Functional Effects of Base Changes Which Further Define the Decoding Center of *Escherichia coli* 16S Ribosomal RNA: Mutation of C1404, G1405, C1496, G1497, and U1498

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ABSTRACT: The existence and functional importance of the tertiary base pair G1401:C1501, which brings together two universally present and highly sequence-conserved single-stranded segments of small subunit ribosomal RNA, was proven recently by mutational analysis [Cunningham, P. R., Nurse, K., Bakin, A., Weitzmann, C. J., Pflumm, M., & Ofengand, J. (1992) *Biochemistry 31*, 12012–12022]. Here we show that the additional nearby tertiary base pairs C1404:G1497 and G1405:C1496 also exist and are functionally important for tRNA binding to the ribosomal A and P sites. Breakage of the base pairs in turn led to a loss of activity at both A and P sites, whereas restoration in the reverse orientation led to recovery of activity. Recovery was incomplete, indicating that base pairing alone is insufficient for full restoration of function. Mutation of U1498 to G created the potential for the tertiary base pair C1403:G1498, which could stack on the aforementioned double base pair, creating a more stable helix longer by one residue. This mutation did not affect subunit association, A- and P-site binding of tRNA to 70S, fMet-tRNA binding to 30S, or poly(Phe) synthesis but did block formation of the first peptide bond, fMet-Val. Mutation of U1498 to A or C did not show this effect. Since the G1498 mutant could make both the 70S initiation complex and the peptide bond, as shown by its ability to form fMet–puromycin, the block in fMet-Val synthesis appears to involve some aspect of A-site function. Codon recognition at the A site seemed normal since the mutant did not miscode in an *in vitro* Leu-tRNA/poly(U) assay, although an effect on frameshifting was not ruled out. Codon–anticodon interaction at the P site also appeared normal as judged by cross-linking of P-site-bound tRNA exclusively to C1400 at a normal rate and yield.

There is now strong evidence that ribosomal RNA plays a major, and perhaps dominant, role in ribosome function (Noller, 1991, 1993; Noller et al., 1992). A considerable amount of this evidence has come from site-specific mutations made in ribosomal RNA. Mutations have been made *in vivo*, and their effect on function has been studied *in vivo* or *in vitro* [reviewed in Leclerc and Brakier-Gingras (1990) and Noller (1991); see also Prescott and Göringer (1990), Hänfler et al. (1990), Bilgin et al. (1990), Prescott and Dahlberg (1990), DeStasio and Dahlberg (1990), Jemiolo et al. (1991), Prescott et al. (1991), Göringer et al. (1991), Tapio and Isaksson (1991), Powers and Noller (1991), Allen and Noller (1991), Leclerc et al. (1991), O'Connor et al. (1992), and Saarma and Remme (1992)]. An alternative approach is to make the mutant ribosomes *in vitro* and to study their function *in vitro*. This latter approach involves *in vitro* synthesis of mutant ribosomal RNA, *in vitro* assembly of the ribosomal subunit from synthetic RNA and isolated ribosomal proteins, and *in vitro* testing of ribosome function (Krzyzosiak et al., 1987; Melançon et al. 1987; Denman et al., 1989a; Ericson et al, 1989; Weitzmann et al, 1990). We term this the "synthetic" ribosome system. It is particularly well suited for the analysis of mutations which are (or are expected to be) dominant lethals *in vivo* since partial reactions of protein synthesis are readily measured. This system has been used by us and others to study several different regions of *Escherichia coli* small subunit ribosomal RNA (Denman et al., 1989b; Gravel et al., 1989; Cunningham et al., 1990a,b, 1991, 1992a,b; Melançon et al., 1990a,b; Ofengand et al., 1993; Weitzmann et al, 1993; Santer et al., 1993; Ringquist et al., 1993; L. Formenoy et al., unpublished experiments).

A region of particular interest is the highly conserved single-stranded sequence 1394–1408 of the *E. coli* small subunit RNA (Figure 1) because C1400, in the middle of this sequence, was shown by cross-linking studies to be at or near the decoding site (Ofengand et al., 1986, 1988). Another feature of this sequence is its likely interaction, by base pairing, with another highly conserved single-stranded sequence, residues 1492–1505. This interaction was first proposed by Gutell et al. (1985) and Gutell and Woese (1990) who, on the basis of phylogenetic sequence analysis, postulated the existence of the tertiary base pairs C1399:G1504, G1401:C1501, and G1405:C1496 (see Table I for a current summary). These base pairs connect the two conserved sequences. Base pairs 1404:1497 and 1407:1494 were also proposed to link the two segments, but inasmuch as they were invariant, their existence could not be verified phylogenetically. Recently, however, an example of a mitochondrial ribosomal RNA sequence with A1404:U1497 replacing the otherwise universal G1404:C1497 has been described (Gutell, 1993).

This set of tertiary interactions is particularly intriguing because current information about the topographical disposition of the two single-stranded regions placed them on either side of the cleft (Stöffler-Meilicke & Stöffler, 1990) or groove (Shatsky *et al.*, 1991) of the subunit, the cleft or groove being that morphological feature which separates the large projection from the head and neck of the particle. Depending on the exact location of the single-stranded segments, tertiary base pair formation may require bridging the cleft or groove in some manner, perhaps transiently, either by movement of

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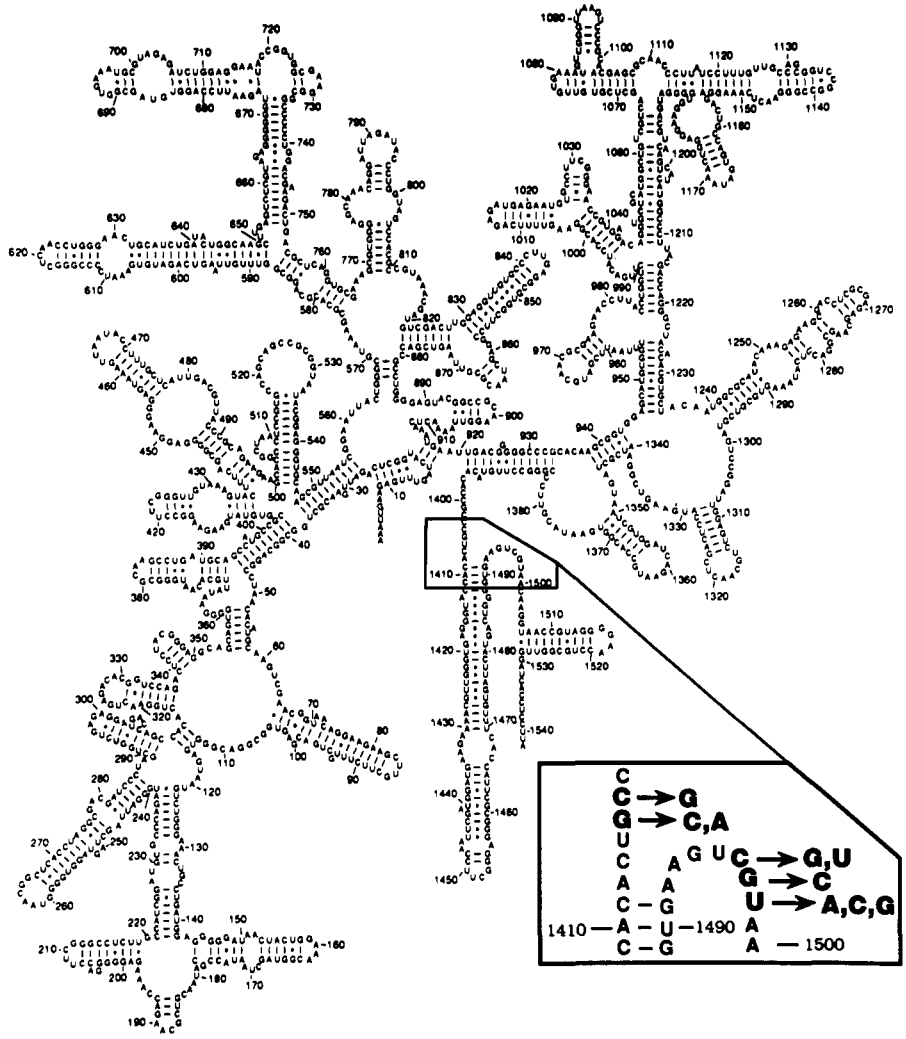

FIGURE 1: Location of the mutated nucleotides. The secondary structure of *E. coli* 16S RNA is according to Stern et al. (1989). The inset shows the sites of the mutations described in this work in boldface type. The two sets of positions, 1404:1497 and 1405:1496, are involved in long-range interactions.

the platform relative to the body or by looping out the RNA sequences so as to span the cleft or groove.

We have begun a series of investigations of the proposed tertiary base pairs by an *in vitro* mutagenesis approach. Specifically, we have asked whether disrupting the base pairs by mutation of each pair in turn has any effect on ribosome function and, if it does, whether that effect can be reversed by reforming the base pairs in the reverse orientation. In a previous study, we tested the G1401:C1501 interaction and found that while mutation either of G1401 to C1401 or of C1501 to G1501 disrupted both tRNA binding and polypeptide synthesis, combining both mutations so as to restore the base pair also restored ribosome function (Cunningham *et al.*, 1992b). In this report, we similarly test the two base pairs 1404:1497 and 1405:1496 for their existence by their effect on ribosome function. In addition, since mutation of m^3U1498 to G would create the possibility for the additional base pair C1403:G1498, which would stack directly over the C1404:G1497-G1405:C1496 minihelix (see Figure 7), the full set of base changes at position 1498 were also made and tested. Preliminary accounts of part of this work have appeared (Cunningham et al., 1990b; Ofengand et al., 1993).

MATERIALS AND METHODS

Materials. All materials were obtained or prepared as described in Cunningham *et al.* (1992b).

In Vitro Preparation of Mutant Ribosomes. Construction of the mutants at positions 1404 and 1405 followed the procedures described by Cunningham *et al.* (1992a) except that the synthetic deoxyoligomers spanning the *Bsm*I and *Nco*I (Krzyzosiak et al., 1987) sites were made in two approximately equal parts, one being constant and the other containing the mutant bases, which were then joined with the double-cut pWK1 vector in a three-way ligation. The mutations at positions 1496, 1497, and 1498 were made similarly by joining pWK1 cut at both the *Nco*I and the *Bst*EII (Krzyzosiak et al., 1987) site with a constant oligomer pair spanning residues 1412–1471 and a mutant oligomer pair covering positions 1472–1504. A separate mutant oligomer pair containing all three base substitutions at the same position in both strands was synthesized for each mutated site. The double mutants were made by isolating the *Kpn*I–*Nco*I fragment (Krzyzosiak et al., 1987) from the 1404 and 1405 single mutants in pWK1 and ligating them into the corresponding plasmids carrying the 1496 and 1497 mutations which had the corresponding *Kpn*I–*Nco*I fragment removed. All regions corresponding to synthetic oligomer inserts and ligation junctions were verified by sequence analysis. Transcription of the linearized plasmid, isolation and characterization of the RNA by denaturing gel electrophoresis and by sequencing, and ribosome reconstitution were performed as described previously (Cunningham *et al.*, 1990, 1992b).

Table I: Proposed Tertiary Base Pairing from Phylogenetic Analysis[a]

organism	1399 1504	1401 1501	1403 1498	1404 1497	1405 1496
E. coli	CG	GC	CU	CG	GC
Daphnia pulex	UG				
Drosophila virilis	UA				
Drosophila yakuba	UA				
Aedes albopictus	UA				AU
Strongylocentratus purpuratus	UA				
Paracentrotus lividus	UA				
Ascaris suum	UA	AU			AU
Coenorhabditis elegans	UA	AU			AU
Paramecium primaurelia			CA		AU
Paramecium tetraurelia			CA		AU
Tetrahymena pyriformis			CA		AU
Chlamydomonas reinhardtii	UG				
Aspergillus nidulans		AU	UA		
Podospora anserina		AU	UA		
Schizosaccharomyces pombe		AU	UA		
Saccharomyces cerevisiae	UA	AU	UA		AU
Mytilus edulis	UG				
Plasmodium falciparum			UU		
Suillus sinuspaulianus			?	UA	

[a] Sequence information was abstracted from Table 1 of Ofengand et al. (1993) and Table 1 of Gutell (1993). *E. coli* is representative of ca. 1000 species of cytoplasmic ribosomes from eukaryotes, archaebacteria, and eubacteria as well as all known chloroplast ribosomes and many from mitochondria. The variants listed are all from mitochondria.

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

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

RESULTS

Rationale for Choice of Mutations. The locations of the mutations studied in this work are shown in Figure 1. Base changes were made in both of the conserved single-stranded segment connecting the 3′-penultimate helix to the rest of the 16S RNA molecule. As stated in the introduction, the specific changes made were designed to test the proposed tertiary base pairing of C1404:G1497 and G1405:C1496 by converting them into C:C or G:G mispairs or the reciprocal C:G pairs. The expectation was that if breaking the putative base pairs inhibited function and restoring them in the reverse way restored function, the results would (a) support the existence of the tertiary base pairs, (b) show that a specific sequence was not required for function, and (c) demonstrate that the tertiary base pairs were required for functional as well as structural reasons. Such an approach had been successfully used to probe the G1401:C1501 tertiary base pair (Cunningham et al., 1992b). For the base pair C1404:G1497, the mutants G1404, C1497, and G1404:C1497 were made. For the base pair G1405:C1496, the mutants C1405, G1496, and C1405:G1496 were made. This latter base pair exists as A1405:U1496 in seven species of mitochondrial ribosomes (Table I). Therefore, A1405, U1496, and A1405:U1496 were also constructed in order to ascertain how well the A:U pair would function in an *E. coli* ribosome context. The double mutants G1404/G1496 and C1405/C1497 were constructed in order to break both putative base pairs simultaneously.

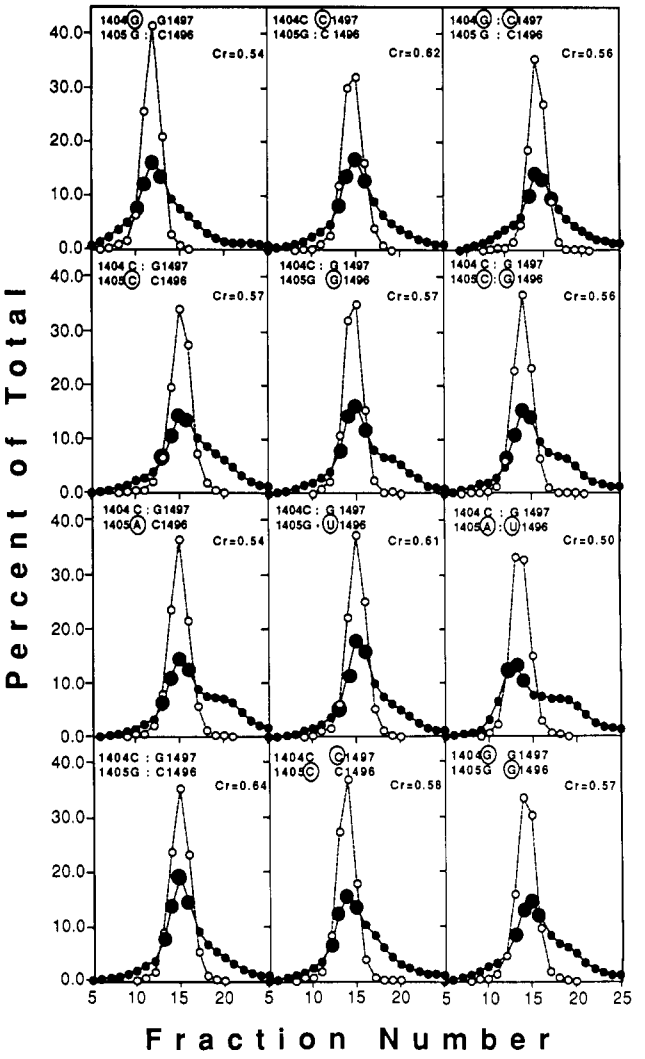

FIGURE 2: *In vitro* reconstitution of wild-type and mutant ribosomes. The two tertiary base pairs are shown in each panel. The mutated bases are circled. Preparation, isolation, and analysis were by velocity centrifugation through a sucrose gradient as described in Materials and Methods. The direction of sedimentation is from right to left: (●) A_{260}; (○) marker [^{32}P]30S ribosomes. Normalization of the curves and calculation of the coefficient of reconstitution, C_r, were as described previously (Cunningham et al., 1992a). The fractions pooled for subsequent functional tests are shown by the larger symbols.

Mutation of U1498 to G formally creates a base pair with C1403 which augments the 1404:1497 and 1405:1496 two base pair putative minihelix (see Figure 7). This mutation thus allowed us to test the effect of creating the possibility to unnaturally increase the length and strength of this minihelix. The A- and C1498 mutations were made as controls to differentiate sequence-specific effects from those due to base pair formation. The existence of U1403:A1498 in mitochondrial ribosomes from four fungi and the noncanonical CA pair in three other mitochondrial species (Table I) provided some support for the possible juxtaposition of positions 1403 and 1498.

Preparation and Characterization of Mutant Ribosomes. Ribosomes were prepared by following the standard procedure in use in our laboratory and purified by velocity gradient centrifugation (Figure 2). The 1404:1497 single and double mutants are in the top row of Figure 2; the next row shows the single and double C and G substitutions at 1405:1496; the third row, the corresponding A:U substitutions; and the bottom row shows on the left the wild-type sequence, followed by the two double mutants, in which both of the proposed base pairs

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

protein	G1404	C1405	G1496	C1497	G1404 G1496	C1405 C1497	G1404 C1497	C1405 G1496	A1405 U1496	A1498	C1498	G1498
S2	0.9	1.1	0.7	1.0	1.2	1.2	1.4	1.4	0.5	0.6	1.7	1.0
S3	1.0	0.9	0.8	0.9	0.9	0.8	0.9	0.8	0.7	1.0	0.9	1.0
S4	1.1	1.0	1.0	0.9	1.0	0.9	1.2	1.0	0.7	1.1	1.1	1.0
S5	1.4	1.6	1.4	0.9	1.0	0.9	1.2	0.9	0.9	1.0[c]	0.9[c]	1.0[c]
S6	1.4[b]	1.2[b]	1.3[b]	1.2	1.1	1.2	1.3	1.2	0.6	0.9	0.8	0.8
S7	1.4[b]	1.2[b]	1.3[b]	1.2	1.1	1.2	1.3	1.2	0.8	1.0	0.9	1.0
S8	1.2	1.2	1.3	1.1	1.0	0.9	1.1	0.9	0.5	1.1	1.0	1.0
S9	1.1	1.3	1.3	1.1	1.1	1.1	1.0	1.1	0.9	1.0[c]	0.9[c]	1.0[c]
S10	1.8	1.2	1.3	1.1	0.9	1.3	1.2	1.1	0.7	0.9	0.9	1.0
S11	0.7	0.9	1.0	1.0	1.0	0.9	1.0	0.9	0.7	1.0	1.0	0.8
S12	0.6	0.9	0.8	0.9	0.9	0.8	0.9	0.9	0.7	1.0	1.0	1.1
S13	1.0	1.0	1.0	1.0	1.2	0.9	1.1	0.9	0.9	1.0	0.9	1.0
S14	0.7	0.8	0.7	1.0	0.9	0.8	0.9	0.9	0.7	1.0	1.0	1.1
S15	0.7	0.7	0.8	0.8	0.9	0.7	0.9	0.9	0.8	1.0	1.0	1.1
S16	0.8	1.0	0.9	1.0	1.0	1.0	1.0	0.9	0.8	0.9	0.9	1.0
S17	0.8	1.1	1.1	1.0	1.1	0.9	1.1	1.1	0.6	1.0	1.0	1.1
S18	1.0	0.9	1.0	0.9	1.1	0.9	1.0	1.0	0.8	1.3	1.2	1.0
S19	0.7	0.9	0.8	1.0	0.9	0.8	0.8	0.8	0.6	0.9	0.8	0.8
S20	0.8	0.9	0.8	1.0	1.1	1.0	1.2	0.9	0.7	1.1	0.9	0.9
S21	0.4	0.7	0.7	0.7	0.9	0.7	0.8	0.7	0.9	1.3	0.9	1.3
n	1	1	1	2	2	2	2	2	1	1	1	2

[a] Values are expressed as protein/RNA molar ratios, normalized to reconstituted nonmutant synthetic 30S ribosomes (Denman et al., 1989a). *n* is the number of analyses. Values <0.6 or >1.4 are underlined. [b,c] Pairs of proteins are sufficiently resolved. The measured protein content was divided equally between both.

Table III: Evidence for Base Pairing between Residues 1404:1497 and 1405:1496[a]

	mutants	tRNA binding P site	tRNA binding A site
(1) wild type	1404C ≡ G1497 1405G ≡ C1496	100	100
(2) single mutant	*G* G $\overline{G}$ ≡ C	35	35
(3) single mutant	C *C* G ≡ $\overline{C}$	30	25
(4) double mutant	*G* ≡ *C* $\overline{G}$ ≡ $\overline{C}$	60	50
calcd:	(2) × (3)	10	10
(5) single mutant	C ≡ G *C* C	20	25
(6) single mutant	$\overline{C}$ ≡ G G *G*	15	15
(7) double mutant	C ≡ $\overline{G}$ *C* ≡ *G*	70	40
calcd:	$(\overline{5})$ × (6)	5	5
(8) single mutant	C ≡ G *A* C	60	60
(9) single mutant	$\overline{C}$ ≡ G G · *U*	60	35
(10) double mutant	C ≡ $\overline{G}$ *A* = *U*	140	160
calcd:	$(\overline{8})$ × $(\overline{9})$	35	20
(11) double mutant	*G* G $\overline{G}$ *G*	<5	15
(12) double mutant	C *C* *C* $\overline{C}$	5	10

[a] Values are expressed as percent of wild-type synthetic 30S subunit activity to the nearest 5%. For the P site, 100% = 0.32 mol of tRNA bound/mol of 30S. For the A site, 100% = 0.25 mol of tRNA bound/mol of 30S. "Calcd" is the product of the percent activity of the two single mutants divided by 100.

were simultaneously broken. The heterogeneity of the mutant ribosomes was in general similar to those reported in previous publications (Cunningham et al., 1990a, 1992a,b), although in some samples the C_r values tended to be slightly lower than for the wild-type control, and in some cases a definite slower sedimenting shoulder was observed. As in our earlier studies, only the fractions sedimenting with the marker ^{32}P-labeled ribosomes were taken for functional analysis. This selection has the effect of compensating for any differential assembly ability in the various mutants.

The ribosomal protein content of each of the mutant preparations is given in Table II. Analysis of the data shows that variation in protein composition of the mutant ribosomes can be excluded as the cause of the functional effects observed in Table III. Although unit stoichiometry of individual proteins in the mutants was not always obtained (underlined values in Table II), there was no correlation of the variation with the protein involved, with the particular class of mutant, or with any specific functional activity. For example, while partial loss of S21 in G1404 could explain its decreased tRNA binding, it does not explain the equivalent loss of activity of C1497. Similar arguments apply to the other cases of nonstoichiometry.

Functional Activity of Mutants at Positions 1404, 1405, 1496, and 1497. The tRNA binding activity at both A and P sites was measured for this series of mutants. The results are shown in Table III. The calculated value was obtained by assuming independence of the two mutations. Thus the activity of a double mutant in which each single mutation resulted in partial inactivation was expected to be lower than that of either of the single mutants. In fact the opposite was observed in all three cases. The activities of the double mutants were not only greater than the calculated values but they were more active than either of the single mutants. It follows from this result that both putative base pairs exist and that both are involved in formation of functional P and A sites. Note that while breaking the 1404:1497 base pair reduced tRNA binding by a factor of 2/3, breaking the 1405:1496 pair was even more inactivating, and breakage of both base pairs simultaneously reduced activity to almost undetectable levels. Replacement of the G1405:C1496 pair by the AU pair found in some mitochondrial ribosomes (Table I) was not deleterious but actually increased tRNA binding at both P and A sites. Although in this case the restoration of activity due to base pair formation in the double mutant was partially obscured by the relatively higher activity of the single mutations compared to the G or C substitutions, the double mutant was

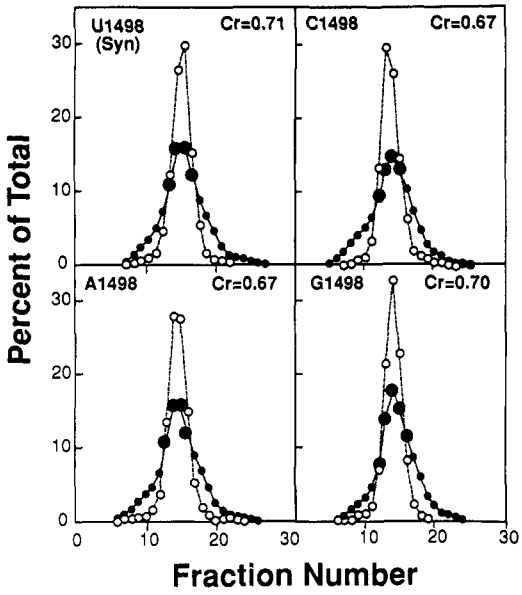

FIGURE 3: *In vitro* reconstitution of ribosomes mutant at U1498. Conditions were as described in the caption of Figure 2.

Table IV: Functional Effects of Site-Specific Nucleotide Substitution at Position 1498[a]

		tRNA binding			peptide synthesis	
mutant	subunit association	P site	A site	I site	fMet-Val	poly-(Phe)
A1498 (R167)[b]	95	75	95	90	110	125
(R173)	110	85	100	85	90	130
av	100	80	100	90	100	130
C1498 (R167)	65	80	85	85	80	105
(R173)	105	50	55	50	30	65
(R176)	85	60		70	45	50
av	85	65	70	70	50	75
G1498 (R167)	90	85	115	85	30	120
(R173)	105	85	105	95	10	95
(R217)		120	85	95	30	95
av	95	95	105	95	25	105

[a] Values are expressed as percent of wild-type synthetic 30S subunit activity to the nearest 5%. 100% corresponds to 80% for subunit association; 0.19, 0.22, and 0.23 pmol of tRNA bound/pmol of 30S for P-, A-, and I-site binding, respectively; 2.4 pmol of Phe/pmol of 30S/10 min for poly(Phe) synthesis; and 3.2 pmol of fMet-Val/pmol of 30S for fMet-Val synthesis. [b] R numbers refer to independent reconstitutions.

still substantially more active than either single mutant. We ascribe the relatively higher activity of the single mutants to the fact that, unlike the G or C substitutions, these can form a GU or a noncanonical AC base pair.

The ability of the G1404:C1497, C1405:G1496, and A1405:U1496 double mutants to cross-link P-site-bound tRNA to C1400 was assessed since cross-linking is a sensitive measure of the stereochemical relationship between C1400 and the anticodon loop of tRNA at the P site (Ofengand et al., 1986, 1988). Cross-linking yields of 84%, 92%, and 130%, respectively, of the wild-type activity were obtained (data not shown). Thus, reversed base pairs 4–5 residues downstream of the cross-link site do not appear to markedly alter the relative orientation of C1400 and the anticodon loop of tRNA at the P site.

Characterization of Ribosome Mutants at U1498. The velocity gradient profiles of these mutants are shown in Figure 3. All three mutants appear similar to the wild type despite the different functional activity of G1498 (see Table IV). The ribosomal protein content of the preparations is listed in Table II. As noted above, the minor variations from unit stoichiometry for the individual proteins did not correlate with functional assays. Thus, while G1498 is the only mutant in this series with a marked functional defect (see below), its content of proteins most closely approaches a unit ratio.

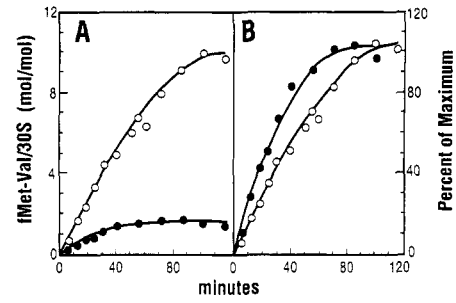

FIGURE 4: Kinetics of fMet-Val synthesis. (A) The rate of synthesis of fMet-Val with nonmutant Syn (○) or mutant G1498 (●) ribosomes. (B) Data of panel A expressed as percent of each plateau value.

Functional Activity of the 1498 Mutants. The activity of these mutants was assessed in a set of protein synthesis assays (Table IV). In addition to A- and P-site tRNA binding, formation of a 30S–fMet-tRNA–mRNA initiation complex (I site) was measured, as was peptide bond formation by two assays. fMet-Val formation measured the ability to form the first peptide bond, while poly(Phe) synthesis was a measure of translocation and internal peptide bond formation. Subunit association ability was a necessary prerequisite to all activities but I-site binding and was therefore directly measured as well.

Several independent reconstitutions were performed for each mutant in order to verify the effects observed. Clearly the A1498 mutation is innocuous. The only effects detectable were a slight decrease in P-site binding and a small increase in the rate of poly(Phe) synthesis. This result is consistent with the natural occurrence of A1498 in a few mitochondrial ribosomes (Table I). Mutation of U1498 to C gave more variable results although there was no correlation with any detectable deficiency in reconstitution profiles (data not shown). Our interpretation of these results is that the U-to-C mutation has caused a subtle perturbation of the subunit structure such that small and undetectable variations in our reconstitution procedure shift the ratio of active to inactive particles in different preparations. Moreover, judging by the lack of correlation among the different assays, the putative perturbation affects the various functional activities in different ways.

In the G1498 mutant, all functions were normal except for fMet-Val formation. Ability to form the first peptide bond was severely inhibited in all three preparations. It is quite striking that only G1498 showed this functional defect and that all other activities of this mutant were normal. In order to analyze this effect in more detail, the kinetics of fMet-Val formation were measured (Figure 4). The rate of the reaction is shown in panel A of Figure 4. Although the kinetics of reaction were similar for both U1498 and G1498, the mutant produced far less fMet-Val than the control, despite full activity in both I- and A-site binding assays. The similarity in kinetics is more clearly shown in panel B of Figure 4 where the data are replotted as a percent of the maximum value obtained in each case. The results suggest that the same series of reactions occurred in both instances but with fewer active particles in the case of the mutant, rather than an initial reaction of the mutant at the same rate as the control which then stops prematurely. Note that the plateau of reaction in panel A, 17% of the control, is similar to the values in Table IV obtained in independent experiments with a different protocol.

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

time (min)	fMet–PM/fMet-tRNA bound	
	U1498	G1498
5	0.82	0.91
10	0.87	0.89
15	0.89	0.84

[a] The 30S–fMet-tRNA initiation complexes that were formed with wild-type and mutant 30S were reacted, after addition of a 1.5-fold excess of 50S subunits and incubation for 5 min at 37 °C, with 1 mM puromycin (PM) at 0 °C for the indicated times. The amount of fMet-tRNA bound to the 30S and 70S complexes was determined by filter binding at 20 mM Mg^{2+} and corrected for the blank value obtained in the absence of 30S subunits. The values were 0.23 and 0.20 mol of fMet-tRNA bound per mol of 30S added for the U1498 and G1498 ribosomes, respectively. 30S-dependent fMet–PM formation was determined by ethyl acetate extraction. There was no fMet–PM found in the absence of 50S subunits, and <7% was found in the absence of puromycin. The ratio of the amount of fMet–PM formed to the amount of fMet-tRNA bound to the 30S subunit is reported.

Table VI: Misincorporation of Leucine Directed by Poly(U) with the G1498 Mutant

ribosome	streptomycin	Leu × 100[a]	Leu + Phe[a]	E^b
30S	–	14.7	10.0	1.5
	+	164.0	9.9	16.6
U1498	–	1.0	4.7	0.2
	+	28.9	6.2	4.7
G1498	–	<0.1	2.6	<0.1
	+	13.6	2.9	4.7

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

Dissection of the Defect in fMet-Val Synthesis in the G1498 Mutant. In order to determine the site of inhibition of fMet-Val synthesis, the ability of the 30S initiation complex (I site) to form a 70S initiation complex and react with puromycin was tested. As pointed out previously (Cunningham *et al.*, 1992b), successful fMet–puromycin synthesis normally can only occur after 70S initiation complex formation and release of initiation factors. As shown in Table V, fMet–puromycin formation was extremely rapid in both the control and the mutant with essentially all of the bound fMet-rRNA being capable of reaction. Clearly, the inability to form fMet-Val is not due to a block in 70S initiation complex formation nor to an effect on peptidyltransferase. The next step in normal fMet-Val formation would be binding of Val-tRNA to the A site and formation of the peptide bond. Direct assay for these two functions showed that the G1498 mutant was capable of both (A-site and poly(Phe) columns in Table IV), yet fMet-Val synthesis did not occur. This apparent paradox will be considered further in the Discussion.

Miscoding. A separate explanation for the failure of fMet-Val synthesis could be miscoding. This would not be tested by either A-site binding or poly(Phe) synthesis as both assays used poly(U) as an mRNA. A direct *in vitro* test for miscoding was applied which is based on the ability of certain Leu-tRNA species to recognize UUU codons (Ruusala et al., 1982). This assay had been used previously by us and by others in the study of different 16S mutants (Allen & Noller, 1991; Cunningham et al., 1992b; Santer et al., 1993). In Table VI the rate of incorporation of Leu residues into a Leu-Phe copolypeptide was measured in the presence and absence of streptomycin, a known inducer of misreading (Ruusala *et al.*, 1984). While neither the wild-type nor the mutant particle showed any evidence of misreading in the absence of streptomycin, both could be stimulated to the same extent when streptomycin was present. We conclude that there is no apparent induction of misreading as a result of changing U1498 to G.

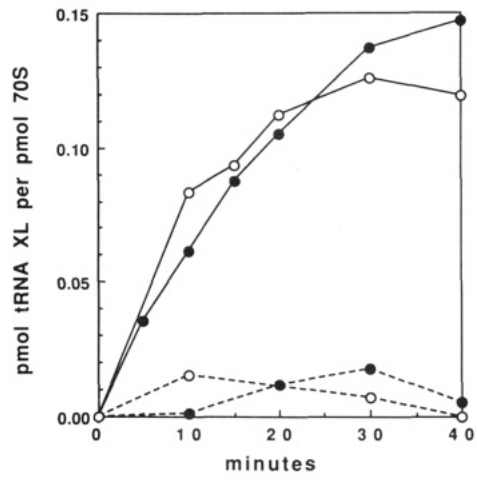

FIGURE 5: Rate and extent of cross-linking of P-site-bound AcVal-tRNA to control U1498 (○) and mutant G1498 (●) 70S ribosomes: (—) plus poly(U_3,G); (- - -) minus poly(U_3,G). Cross-linking was performed and assayed as described in Materials and Methods. Values are expressed as pmol of AcVal-tRNA cross-linked per pmol of ribosomes added with 0-time values subtracted. Poly(U_3,G)-dependent noncovalent binding was 0.28 and 0.27 pmol/pmol for U1498 and G1498, respectively. Therefore, mRNA-dependent cross-linking at the plateau of reaction was 42% and 49%, respectively.

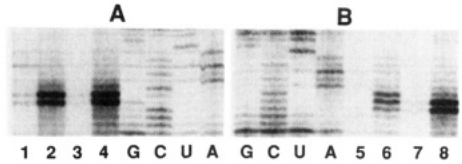

FIGURE 6: Site of cross-linking in mutant G1498 30S ribosomes. Analysis was by reverse transcription arrest as described in Materials and Methods. (A) Lane 1, wild-type control minus poly(U_3,G) during UV irradiation; lane 2, as lane 1 plus poly(U_3,G); lane 3, mutant G1498 minus poly(U_3,G); lane 4, mutant G1498 plus poly(U_3,G); lanes G, C, U, and A, sequencing lanes for the wild type. The gel region from positions 1391–1405 is shown. (B) Lanes G, C, U, and A, sequencing lanes for the mutant C1401:G1501; the gel region from positions 1390–1404 is shown. Lane 5, mutant C1401:G1501 minus poly(U_3,G); lane 6, mutant C1401:G1501 plus poly(U_3,G); lane 7, wild-type control minus poly(U_3,G); lane 8, as lane 7 plus poly(U_3,G).

Cross-Linking to C1400. Another way to detect effects on the codon–anticodon interaction is to measure the ability of the mutation to perturb the stereochemical relationship between the anticodon of P-site-bound tRNA and C1400 of 16S RNA. This relationship, measured by the ability to cross-link the 5′ anticodon base of tRNA to C1400 by cyclobutane dimer formation, is very sensitive to the nature of the codon present in the P site (Ofengand & Liou, 1981; Ofengand *et al.*, 1986, 1988). The rate and extent of cross-linking for the control U1498 and mutant G1498 ribosomes are shown in Figure 5. Cross-linking was dependent on mRNA in both cases, and both the rate and the extent of reaction were the same. However, the site of cross-linking could have been shifted, for example, to C1399, without being detected by this assay. Therefore, the site was determined by reverse transcription arrest (Nurse et al., 1987; Denman et al., 1988, 1989a). The results are shown in Figure 6. Normally two bands are seen. The lower band occurs one base 3′ to the cross-link, and the second, or "stutter", band (Nurse et al., 1987, Denman et al., 1988; Ericson & Wollenzien, 1988)

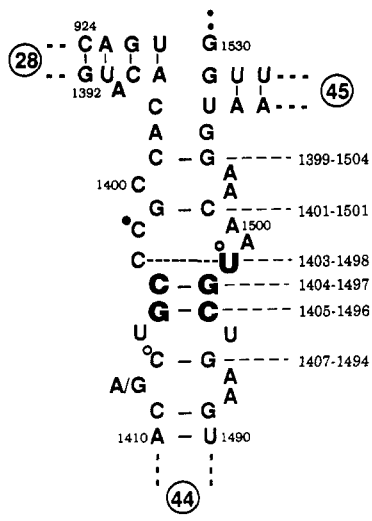

FIGURE 7: Tertiary interactions proposed to occur between the 1397–1408 and 1492–1505 conserved single-stranded sequences of *E. coli* 16S ribosomal RNA. The relationship of helices 28, 44, and 45 to the rest of the 16S RNA molecule is shown in Figure 1. Helix 28 connects to the 1397 end of sequence 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 nucleotides in boldface type are the ones mutated in this work. The tertiary interactions shown as solid lines are those proposed by Gutell et al. (1985) and Gutell and Woese (1990). The dashed line indicates the potential base pair created by the G1498 mutation. •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.

occurs at the cross-link site itself. Both control samples as well as the G1498 mutant showed two bands, at G1401 and C1400. Conseqeuntly, cross-linking to this mutant occurs exclusively to C1400. Figure 6 also shows results with the mutant C1401:G1501 employed as a positive control. As reported previously (Cunningham et al., 1992b), this mutant gives three bands, at G1401, C1400, and C1399, indicative of cross-linking to both C1399 and C1400. In all cases, appearance of the bands was dependent on the presence of mRNA in the cross-linking reaction mixtures.

DISCUSSION

"Base-Pair" Mutations. The results presented here show that the base pairs C1404:G1497 and G1405:C1496 (Figure 7) are important for the binding of tRNA to the A and P sites. When either base pair was broken, both binding activities were severely inhibited but could be largely recovered when base pairing was restored in the double mutant, despite the changes in primary sequence of both single-stranded regions associated with the reversed base pairs. The inhibition and subsequent restoration of binding activity could not be ascribed to the absence of any specific protein, as shown by quantitative HPLC analysis of the protein content of the mutant ribosomes, nor to gross structural defects which would have affected the sedimentation profiles. Both A- and P-site activities were affected similarly, suggesting either that some element common to both sites was perturbed or, more likely, that these bases are at the region of junction of the A and P binding sites on the 30S subunit. The latter hypothesis is consistent with the tRNA protection studies of Moazed and Noller (1990), who found P-site protection of residues 1399–1401 and A-site protection of bases 1405, 1408, and 1492–1494. Like the base pair G1401:C1501 (Cunningham et al., 1992b), existence of these two base pairs may be transient, as suggested by the accessibility of the N_2 of G1405 in the 30S subunit to kethoxal attack (Moazed et al., 1986).

The fact that P- and A-site binding activity was not fully recovered in the double mutants indicates a partial dependence on primary sequence as well as on base pairing. The complete failure of the C1405:G1496 double mutant to restore fMet-Val synthesis activity (data not shown) also illustrates this point. The single mutants at positions 1405 and 1496 as well as the G1404/G1496 and C1405/C1497 double mutants also failed to make fMet-Val. Synthesis of fMet-Val appears to be perturbed by any change in sequence at positions 1405 and 1496, which may explain why reverse base pair mutants have not been found in nature. In this regard, it will be interesting to examine the fMet-Val activity of the A1405:U1496 double mutant in *E. coli*, in view of the natural occurrence of this base pair in some mitochondrial ribosomes.

Other kinds of evidence link residues 1404, 1405, 1496, and 1497 to aspects of ribosome function: the N_7 of G1497 is protected by P-site-bound tRNA (Meier & Wagner, 1984); methylation of G1405 at the N_7 position leads to kanamycin resistance (Beauclerk & Cundliffe, 1987); the N_1 and N_2 of G1405 and G1497 are protected from kethoxal by 70S formation (Herr et al., 1979); and the entire region from 1490–1505 is unavailable for hybridization to a complementary deoxyoligomer (Weller & Hill, 1992), suggesting that it may be involved in some other interaction.

Mutation of U1498 to G, A, or C. U1498 is normally m^3U in *E. coli* 16S RNA. Mutation to G was designed to generate a putative base pair with C1403 which would augment the C1404:G1497-G1405:C1496 2-bp helix (Figure 7), making the helix both longer and stronger. Natural occurrence of a base pair at this position is rare (Table I), and in no case is it C:G. Formation of this base pair would also have other consequences. Both the C1402-C1403 loop and the U1498-A1499-A1500 loops would be reduced in size by one base each, and the orientation of the loop would also be altered. For any or all of these reasons, it seemed reasonable to expect functional changes from this mutation. Although the A1498 and C1498 mutants were made as controls, A1498 allows formation of a C:A base pair, several examples of which are now believed to occur in ribosomal RNAs (Gutell, 1993).

As was the case with the "pairing" mutants, ribosome assembly from these three mutant RNAs was normal and all the ribosomal proteins were present in normal amounts as well. Therefore, the functional effects observed are interpreted as a direct consequence of the RNA base change. Since replacement of U by A had essentially no effect on any of the assays, C:A base pair formation, if it occurs, must be without functional effect. Mutation to C created particles with a partial decrease in all activities that was somewhat obscured by increased variability in the functional assays. The G1498 mutation, however, produced a striking effect on function. fMet-Val formation was strongly inhibited, but no other assay was perturbed, not even poly(Phe) synthesis. This is more clearly shown by the ratios of fMet-Val activity to poly(Phe) activity, which were, for the A-, C-, and G1498 mutants, 0.76, 0.66, and 0.24, respectively.

The inability to form fMet-Val while peptidyl transferase activity, A-site binding activity, and the ability to form a 70S initiation complex are retained is at first glance paradoxical, since once the 70S initiation complex is formed, all that remains is for Val-tRNA to bind to the A site and for fMet-Val peptide bond formation to occur. A trivial explanation for this effect could be that, at the Mg^{2+} concentration at which the fMet-Val assay was done (7.5 mM), A-site binding of the G1498

mutant was much decreased relative to that of the wild type. This might not have been detected in the standard A-site binding assay which was performed at 12 mM Mg^{2+}. However, when A-site binding was assayed over the range 6.5–10 mM Mg^{2+} by the method previously described (Santer et al., 1993), no difference in binding between the wild-type and G1498 particles could be detected (data not shown). Thus, weaker binding of G1498 to the A site is an unlikely explanation for the G1498 defect in fMet-Val synthesis.

Interestingly, two other mutants, the C1401:G1501 double mutant (Cunningham et al., 1992b) and U530 (Ofengand et al., 1993; Santer et al., 1993), show this same set of functional properties. It appears that all three mutants have suffered an insult to normal A-site function which is not revealed by poly(U)-dependent A-site binding assays. One explanation, which makes use of the concept of A_i and A_e sites (Nierhaus, 1990), postulates that the three mutations have in some way impaired the ability of the ribosome to correctly create an A_i site while not affecting the A_e site. Alternatively, the fidelity of the decoding component of the A site may have been compromised. This would not have been detected by the poly(U)/Phe-tRNA system used in the A-site and poly(Phe) synthesis assays but could manifest itself as an inability of Val-tRNA to correctly recognize the GUU codon. Insofar as the *in vitro* miscoding assay we used would detect such infidelity, this does not seem to have occurred. However, a deficiency in proper frame registration at the A site, i.e., a frameshifting problem, would not have been detected in any of these assays since they all used poly(U) and either Phe-tRNA or Leu-tRNA for recognition. Thus, although miscoding and frameshifting are usually correlated, a defect in frameshifting remains a possibility.

Although the three mutations G1498, C1401:G1501, and U530 show the same functional pattern, they are not alike in all aspects. Cross-linking of P-site-bound tRNA to C1400 showed some differences. Whereas G1498 and U530 (Santer et al., 1993) cross-linked exclusiveley to C1400 with the same rate and yield as wild type, C1401:G1501 cross-linked about equally to both C1399 and C1400. While the former two mutants do not disturb the cross-link site, the latter one does, presumably because the reversed base pair is adjacent to the site of cross-linking.

The fact that both forming a reversed base pair at positions 1401/1501 and creating a situation that allows for base pairing at 1403/1498 should have the same rather specific functional effect is perhaps not surprising in view of the stereochemical relationship illustrated in Figure 7. What is surprising is that U530 shows the same pattern of behavior. As we have commented elsewhere (Ofengand et al., 1993; Santer et al., 1993), this may mean that the U530 region is physically close to, and part of, the decoding site. Such a situation would be in accordance with recent cross-linking results (Dontsova et al., 1992).

Conclusions. The tertiary base pairs shown in Figure 7, originally proposed only on the basis of weak phylogenetic evidence, have now been substantiated by several experimental results. The functional effects of breaking and reforming base pairs in the reverse way have substantiated the G1401:C1501 interaction (Cunningham et al., 1992b), and this work provides evidence for the C1404:G1497 and G1405:C1496 pairs. The behavior of the G1498 mutant is also consistent with apposition of residues 1403 and 1498, shown as a dashed line in Figure 7. In a more general way, the three zero-length cross-links reported by Döring et al. (1992), namely, segment 1402–1403 to segment 1498–1501, 1393–1401 to 1531–1532, and 1393–1401 to 1506, are also consistent with the structure shown in Figure 7. What is the mechanistic rationale for such a structure? The answer to this important question is not yet at hand. Nevertheless, as the previously proposed (Cunningham et al., 1992b) schematic arrangement of tRNAs, mRNA, and rRNA in the vicinity of the decoding site of the ribosome illustrates, the structure is likely to be involved in organizing codon–anticodon interaction at both A and P sites.

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