein synthesis, however, and it is likely that the specialized mRNA may not be sufficiently discriminatory in vitro (Calogero et al., 1988). The insertion of an RNA "handle" at an innocuous site to allow specific recognition of the plasmid-borne RNA and its mutants has been a successful method for studying rRNA processing, ribosome assembly, and the ability to form polysomes in vivo (Musters et al., 1991) but has not yet been used to isolate purely mutant ribosomes for in vitro study.

Another advantage of the totally in vitro system described in this paper is that as ribosome reconstitution is carried out in the test tube, assembly is physically and temporally separated from function. This proved to be useful in the case of G1400, which was more difficult to assemble than the other mutants under the conditions used (Denman et al., 1989a). However, when the conditions were modified, assembly improved and functional testing could proceed. In the in vivo systems, this decoupling of assembly and function is not possible.

An additional feature of our system is the ability to directly determine whether mutation has caused the loss of a ribosomal protein. It is clearly important to know that the mutant particles retain all of their proteins if functional effects are to be ascribed directly to base changes in the RNA, yet this has not been examined in any in vivo assays of function and is difficult to do even after isolation of the ribosomes because of the approximately 50% background of wild-type particles. Moreover, quantitative HPLC analysis as performed in this work is preferable to the usual two-dimensional gel analysis, which relies on a visual comparison of the staining pattern between control and mutant ribosomes.

Perhaps the most important advantage, however, is the ability to assay the partial reactions of protein synthesis, unencumbered by high background values from host cell ribosomes or complicated by the use of antibiotics or specialized mRNAs. This is amply illustrated by our dissection of the effect of the 1400–1402 mutants as well as by our previous studies (Krzyzosiak et al., 1987; Denman et al., 1989a; Cunningham et al., 1990). These studies would not have been possible by the other approaches.

The in vitro system does have problems of its own. Because the rRNA is made in vitro, it lacks the base modifications, methylation and pseudouridylate formation, found in vivo (Krzyzosiak et al., 1987). The lack of these modifications is responsible both for the increased size heterogeneity of the reconstituted ribosomes as seen in Figure 2 and for their lower functional activity (Cunningham et al., 1991). Despite this, structure probing of synthetic 16S RNA in the 30S subunit did not show any deviation from wild type (Ericson et al., 1989), and synthetic 30S is capable of the synthesis of a complete protein (Melancon et al., 1990) in addition to carrying out the partial reactions of protein synthesis described in this work. Moreover, since the mutants were always compared to an unmodified wild-type control, the lack of modification should not perturb the results.

G1401 but Not C1400 or C1402 Is Crucial for Ribosome Function in Vitro. We previously showed that of the five single deletions C1397, A1398, C1400 (or C1399), G1401, and C1402 (or C1403 or C1404), only deletion of G1401 caused the loss of all ribosomal functions (Denman et al., 1989a). The most striking effect was in poly(Phe,Val) synthesis, where all of the deletions were quite active with the exception of ΔG1401. We have extended that initial observation by examining the effect of base substitution at G1401 and find that no other base but G is tolerated at that position in any of the assays employed. The only exception was A1401 in the 30S initiation complex formation (I site) assay. However, even in that case the activity of A1401 was transient and did not survive storage at liquid nitrogen temperatures. Interestingly, the only assay to show this effect is the only one which does not require prior association with the 50S subunit. A similar behavior has recently been observed in studies on mutations at positions 1512, 1523, and 1524. Here also the reactivity of certain mutant ribosomes was unstable to storage, losing up to 70% of the initial activity after 1 month in a liquid nitrogen freezer. In this case, however, activity was lost in poly(Phe) synthesis as well as in I site binding (L. Formenoy, K. Nurse, and J. Ofengand, unpublished results).

Although G1401 could not be replaced by any other base, substitution of C1400 or C1402 by other nucleotides had little effect. It is quite striking that G1401 should be so crucial for ribosome function while nucleotides on either side could be freely substituted. To our knowledge, G1401 is the only nucleotide in rRNA which has been shown to block all ribosomal tRNA binding and peptide bond formation functions when substituted by any other base. The demonstrated

importance of G1401 is in agreement with cross-linking studies reported earlier in which the insertion of C or U between C1400 and G1401 resulted in a shift of cross-link site from C1400 to the inserted base (Denman et al., 1989b). This result implies that being 5′-adjacent to G1401 is the key element in cross-link formation. Our results do not explain, however, the high degree of conservation of all three residues documented in Table I. Either the conserved C1400 and C1402 residues are favored but not essential and our assays are not able to measure these more subtle effects or they are required for some function we have not measured in the in vitro assays.

Agreement with Other Results. The double mutants A1399/U1401 and A1399/C1401, studied in vivo by Rottmann et al. (1988), were defective in association and could not be found in polysomes, in agreement with our results with U1401 and C1401. Hui et al. (1988), using the specialized ribosome system, observed that U1400 had no effect, A1400 inhibited protein synthesis partially, and G1400 was strongly inhibitory. These results agree with ours for U1400 and probably with the others as well when the effect of the base changes on assembly as distinct from function are taken into account. As pointed out above, in vivo assays cannot separate these two events. Hui et al. (1988) also reported that deletions and substitutions spanning 1400–1404 were inhibitory. These results can all be accounted for by the effects we have reported above for G1401, even without considering effects due to other bases or the stereochemical effects of such a massive change. The one result in disagreement with our work is their finding that mutation of C1402 to A was inhibitory. We did not see such an effect (Table III) and have no explanation for the discrepancy. In agreement with our results, Thomas et al. (1988) found that changing C1399 or C1400 to U had no effect in vivo but that deletion of C1400 created a dominant lethal phenotype. These authors also studied deletion of G1401 and replacement of C1400–G1401 by a single A residue. In both cases, the growth rate of cells containing mutant ribosomes plus host cell ribosomes was only slightly affected. These results also agree with those in this work if a recessive lethal phenotype is assumed for these in vivo mutants.

Functional involvement of G1401 has also been shown by protection of its N_7 position when tRNA is bound to the ribosomal P site (Meier & Wagner, 1984; Moazed & Noller, 1990), by the dramatic exposure of the N_7 position when 30S ribosomes undergo the transition from inactive to active (Moazed et al., 1986), by the inhibitory effects on tRNA binding to the P site of complementary deoxyoligonucleotides which bind to the 1400 region (Hill et al., 1990), and by the loss of protein synthesizing ability as a result of site-specific cleavage in the 1392–1408 region (Afonina et al., 1991). In contrast to this general agreement is the recently reported finding that deletion of residues 1401–1404 does not affect in vitro assembly of the 30S subunit and only inhibits the ability of the ribosome to synthesize MS2 coat protein by 50% or less (Almehdi et al., 1991). The reason for the discrepancy between this result and those cited above is not obvious.

Association with the 50S Subunit. It was initially surprising to find that mutation of G1401 strongly affected subunit association as well as 30S-fMet-tRNA-mRNA complex formation (I site assay), a reaction which is not dependent on prior subunit association. Although studies have indicated that residues in the vicinity of G1401 are involved in subunit–subunit association (Herr et al., 1979; Baudin et al., 1987, 1989), the region of the cleft wherein G1401 is thought to reside (Gornicki et al., 1984) has not been directly implicated

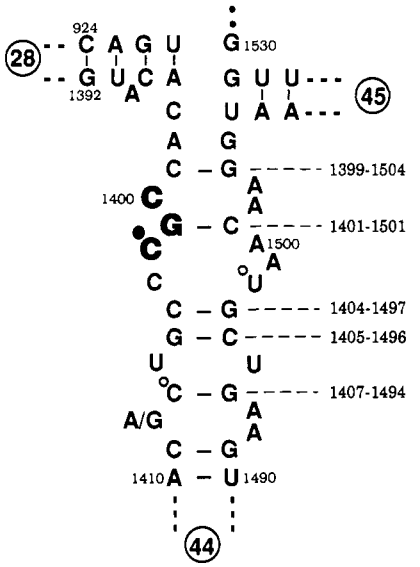

FIGURE 3: Proposed tertiary interactions between the 1397–1408 and 1492–1505 conserved single-stranded sequences of *E. coli* 16S ribosomal RNA. The relationship of helices 28, 44, and 45 to the rest of the 16S RNA molecule is shown in Figure 1. Helix 28 connects to the 1397 end of sequence 1397–1408, helix 44 is the 3′-penultimate stem connecting the two single-stranded sequences, and helix 45, the 3′-ultimate stem, connects the 1505 end of sequence 1492–1505 to the 3′-terminal single-stranded region containing the Shine–Dalgarno sequence. The tertiary interactions, shown as solid lines, are those proposed by Gutell et al (1985) and Gutell and Woese (1990). The nucleotides in boldface type are the ones mutated in this work. $^{+}$C, °C, and °U are m^4Cm, m^5C, and m^3U, respectively, in isolated 16S RNA. They are unmodified in the in vitro transcripts.

previously and would appear to be spatially distant from direct contact with the 50S subunit. Nevertheless, association with the 50S subunit was reported to increase exposure of the N_7 position of G1401 (Meier & Wagner, 1985), and Noller has pointed out that there are a number of subtle indications of a connection between tRNA binding and subunit association (Moazed & Noller, 1990; Noller, 1991). Our results reinforce that view. Recently, it has been reported that a striking conformational change in the 1400 region of 16S RNA in the 30S subunit occurs upon subunit association (Hill et al., 1992).

A Model for the Importance of G1401. A rationale for the importance of G1401 in all ribosomal functions is illustrated in Figure 3. In this figure, the 3′-minor domain is stripped to its essentials, namely, the two single-stranded regions connecting helices 28, 44, and 45. The mutated residues described in this work are indicated by boldface type. Gutell et al. (1985) and Gutell and Woese (1990), on the basis of comparative sequence analysis of a large number of small subunit RNAs, have proposed the base pair interactions indicated in the figure. In all known prokaryotes, eukaryotes, and chloroplasts, the base pairs shown are invariant. Only in a few mitochondrial ribosomal RNAs are there UA or UG replacements for the CG pairs, although the orientation Pu-Py is never reversed. Two pairs, 1404-1497 and 1407-1494, are invariant even in mitochondria. When our results are interpreted according to this scheme, G1401 cannot be changed because doing so would break the base pairing with C1501, which is in some still mysterious way essential to the well-being of the ribosome. Mutation of C1400 and C1402, on the other hand, might not be expected to be deleterious as these residues are not involved in holding the two strands together, and indeed they were not. This model could also explain why C1400 but not C1399 is available for cross-linking to the

anticodon of tRNA (Ehresmann & Ofengand, 1984) since C1400 is looped out while C1399 interacts with G1504.

ACKNOWLEDGMENT

We thank Joseph Sinning for his participation as an undergraduate summer student in the cloning of some of the mutants described in this work.

REFERENCES

Afonina, E., Chichkova, N., Bogdanova, S., & Bogdanov, A. (1991) *Biochimie 73*, 777.
Almehdi, M., Yoo, Y. S., & Schaup, H. W. (1991) *Nucleic Acids Res. 19*, 6895.
Baudin, F., Ehresmann, C., Romby, P., Mougel, M., Colin, J., Lempereur, L., Bachellerie, J.-P., Ebel, J.-P., & Ehresmann, B. (1987) *Biochimie 69*, 1081.
Baudin, F., Mougel, M., Romby, P., Eyermann, F., Ebel, J.-P., Ehresmann, B., & Ehresmann, C. (1989) *Biochemistry 28*, 5847.
Brimacombe, R., Atmadja, J., Stiege, W., & Schüler, D. (1988) *J. Mol. Biol. 199*, 115.
Calogero, R. A., Pon, C. L., Canonaco, M. A., & Gualerzi, C. O. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 6427.
Cunningham, P. R., Nègre, D., Weitzmann, C., Denman, R., Nurse, K., & Ofengand, J. (1988) *Arch. Biol. Med. Exp. 21*, 393.
Cunningham, P. R., Weitzmann, C. J., Nurse, K., Masurel, R., van Knippenberg, P. H., & Ofengand, J. (1990) *Biochim. Biophys. Acta 1050*, 18.
Cunningham, P. R., Richard, R. B., Weitzmann, C. J., Nurse, K., & Ofengand, J. (1991) *Biochimie 73*, 789.
Dahlberg, A. E. (1989) *Cell 57*, 525.
de Boer, H. A., Ng, P., & Hui, A. (1985) in *Sequence Specificity in Transcription and Translation. UCLA Symposia on Molecular and Cellular Biology* (Calender, R., & Gold, L., Eds.) Vol 30, pp 419–437, Liss, New York.
Delaney, T. P., & Cattolico, R. A. (1991) *Nucleic Acids Res. 19*, 6328.
Denman, R., Nègre, D., Cunningham, P. R., Nurse, K., Colgan, J., Weitzmann, C., & Ofengand, J. (1989a) *Biochemistry 28*, 1012.
Denman, R., Weitzmann, C., Cunningham, P. R., Nègre, D., Nurse, K., Cogan, J., Pan, Y. C., Miedel, M., & Ofengand, J. (1989b) *Biochemistry 28*, 1002.
Desjardins, P., & Morais, R. (1991) *J. Mol. Biol. 32*, 153.
Dubin, D. T., & Hsuchen, C. C. (1983) *Plasmid 9*, 307.
Ehresmann, C., & Ofengand, J. (1984) *Biochemistry 23*, 438.
Ehresmann, C., Ehresmann, B., Millon, R., Ebel, J. P., Nurse, K., & Ofengand, J. (1984) *Biochemistry 23*, 429.
Ericson, G., Chevli, K., & Wollenzien, P. (1989) *Biochemistry 28*, 6446.
Gornicki, P., Nurse, K., Hellmann, W., Boublik, M., & Ofengand, J. (1984) *J. Biol. Chem. 259*, 10493–10498.
Gutell, R. R., & Woese, C. R. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 663.
Gutell, R. R., Weiser, B., Woese, C. R., & Noller, H. J. (1985) in *Nucleic Acid Research and Molecular Biology* (Cohn, W. E., & Moldave, K., Eds.) Vol. 32, pp 155–216, Academic Press, New York.
Herr, W., Chapman, N. M., & Noller, H. F. (1979) *J. Mol. Biol. 130*, 433–449.
Hill, W. E., Weller, J., Gluick, T., Merryman, C., Marconi, R. T., Tassanakajohn, A., & Tapprich, W. E. (1990) in *The Ribosome. Structure, Function*, and *Evolution* (Hill, W., Dahlberg, R. Garrett, P., Moore, D., Schlessinger, D., & Warner, J., Eds.) pp 253–261, American Society for Microbiology, Washington, DC.
Hill, W. E., Weller, J. W., & Lodmell, J. S. (1992) *Fed. Proc. 6*, A65.
Hui, A., & de Boer, H. A. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 4762.
Hui, A. S., Eaton, D. H., & de Boer, H. A. (1988) *EMBO J. 7*, 4383.
Jacob, W. F., Santer, M., & Dahlberg, A. E. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 4757.
Krzyzosiak, W., Denman, R., Nurse, K., Hellmann, W., Boublik, M., Gehrke, C. W., Agris, P. F., & Ofengand, J. (1987) *Biochemistry 26*, 2353.
Krzyzosiak, W. J., Denman, R., Cunningham, P. R., & Ofengand, J. (1988) *Anal. Biochem. 175*, 373.
Leclerc, D., & Brakier-Gingras, L. (1990) *Biochem. Cell Biol. 68*, 169.
Meier, N., & Wagner, R. (1984) *Nucleic Acids Res. 12*, 1473.
Meier, N., & Wagner, R. (1985) *Eur. J. Biochem. 146*, 83.
Melançon, P., Leclerc, D., Destroismaisons, N., & Brakier-Gingras, L. (1990) *Biochemistry 29*, 3402.
Moazed, D., & Noller, H. F. (1989) *Cell 57*, 585.
Moazed, D., & Noller, H. F. (1990) *J. Mol. Biol. 211*, 135.
Moazed, D., Van Stolk, B. J., Douthwaite, S., & Noller, H. F. (1986) *J. Mol. Biol. 191*, 483.
Musters, W., Gonçalves, P. M., Boon, K., Raué, H. A., & van Heerikhuizen, H. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 1469.
Neefs, J.-M., Van de Peer, Y., De Rijk, P., Goris, A., & De Wachter, R. (1991) *Nucleic Acids Res. 19*, 1987–2007.
Noller, H. F. (1991) *Annu. Rev. Biochem. 60*, 191.
Nurse, K., Colgan, J., Denman, R., Wilhelm, J., & Ofengand, J. (1987) *Biochimie 69*, 1105.
Ofengand, J., Liou, R., Kohut, J., III, Schwartz, I., & Zimmermann, A. (1979) *Biochemistry 18*, 4322.
Ofengand, J., Ciesiolka, J., & Nurse, K. (1986) in *Structure and Dynamics of RNA* (van Knippenberg, P. H., & Hilbers, C. W., Eds.) pp 273–287, Plenum Publishing, New York.
Okimoto, R., MacFarlane, J. L., Clary, D. O., & Wolstenholme, D. R. (1992) *Genetics 130*, 471.
Raué, H. A., Klootwijk, J., & Musters, W. (1988) *Prog. Biophys. Mol. Biol. 51*, 77.
Raué, H. A., Musters, W., Rutgers, C. A., van't Riet, J., & Planta, R. J. (1990) in *The Ribosome. Structure, Function, and Evolution* (Hill, W., Dahlberg, A., Garrett, R., Moore, P., Schlessinger, D., & Warner, J., Eds.) pp 217–235, American Society for Microbiology, Washington, D.C.
Rottmann, N., Kleuvers, B., Atmadja, J., & Wagner, R. (1988) *Eur. J. Biochem. 177*, 81.
Stark, M. J. R., Gourse, R. L., & Dahlberg, A. E. (1982) *J. Mol. Biol. 159*, 417.
Stern, S., Weiser, B., & Noller, H. F. (1988) *J. Mol. Biol. 204*, 447.
Stern, S., Powers, T., Changchien, L.-M., & Noller, H. F. (1989) *Science 44*, 783.
Tapprich, W. E., Göringer, H. U., De Stasio, E., Prescott, C., & Dahlberg, A. E. (1990) in *The Ribosome. Structure, Function, and Evolution* (Hill, W., Dahlberg, A., Garrett, R., Moore, P., Schlessinger, D., & Warner, J., Eds.) pp 236–242, American Society for Microbiology, Washington, DC.
Thomas, G., Gregory, R. J., Winslow, G., Muto, A., & Zimmermann, R. A. (1988) *Nucleic Acids Res. 16*, 8129.
Triman, K., Becker, E., Dammel, C., Katz, J., Mori, H., Douthwaite, S., Yapijakis, C., Yoast, S., & Noller, H. F. (1989) *J. Mol. Biol. 204*, 645.
Zimmermann, R. A., Thomas, C. L., & Wower, J. (1990) in *The Ribosome. Structure, Function, and Evolution* (Hill, W., Dahlberg, A., Garrett, R., Moore, P., Schlessinger, D., & Warner, J., Eds.) pp 331–347, American Society for Microbiology, Washington, DC.

Registry No. Guanine, 73-40-5.