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In Vitro Assembly of 30S and 70S Bacterial Ribosomes from 16S RNA Containing Single Base Substitutions, Insertions, and Deletions around the Decoding Site (C1400)

Robert Denman,[‡] Carl Weitzmann,[‡] Philip R. Cunningham,[‡] Didier Nègre,[‡] Kelvin Nurse,[‡] John Colgan,[‡] Yu-Ching Pan,[§] May Miedel,[§] and James Ofengand*,[‡]

Roche Institute of Molecular Biology, Roche Research Center, and Department of Protein Biochemistry, Hoffmann-La Roche Inc., Nutley, New Jersey 07110

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ABSTRACT: An in vitro system developed for the site-specific mutagenesis of 16S RNA of *Escherichia coli* ribosomes [Krzyzosiak et al. (1987) *Biochemistry 26*, 2353-2364] was used to make 10 single base changes around C1400, a residue known to be at the decoding site. C1400 was replaced by U, A, or G, five single base deletions at and to either side of C1400 were made, and C or U was inserted next to C1400. Another mutant possessed seven additional nucleotides at the 3′ end of the 16S RNA such that a stem and loop involving the anti-Shine–Dalgarno sequence could form. Each of the mutant RNAs was reconstituted with a complete mixture of 30S proteins to yield 30S ribosomes. Modified in vitro reconstitution conditions were required to obtain assembly of all of the synthetic ribosomes. Quantitative HPLC analysis of the protein content of each mutant showed that all of the proteins were present. The ability of synthetic 30S to form 70S particles under functional assay conditions was about 75% that of natural 30S and was unchanged by any of the mutations except for the deletion of G1401, which decreased the association activity under the standard conditions to 35–40% of synthetic 30S. That part of the ribosomal P site which interacts with the anticodon loop of tRNA was investigated by near-UV (>300 nm) induced cross-linking of AcVal-tRNA. Cross-linking depended on both 30S subunits and the correct codon. The cross-linking yield of all mutants with a pyrimidine at position 1400 was equal to control isolated 30S, and the first-order rate constants for cross-linking of those mutants tested were like reconstituted natural 30S. The site of cross-linking for mutants with a C or U insertion between C1400 and G1401 was shifted to the inserted residue. Cross-linking to the base 5′ to G1401 rather than to the residue 3′ to C1399 indicates that G1401 is an important structural determinant of the P site.

In a previous publication, we described a novel system for creating and studying *Escherichia coli* ribosomes mutant in their RNA moiety (Krzyzosiak et al., 1987). This was accomplished by modifying a 16S RNA gene to allow synthesis in vitro by T7 RNA polymerase. The transcription product contained three additional 5′-G residues with a presumed 5′-triphosphate terminus, a base change from A → G at position 2, and, in 20% of the molecules, an additional residue at the 3′ end. Otherwise, the transcript was a faithful copy of the 1542 nucleotides of 16S RNA, except that the 10 modified bases known to be present in that RNA (Noller, 1984) were lacking. The RNA could be reconstituted into 30S particles by using a modification of the procedures described by Held et al. (1973). The particles were morphologically indistinguishable from 30S ribosomes and were shown to possess considerable tRNA binding activity.

Two mutant ribosomes, A1400 and G1400, were also described in that work. This position, normally a C residue, was chosen for initial study because a considerable body of evidence from our laboratory had implicated this position in the decoding site function of the ribosome (Ehresmann & Ofengand, 1984; Gornicki et al., 1984; Ciesiolka et al., 1985; Nurse et al., 1987). We have now made eight additional mutations in the vicinity of C1400 plus one at the 3′ end and have studied

* To whom correspondence should be addressed.
‡ Roche Institute of Molecular Biology.
§ Hoffmann-La Roche, Inc.

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the properties of the mutant ribosomes. In this report, we describe the formation of the 11 mutant ribosomes, their ribosomal protein content, and their ability to associate with 50S subunits to form 70S ribosomes. We have also examined their ability to cross-link to P site bound tRNA via 16S RNA (Ofengand et al., 1988). In the following paper, we describe the activity of these mutant particles in a battery of assays which test their ability to carry out the various partial reactions of protein synthesis (Denman et al., 1988b).

Experimental Procedures

Buffers. RD buffer is 20 mM Hepes, pH 7.5, 100 mM NH_4Cl, 20 mM $Mg(OAc)_2$, and 5 mM mercaptoethanol. RDm is RD with dithiothreitol (DTT)[1] replacing mercaptoethanol. SG buffer is 20 mM Hepes, pH 7.5, 100 mM NH_4Cl, and 15 mM $Mg(OAc)_2$. Rec-20 buffer is 20 mM Hepes, pH 7.5, 400 mM NH_4Cl, 20 mM $Mg(OAc)_2$, and 4 mM mercaptoethanol.

Materials. Plasmids and RNA transcripts were prepared as described by Krzyzosiak et al. (1987, 1988) except as follows. For plasmid linearization, *Mst*II was replaced by *Bsu*36I (1 unit/μg of DNA) and incubated at 37 °C for 2 h in 10 mM Tris (pH 7.5), 100 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, and 0.1 mg/mL BSA. A second unit of enzyme per microgram of DNA was added and incubation continued for 2 h more. DNase treatment was with 1 μg/mL DNase I (Worthington) for 15 min at 38 °C. The transcription buffer contained 20 mM instead of 10 mM DTT. The final dialysis was versus 5 mM KOAc, pH 5.0, and 1 mM $Mg(OAc)_2$ instead of Rec-20 buffer. All RNA preparations were checked for sequence in the modified regions by primed reverse transcription (Krzyzosiak et al., 1987) and for size by glyoxal–Me_2SO denaturation (Maniatis et al., 1982) at 70 °C for 10 min, followed by gel electrophoresis (1.5% agarose–0.2% iodoacetate) in 10 mM phosphate buffer, pH 7.0.

Ribosomal subunits were prepared as described previously (Krzyzosiak et al., 1987). The mixture of 30S ribosomal proteins (TP30) was prepared as described previously (Krzyzosiak et al., 1987) except that in some cases the exposure to acetic acid was reduced from 45 min to 10 min. Both types of preparations behaved identically. For HPLC analysis, proteins were extracted as follows: 670 nM 30S subunits in RD buffer were adjusted to 0.12 M $Mg(OAc)_2$ by addition of 0.1 volume of 1 M $Mg(OAc)_2$ and then extracted by addition of 1.1 volumes of chilled glacial acetic acid, vortexed, and incubated at 0 °C for 15 min with occasional mixing. RNA was removed by centrifugation. The supernatant volume was measured and diluted with 6.5 volumes of 0.1% (w/v) TFA to reduce the acetic acid concentration to 6.7%, because in our system S12 elutes in the void volume at 10% acetic acid. The sample was applied to the HPLC column within 6 h of preparation. Small subunit protein standards (the kind gift of V. Mandiyan, Roche Institute of Molecular Biology, Nutley, NJ) were prepared by elution from phosphocellulose as described by Zimmermann (1979). Proteins S4, S6, S8, S15, and S20 were characterized by their elution behavior and by two-dimensional gel electrophoresis (Madjar et al., 1979). Protein S5, S7, S9, S13, and S18 were characterized by their elution behavior and by their molecular weight on 0.1% SDS–8 M urea–18% polyacrylamide gels. Acetonitrile and 2-propanol were HPLC grade from Fisher and had an absorbance of less than 1 at 190 and 210 nm, respectively. Water was purified by ion-exchange and activated carbon cartridges. TFA was from Sigma. BSA (catalog no. 238-031) was from Boehringer-Mannheim. Hen egg lysozyme (catalog no. L6876) was from Sigma. The reverse transcriptase primer, complementary to residues 1431–1453 of 16S RNA, was prepared according to Denman et al. (1988a). Reagents for the reverse transcription assay were obtained as described by Nurse et al. (1987).

Methods. Ribosome reconstitution conditions were as described in Table II and the individual experiments. Other reconstitution procedures were as described by Krzyzosiak et al. (1987). Formation of 70S from 30S and 50S was assayed by sucrose gradient centrifugation. Reaction conditions for the formation of 70S are given in the legend to Table V. 30S and 50S subunits were combined and incubated at 37 °C for 20 min. The 70S ribosomes and subunits were resolved by centrifugation of 0.1–0.2 A_{260} unit (0.1–0.2 mL). Gradients were prepared by layering 1 mL of 7.5% sucrose in SG buffer on top of a 36-mL, 15–30% linear sucrose gradient also in SG buffer. Centrifugation was in a VTi50 rotor at 4 °C for 1.1 h at 50 000 rpm. Samples were pumped through a Gilson model HM/HPLC Holochrome flow detector set at 260 nm. The tracings were integrated by weighing the peak areas cut from photocopies. The percent 30S as 70S was calculated as 37 times the 70S area divided by the sum of the free 30S area plus 0.37 times the 70S area.

N-Terminal sequence analysis of ribosomal proteins was performed by using a Model 470A gas-phase sequencer (Applied Biosystems, Foster City, CA) (Hewick et al., 1981). Phenylthiohydantoin (PTH)-amino acid derivatives were identified on-line with a PTH Model 120 analyzer (Applied Biosystems) (Hunkapillar, 1985) or off-line with a Waters HPLC system using an Altex Ultrasphere C-18 column (4.6 mm × 25 cm) (Hawke et al., 1982).

HPLC analysis and isolation of proteins for sequencing were performed essentially as described by Kerlavage et al. (1983) on a system consisting of two Waters M6000A pumps, a Waters 710 WISP autoinjector, a Waters Model 721 system controller, and a Waters 990A diode array detector. Separations were performed on 4 × 50 mm RP-P columns from Synchrom (C18 reversed-phase, 6.5-μm particle size, 300-Å pore size). Solvents were kept under helium; solvent A = 0.1% w/v TFA in water; solvent B = 0.067% w/v TFA in acetonitrile. The gradient was from 19% to 38.5% B in 120 min (convex upward, Waters curve 5) and then from 38.5% to 50% B in 20 min (linear) or from 21.5% to 37.2% B in 120 min (convex upward, Waters curve 5), from 37.2% to 39% B in 25 min (linear), from 39% to 47% B in 15 min (linear), and from 47% to 50% B in 5 min (linear).

The protein content of mutant ribosomes was determined relative to 30S subunits. The amount of 30S subunit standard or mutant subunits was determined by the absorbance at 260 nm assuming a value of 1 equal to 67 nM. The proteins were extracted and separated by HPLC as described above. The area of each peak was integrated by using the Waters 990A manual edit mode as A_{214} × minutes and normalized to the number of picomoles extracted. The protein content of mutant subunits was expressed as a ratio of the area per picomole of that peak in the mutant to the area per picomole of the same peak in standard (native) 30S subunits. Variations in pattern and recovery were controlled by periodic analysis of the standard 30S subunits.

The amount of each protein in 30S subunits was estimated by the method of Buck (1988; M. Buck, T. Olah, C. Weitz-

[1] Abbreviations: TFA, trifluoroacetic acid; BSA, bovine serum albumin; Me_2SO, dimethyl sulfoxide; TP30, complete mixture of ribosomal proteins extracted from 30S subunits; DTT, dithiothreitol; 30S and 50S, small and large ribosomal subunits of *E. coli*, respectively.

mann, and B. S. Cooperman, unpublished experiments). Briefly, each peak area was converted to absorbance units by multiplication by the flow rate (milliliters per minute) and divided by its ϵ_{214} value to obtain a value of picomoles eluted for each protein peak. This value was then divided by the fractional recovery factors for 30S proteins determined by Kerlavage et al. (1983) and divided again by the amount of subunits extracted as determined by A_{260}. For those proteins which sometimes eluted together, S5–S9, S6–S7, S8–S13, and S15–S18, an average value of ϵ_{214} and fractional recovery was used (the difference was <7%), and the amount was divided equally between both proteins. The ϵ value was calculated for each ribosomal protein as $\sum(\epsilon_{214})$ in 30% acetonitrile–0.1% TFA for each aromatic amino acid in the protein plus the contribution from each peptide bond (Buck, 1988; M. Buck, T. Olah, C. Weitzmann, and B. S. Cooperman, unpublished results). This value was corrected for systematic errors by HPLC analysis of BSA and hen egg lysozyme in the same solvent used for ribosomal proteins. Division of the protein peak area times the flow rate by the actual amount of protein eluted yielded an experimental value for ϵ_{214} which could be compared to that calculated as the sum of ϵ_{214} for each amino acid as described above for ribosomal proteins. The amount of eluted BSA was determined colorimetrically with reference to a gravimetric standard and by the ϵ_{280} value (Tanford & Roberts, 1952). The amount of lysozyme was determined by using the known ϵ_{282} value (Canfield, 1963). Both ϵ values were confirmed within 4% by gravimetric analysis of the proteins. The calculated ϵ_{214} was 2.05 times the experimental value for BSA and 1.98 times the value measured for lysozyme. Therefore, these calculated ϵ_{214} values were divided by 2.0 to obtain the corrected values.

Ribosomal protein spectral data for the complete elution profile were obtained from the diode array detector. Spectral comparisons from 214 to 300 nm (6-nm resolution) of the proteins in question indicated which wavelength ratios were optimal. Values were taken at the chromatographic peak maxima. Base-line subtraction was performed by using the values between peaks.

P site binding was done as described (Denman et al., 1988b), and P site cross-linking was according to Denman et al. (1988a). Blank values were those obtained with 50S subunits only. Total rRNA was isolated from 0.5 mL of the various P site binding reaction mixtures by extraction with 1 volume of phenol equilibrated with buffer RDm. The phenol phase was then reextracted with 0.5 volume of buffer RDm, and the two aqueous phases were combined and extracted 4 times with 0.5 volume of ether. The rRNA was precipitated twice from the aqueous phase with 2 volumes of EtOH at −70 °C for 10 min. The final pellet was dissolved in 25 μL of H_2O and dialyzed against 5 mL of H_2O for 15 min as described by Marusyk et al. (1980). The average recovery of rRNA for the nine samples used in this study was 50–70%.

Primer extension was done as described by Nurse et al. (1987) but with 8.4 pmol of cross-linked total rRNA and 3 μL of [^{35}S]αSdATP (1000 Ci/mmol, Amersham) in place of the [^{32}P]dATP, and an extension time of 35 min. Sanger dideoxy sequencing was performed as described previously (Nurse et al., 1987). One A_{260} unit of 30S or 16S RNA was assumed equal to 67 pmol, 1 A_{260} unit of 50S equal to 40 pmol, and 1 A_{260} unit of 70S equal to 25 pmol.

Results

Reconstitution of Mutant Ribosomes. The mutant RNAs listed in Table I were made by cassette mutagenesis as described by Krzyzosiak et al. (1987, 1988). Mutants A1400, G1400, and Δ1400 were made by single cassette insertion while U1400, the insertions, and the other deletions were made by the double cassette method. Mutant 3′(+7) was created by linearization of pWK1 (Krzyzosiak et al., 1987) with *Xba*I instead of *Bsu*36I.

Table I: Mutant Ribosomes Constructed[a]

	1	1393	1400	1408	1542
NAT	A A A	U A C A C A C C G	m^{4}Cm C C G U m^{5}C A	A	
C1400	G G G · G ·	· · · · · · · · · ·	C · · · ·	C ·	·
U1400	G G G · G ·	· · · · · · · U ·	C · · · ·	C ·	·
A1400	G G G · G ·	· · · · · · · A ·	C · · · ·	C ·	·
G1400	G G G · G ·	· · · · · · · G ·	C · · · ·	C ·	·
Δ1397	G G G · G ·	· · · · ▒ · · · ·	C · · · ·	C ·	·
Δ1398	G G G · G ·	· · · · · ▒ · · ·	C · · · ·	C ·	·
Δ1400	G G G · G ·	· · · · · · · ▒ ·	C · · · ·	C ·	·
Δ1401	G G G · G ·	· · · · · · · · ▒	C · · · ·	C ·	·
Δ1402	G G G · G ·	· · · · · · · · ·	▒ · · · ·	C ·	·
U1400.1	G G G · G ·	· · · · · · · U ·	C · · · ·	C ·	·
C1400.1	G G G · G ·	· · · · · · · C ·	C · · · ·	· ·	·
3′(+7)	G G G · G ·	· · · · · · · · ·	C · · · ·	C ·	· G G U C U A G

[a] Numbers refer to the sequence of natural 16S RNA (Noller, 1984). NAT, 16S RNA isolated from 30S subunits; C1400, 16S RNA transcribed from pWK1 in vitro (Krzyzosiak et al., 1987); Δ indicates a deletion of that residue, also noted by a dotted rectangle; 1400.1 indicates the insertion of a single additional base 3′ to the 1400 residue.

Table II: Reconstitution Conditions[a]

reaction components	A	B	C	D
Hepes, pH 7.5	30	20	20	20
NH_4Cl		400	500	500
KCl	330			
$Mg(OAc)_2$	20	20	20	16
mercapto-ethanol	6	4	5	5
spermidine				3
RNasin				100 units/mL
rRNA	0.2 μM	0.2 μM	0.2 μM	0.4 μM
30S proteins (TP30)	0.4 μM	0.4 μM	0.4–0.8 μM	0.8 μM
incubation	40 °C, 60 min	40 °C, 30 min	40 °C, 15 min 43 °C, 15 min 46 °C, 15 min 48 °C, 15 min 50 °C, 20 min	
internal control	[^{14}C]uracil–30S	[^{32}P]pCp–30S	[^{32}P]pCp–30S	

[a] Ribosome reconstitution conditions: column A, Held et al. (1973); column B, K. Nierhaus (personal communication); column C, Krzyzosiak et al. (1987); column D, this work. All concentrations are millimolar, except as indicated.

Transcription of mutated plasmids in vitro with T7 RNA polymerase yielded 416–648 mol of RNA/mol of DNA. As approximately 20% of the transcript was lost during the workup, the actual transcription copy number was 500–800. In practical terms, this means that ca. 1 mg of 16S RNA could be obtained from 10 μg of plasmid DNA. Since, in our hands, 2–4 mg of plasmid DNA can be purified from 100 mL of culture by the method of Tartof and Hobbs (1987), large amounts of 16S RNA of defined sequence were readily available.

Conditions for reconstitution of the RNA transcript with a mixture of 30S proteins (TP30) were explored by using the pWK1 transcript and conditions slightly modified (Table II, column B) from Held et al. (1973) as a starting point. Although the synthetic (C1400) RNA yielded material with a

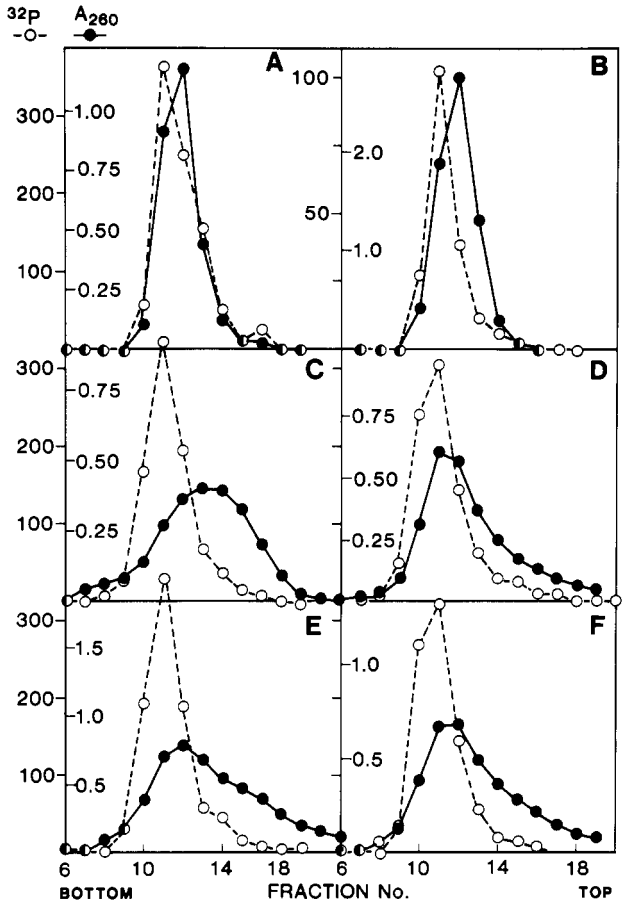

FIGURE 1: Sucrose gradient analysis of reconstituted 30S ribosomes. Panels A and B, natural RNA extracted from 30S particles (NAT); panels C–F, synthetic RNA (C1400). Panels A and C, incubation as in Table II, column B; panels B, D. E, and F, incubation as in Table II, column C. Ratio of complete 30S ribosomal protein mixture (TP30) to RNA was 2 in panels A, C, and E, 3 in panel F, and 4 in panels B and D. Sucrose gradient analysis was done according to Krzyzosiak et al. (1987). A total of 26 0.5-mL fractions were collected. The percent recovery for panels A–F was 72, 69, 61, 41, 76, and 54, respectively. Closed circles, A_{260}; open circles, [^{32}P]pCp–30S added as a position marker.

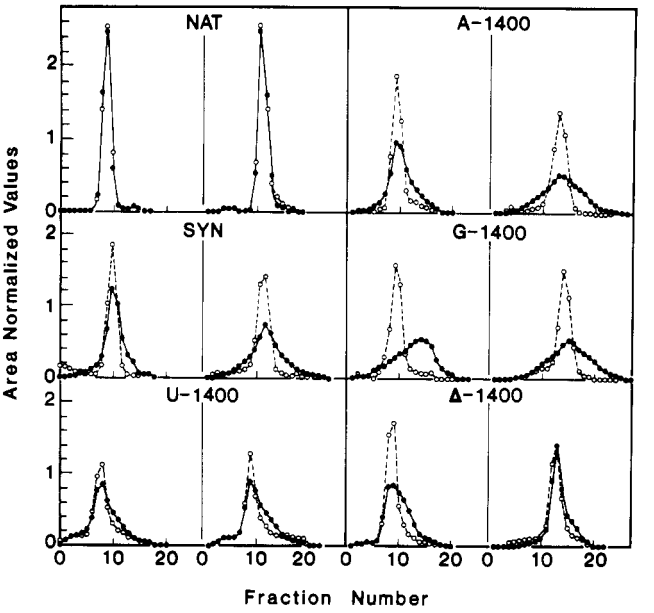

FIGURE 2: Comparison of 30S reconstitution under different conditions by sucrose gradient analysis. Mutant designations are as in Table I. SYN is C1400. The left panel of each pair of gradients shows reconstitution according to Table II, column C, with 0.8 μM TP30, except for U1400 which was condition D at 0.2 μM rRNA instead of 0.4 μM. The right panel reconstitution was done according to Table II, column D. Analysis was as in Figure 1 except that the $Mg(OAc)_2$ concentration in the sucrose gradient was raised from 10 mM to 15 mM. Sedimentation was from right to left. Closed circles, A_{260} of reconstituted particles; open circles, [^{32}P]pCp–30S added as a marker. All profiles were normalized to the same recovered peak area. Actual recoveries ranged from 37 to 70% for A_{260} and from 75 to 95% for ^{32}P.

mean sedimentation value of 25 S (Figure 1, panel C), it had no tRNA binding capacity. Other experiments using ^{32}P-labeled RNA showed that all of the RNA was taken up into this particle. By contrast, natural RNA (NAT) isolated by SDS extraction from 30S particles formed a complete 30S particle (panel A) which was functionally active. Variation of the reaction parameters eventually led to a set of conditions (Table II, column C) which allowed reconstitution of C1400 RNA (panels D–F) without affecting the ability of natural RNA to reconstitute (panel B). As shown in panels E, F, and D, increasing the TP30/RNA ratio from 2 to 4 increased the amount of reconstituted synthetic ribosomes which sedimented under the ^{32}P peak of marker natural 30S. It was previously shown that the [^{32}P]pCp–30S marker sedimented exactly like natural isolated 30S (Krzyzosiak et al., 1987).

While the parameters of Table II, column C (TP30/RNA ratio of 4), were satisfactory for wild-type synthetic ribosomes and the A1400 mutant, the G1400 mutant did not assemble well under these conditions (Krzyzosiak et al., 1987). Further experimentation led to the development of modified conditions (Table II, column D). As shown in Figure 2, the modified conditions markedly improved the ability of the G1400 mutant to assemble. Note that simply raising the Mg^{2+} concentration in the sucrose gradient from 10 mM (Krzyzosiak et al., 1987) to 15 mM (this work) was insufficient to allow good assembly of G1400. The modified conditions also improved assembly of Δ1400 but were less effective for A1400 and C1400 (SYN) assembly. A consistent broadening of the peaks was found with both of these latter two particles. Natural 16S RNA assembly was again unaffected. In a series of experiments not shown, the improvement in G1400 assembly was found to be primarily due to the 2-fold increase in RNA concentration rather than to the inclusion of spermidine and RNasin which were added primarily as prophylactic measures.

A sucrose gradient analysis of a complete reconstitution series for all 11 mutants using the conditions of Table II, column D, is shown in Figure 3. This set of reconstitutions was performed on a large scale with a single preparation of TP30. Some of the mutants show clear evidence of a slower sedimenting peak at approximately 22 S. In order to obtain as homogeneous a preparation as possible for all 11 mutants, only those fractions sedimenting with the ^{32}P-labeled marker 30S were pooled for functional analysis. This selection procedure was adopted since it was known from previous work (Krzyzosiak et al., 1987) that the slower sedimenting fractions were only poorly able to bind tRNA to the P site, and the same was true for the faster sedimenting fractions (data not shown).

Protein Content of Reconstituted Ribosomes. Total ribosomal proteins from native and reconstituted 30S particles were analyzed by HPLC (Figure 4). All of the protein peaks in the chromatogram were identified by sequencing, except for S6 and S7, and S5 and S18 whose N-termini are blocked. In these four cases, the positions were established by addition of a large amount of the standard protein to a small amount of TP30. Identification was confirmed by comparison with published elution profiles (Kerlavage et al., 1983). Position changes in the elution profile of native 30S which occurred when the HPLC column was changed or as it aged were

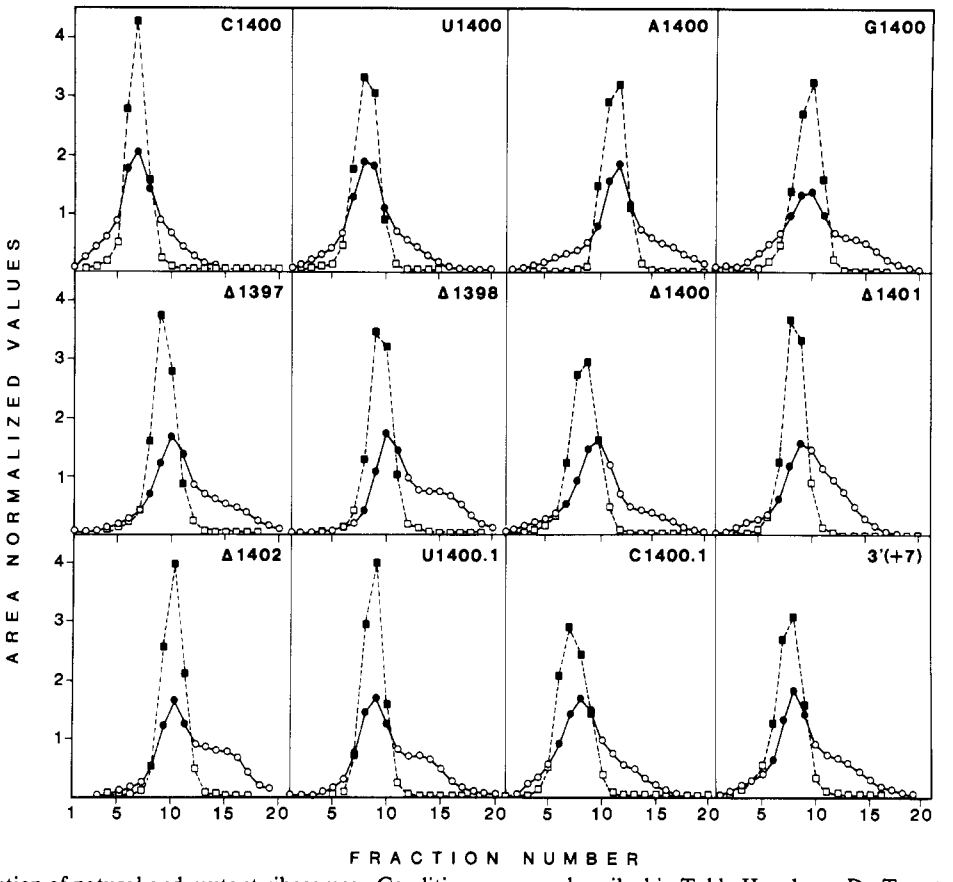

FIGURE 3: Reconstitution of natural and mutant ribosomes. Conditions were as described in Table II, column D. Twenty A_{260} units of RNA and 40 equivalent units of TP30 in 3.6 mL of buffer were equally divided among five 1.5-mL Eppendorf tubes and annealed. After pooling and addition of [^{32}P]pCp–30S, the sample was applied to a single sucrose gradient and centrifuged (SW28 rotor, 26 200 rpm, 20 h, 4 °C). Analysis was as in Figure 2. Circles, A_{260}; squares, [^{32}P]pCp–30S. Closed symbols, *fractions pooled for functional assays*; see Denman et al. (1988b). Recoveries ranged from 55 to 88%. A total of 33–36 approximately 1-mL fractions were collected per gradient.

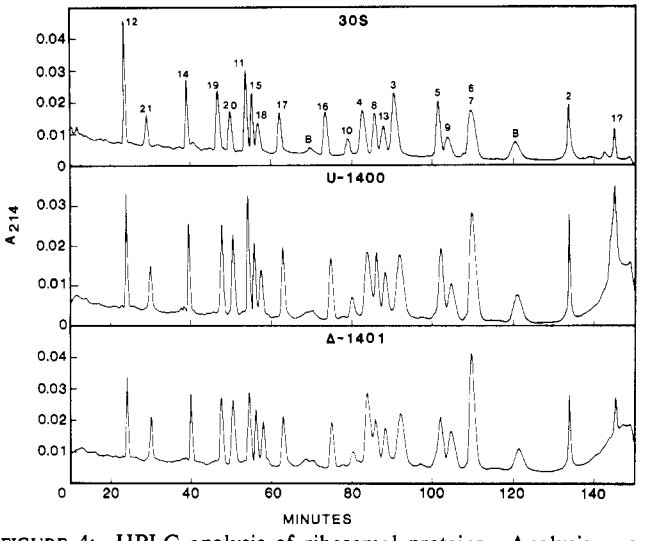

FIGURE 4: HPLC analysis of ribosomal proteins. Analysis was performed as described under Experimental Procedures.

controlled by the use of standard proteins and by comparing the spectral properties of the peaks in question. For example, the region of the chromatogram containing S20, S11, S15, and S18 (region I), that containing S8, S13, and S3 (region II), and that containing S5, S9, S6, and S7 (region III) exhibited variable peak patterns. In region I, three or four peaks were found for the four proteins, the degree of resolution and order of elution of S15 and S18 varying with column age and from column to column. S20 and S11 were assigned to the first and second peaks, respectively, by sequence analysis of the isolated peaks and by their characteristic spectral properties (Table III) among the proteins of region I. When four peaks were found (Figure 4), the order of elution of S15 and S18 could be determined by the use of protein standards and by the higher 278/214-nm ratio of S18. S18 and S11 could be distinguished by the lower absorbance at 295 nm of S18 and by a higher value at 278 nm. When three peaks were found,

Table III: Spectral Properties of Some 30S Ribosomal Proteins[a]

		278/214		295/214	
region	protein	test protein	standard protein	test protein	standard protein
I	S19	42	44	17	26
	S20	20	17	<5	7
	S11	37	35	15	17
	S15	26	32	3	11
	S18	73	72	8	11
	S17	53	51	21	25
II	S4	58	57	12	15
	S8	30	35	<7	12
	S13	36	40	<9	<10
	S3	67	68	27	30
III	S5	21	24	4	7
	S9	39	47	8	<20
	S6	46	52	15	17
	S7	62	63	21	26

[a] Regions I, II, and III are defined in the text. All values are absorbance ratios × 10^3 and were obtained as described under Experimental Procedures. At least three independent measurements were made, either from independent elution profiles or from different parts of the same peak, and averaged. Test protein, injection of TP30 from 3–5 A_{260} units of 30S; standard protein, injection of a known standard or a peak from a preparative chromatogram which was subsequently sequenced.

S15 was found in the third peak by sequence analysis (S18 is N-acetylated), and S18 was found there by use of an S18 standard.

In region II, S8, S13, and S3 were found as two or three peaks. When two peaks were found, sequencing analysis of the peaks showed the first peak to be S8 and the second to contain S13 and S3. In some cases, detected as an altered ratio of the two peaks to each other, S8 and S13 eluted together (confirmed by the use of standard preparations of S8 and S13), with S3 eluting separately. When three peaks were visible (Figure 4), the order of elution was determined by using S8 and S13 standards and from the fact already noted that S13 was sometimes found with S8 and sometimes with S3. Discrimination between S13 and S3 and between S4 and S8 could also be made on the basis of their spectral properties (Table III). In region III, the four proteins S5, S9, S6, and S7 eluted as two to four peaks. The order of elution was determined by using protein standards. Spectral ratios were also used to distinguish between S5 and S9 (278/214 nm) and between S6 and S7 (295/214 nm). Sequence analysis on an example with only two peaks confirmed the presence of S9 in the first peak (S5 is N-acetylated), and use of S6 and S7 standards confirmed that both proteins coeluted in the second peak. The peak assignments given here (Figure 4) agree with those of Kerlavage et al. (1983), who used the same HPLC system.

The protein content of each of the synthetic reconstituted mutant ribosomes relative to reference 30S particles is given in Table IV. Thus, the values in Table IV are independent of errors in determining the absolute amount of protein present, which ranged from 0.8 to 1.5 for the different proteins. No protein is absent in any of the mutants. Except for the underlined values in the table, all the proteins are present at unit stoichiometry ±30%. There is a general trend for S20 to be elevated in all of the preparations. Also, all of the C1400.1 values tend to be high, suggesting a slight systematic error in this single analysis.

Formation of 70S Ribosomes. An essential parameter for protein synthesis activity of 30S ribosomes is the ability to associate with 50S ribosomes since all functional activities for the initial association of mRNA and fMet-tRNA with 30S subunits take place on a 70S ribosome. It was therefore important to know if the mutant 30S subunits could associate with 50S subunits to make a 70S ribosome. Otherwise, lack of activity in the functional assays applied (Denman et al., 1988b) might simply be due to a failure to associate. For this reason, rather than applying stringent conditions for the association assay such as the use of 6 mM Mg^{2+} as is used for the detection of "tight couples" (Noll et al., 1973), reaction conditions were chosen which closely mimicked those used for the P site binding assay and initiation-independent polypeptide assay (Denman et al., 1988b). As described in Table V, system A corresponds to the P site binding assay and system B to the polypeptide synthesis assay in terms of the Mg^{2+} concentration and the salt mixture used. Association of subunits was analyzed by a velocity centrifugal separation of 30S and 50S subunits from 70S ribosomes with appropriate quantitation. The results are expressed in Table V as the actual percent of 30S subunits which associated and also as a relative percent of the reconstituted, nonmutated synthetic 30S (C1400) ribosome. Table V shows that all of the mutants, except for Δ1401, associated well under either set of conditions. Deletion of G1401 resulted in a decrease in association activity to one-third that of C1400, but this sharp drop could be alleviated by an increase in the 50S subunit concentration (series II). The relatively modest decrease in association activity seen with

Table IV: Protein Content of Natural, Synthetic, and Mutant Reconstituted 30S Ribosomes[a]

protein	NAT	C1400	U1400	A1400	G1400	Δ1397	Δ1398	Δ1400	Δ1401	Δ1402	U1400.1	C1400.1	3′(+7)
S2	1.0 (0.3)	0.7 (0.1)	1.0 (<0.1)	0.9 (0.1)	0.8 (0.2)	0.8 (0.3)	0.8	0.5 (0.2)	0.7 (0.1)	0.6	0.7	1.0	0.5 (0.1)
S3	0.8 (0.1)	0.8 (0.1)	0.9 (0.1)	0.9 (0.1)	0.8 (0.1)	0.8 (0.1)	1.0	0.8 (<0.1)	0.7 (0.1)	0.7	0.6	1.1	0.7 (0.1)
S4	0.9 (0.1)	1.2 (0.3)	1.1 (0.3)	1.2 (0.2)	1.3 (0.2)	0.9 (0.2)	1.2	1.1 (0.2)	1.0 (0.2)	1.0	0.9	1.3	1.0 (0.2)
S5	0.9 (0.1)	1.0 (0.3)[b]	1.2 (0.2)	1.3 (<0.1)[b]	1.2 (0.2)[b]	0.9 (0.2)	1.1[b]	1.1 (0.2)[b]	0.9 (0.1)[b]	0.9[b]	0.8[b]	1.2[b]	0.7 (<0.1)
S6	0.8 (0.1)	0.9 (0.2)	1.0 (0.2)	1.1 (0.1)	1.1 (0.1)	0.9 (0.2)	1.2	0.9 (<0.1)	1.0 (0.2)	0.8	0.7	1.3	0.9 (<0.1)
S7	0.9 (0.1)	0.9 (0.2)	0.9 (0.1)	1.0 (0.1)	1.0 (0.1)	0.9 (0.2)	1.0	0.9 (0.1)	0.9 (0.1)	0.9	0.6	1.1	0.6 (<0.1)
S8	0.9 (0.1)	1.0 (0.2)[c]	1.3 (0.2)	1.1 (0.2)[c]	1.2 (0.2)[c]	1.0 (0.2)	1.2[c]	1.0 (0.1)[c]	1.0 (0.1)	0.9[c]	0.8[c]	1.3[c]	0.8 (0.2)
S9	0.8[b]	1.0 (0.3)[b]	0.8 (0.2)	1.3 (<0.1)	1.2 (0.1)[b]	0.8 (0.2)	1.1[b]	1.1 (0.2)[b]	0.8 (0.1)[b]	0.9[b]	0.8[b]	1.2[b]	0.9 (0.1)
S10	1.3 (0.4)	0.9 (0.1)	1.1 (0.2)	0.9 (<0.1)[b]	0.9 (0.1)	1.0 (0.3)	1.1	0.8 (<0.1)	1.0 (0.1)	0.9	0.7	1.2	0.9 (0.2)
S11	0.9 (<0.1)	1.0 (0.3)	1.1 (0.2)	1.1 (0.1)	1.2 (0.1)	0.7 (0.2)	1.1	1.0 (0.2)	0.9 (0.2)	1.0	0.8	1.2	0.8 (0.2)
S12	0.8 (0.1)	0.8 (0.2)	0.8 (0.2)	0.9 (<0.1)	1.0 (0.1)	0.7 (<0.1)	0.9	0.7 (<0.1)	0.6 (0.2)	0.8	0.7	0.9	0.7 (<0.1)
S13	1.2 (<0.1)	1.0 (0.2)[c]	1.0 (0.1)	1.1 (0.1)[c]	1.2 (0.2)[c]	1.0 (0.2)	1.2[c]	1.0 (0.1)[c]	0.9 (0.1)	0.9[c]	0.8[c]	1.3[c]	0.8 (0.2)
S14	0.8 (<0.1)	0.9 (0.1)	0.9 (0.2)	0.9 (0.1)	1.0 (0.1)	0.7 (0.1)	0.9	0.9 (0.1)	0.7 (0.2)	0.8	0.7	1.1	0.6 (0.1)
S15	0.8 (0.2)	0.8 (0.3)	1.0 (0.1)	0.9 (0.2)	1.0 (0.2)	0.8 (0.2)	1.0	1.0 (0.2)	0.8 (0.1)	0.7	0.6	1.1	0.8 (<0.1)
S16	0.9 (<0.1)	1.0 (0.2)	1.0 (0.1)	1.0 (0.1)	1.1 (0.1)	0.8 (0.2)	1.1	1.0 (0.1)	0.9 (0.1)	0.9	0.7	1.1	1.0 (<0.1)
S17	0.9 (0.1)	1.1 (0.3)	1.1 (0.1)	1.1 (0.2)	1.3 (0.1)	0.9 (0.1)	1.2	1.1 (0.1)	1.0 (0.2)	1.0	0.8	1.3	1.1 (<0.1)
S18	0.8 (0.1)	1.0 (0.2)	1.0 (0.1)	1.0 (0.2)	1.2 (0.2)	0.7 (0.1)	1.2	0.9 (0.2)	0.9 (0.2)	1.0	0.7	1.0	0.7 (0.3)
S19	0.9 (<0.1)	1.0 (0.2)	0.9 (0.1)	1.0 (<0.1)	1.1 (<0.1)	0.7 (0.2)	1.0	1.0 (0.2)	0.7 (0.1)	0.9	0.8	1.1	0.7 (<0.1)
S20	1.2 (0.2)	1.5 (0.4)	1.4 (0.2)	1.4 (0.2)	1.6 (0.3)	1.2 (0.2)	1.2	1.2 (0.2)	1.3 (<0.1)	1.2	1.1	1.8	1.4 (0.1)
S21	0.9 (0.1)	0.9 (0.1)	0.9 (<0.1)	1.0 (<0.1)	1.1 (0.1)	0.7 (0.1)	0.9	0.8 (0.2)	0.9 (0.1)	0.9	0.6	0.9	0.7 (0.2)
n	3	4	2	2	2	2	1	2	2	1	1	1	2

[a] Analyses were as described under Experimental Procedures. Reconstituted ribosome designations are described in Table I. Two or more values (*n*) from at least two separate reconstitutions were averaged. Values in parentheses are standard deviations ($n = 3$ or 4) or average deviations ($n = 2$). [b,c] Pairs of proteins which did not separate clearly from each other. Underlined values are those <0.7 or >1.3.

Table V: Formation of 70S Ribosomes by Reconstituted Mutant 30S Subunits[a]

series	30S	% 30S as 70S		% of C1400		P site
		system A	system B	system A	system B	binding
I	NAT		96		133	210
	C1400	74	72	100	100	100
	U1400	67	67	91	93	135
	A1400	81	78	109	108	138
	G1400	77	68	104	94	113
	Δ1397	57	64	77	89	50
	Δ1398	66	56	89	78	43
	Δ1400	61	59	82	82	18
	Δ1401	27	28	36	39	<5
	Δ1402	72	71	97	99	8
	U1400.1	64	65	86	90	45
	C1400.1	67	66	91	92	48
	3′(+7)	87	77	118	107	98
II	C1400	89	81	100	100	100
	Δ1401	68	55	76	68	<5

[a] Formation of 70S was analyzed as described under Experimental Procedures. Series I, system A was 50 mM Hepes, pH 7.5, 100 mM NH_4Cl, 15 mM $Mg(OAc)_2$, 5 mM DTT, 20 μg/mL poly(U_2,G), 67 nM 30S, 60 nM 50S, and 100 nM uncharged $tRNA^{Val}$. Series I, system B was as in A except for the use of 50 mM NH_4Cl and 20 mM $Mg(OAc)_2$. Series II was like series I except with 100 nM 50S. P site binding values as percent of C1400 were calculated from Table I of Denman et al. (1988b).

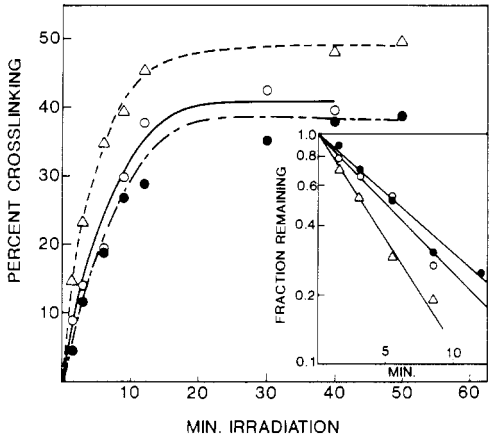

FIGURE 5: Kinetics of P site cross-linking to 70S ribosomes. P site binding and cross-linking were done as described under Experimental Procedures. Percent cross-linking is the amount of tRNA cross-linked × 100 divided by the amount of 30S-dependent noncovalent binding which was 0.55, 0.27, and 0.31 pmol of tRNA/pmol of ribosomes for isolated 30S, C1400, and U1400, respectively. Triangles, isolated 30S; open circles, C1400; closed circles, U1400. Inset: First-order semilogarithmic plot of the fraction remaining un-cross-linked calculated as (plateau cross-linking value minus the value at time t)/plateau value.

Δ1401 is in marked contrast to the lack of detectable activity in P site binding (last column) and in other functional activities (Denman et al., 1988b). There is also a lack of correlation to P site binding with the other mutants. The most striking case is Δ1402 which completely retained its 50S subunit association ability while losing most of its detectable P site binding activity.

Cross-Linking Kinetics of Mutant Ribosomes. The initial rationale for examining the rate of cross-linking was to see the effect of a C1400 → U1400 substitution, since uridine is normally an order of magnitude better than cytidine in cyclobutane dimer formation (Fisher & Johns, 1976). Unexpectedly, U1400 and C1400 cross-linked to tRNA with the same rate and yield (Figure 5). This may be a result of the hydrophobic environment of the ribosome in the vicinity of C1400 (Gornicki et al., 1985) since it is believed that the low quantum yield of cytidine in dimer formation is due to the ease with which it forms hydrates (Fisher & Johns, 1976). This result prompted us to examine the remaining mutants which had retained sufficient P site binding activity (Table VI). All of the reconstituted ribosomes cross-linked with similar kinetics. The average rate constant value for the C1400 and 3′(+7) 30S was 0.165 min^{-1} compared to a value of 0.17 min^{-1} for natural reconstituted 30S, and 0.12 and 0.14 min^{-1} for the two mutants around position 1400. The isolated 30S value was 0.22 min^{-1}. The differences in rate constants observed are probably not due to the absence of methyl groups in the synthetic 30S because the rate constant for natural reconstituted 30S was like the nonmutant synthetic particles. Thus, the double methylation of C1402 to m^4Cm appears not to influence cross-linking to C1400. The yield of cross-linking was essentially the same for all the ribosomes with a pyrimidine at position 1400. This was true even though the relative amounts of P site binding differed by over 5-fold.

Table VI: P Site Cross-Linking Rate and Yield for Mutant Ribosomes

30S ribosome	k (min^{-1})[a]	yield (% of control 30S)[b]	P site binding[c]
isolated	0.22 ± 0.01 (3)	100	100
NAT	0.17	108	84
C1400	0.15 ± 0.02 (4)	93 ± 14 (5)	40
3′(+7)	0.18	83	39
U1400	0.12 ± 0.01 (2)	80	54
C1400.1	0.14	118	19
U1400.1	ND	98	18
Δ1397	ND	105	20
Δ1398	ND	114	17
A1400	ND	18	55
G1400	ND	15	45

[a] Calculated as $0.69/T_{1/2}$. Half-times were obtained from the semilogarithmic plots of Figure 5 (inset). Average deviations from the mean are indicated. The number of determinations is in parentheses. ND, not determined. [b] Control 30S ranged between 41 and 62% cross-linking defined as 100(pmol of tRNA cross-linked/pmol of tRNA bound). [c] Recalculated from Denman et al. (1988b).

A different situation was found when purines were present at position 1400. No cross-linking was expected since cyclobutane dimer formation is pyrimidine specific (Fisher & Johns, 1976). Nevertheless, by this assay, a low but consistent level of cross-linking was detected for both the A1400 and G1400 mutants. The amounts found, 18% and 15%, respectively, were in good agreement with values of 19% and 12% reported previously (Ofengand et al., 1988).

Characterization of the Cross-Linked Products. Since both A1400 and G1400 unexpectedly showed UV-induced cross-linking in the filter binding assay, the nature and location of the cross-links were examined. C1400 was included to verify that cross-linking of 30S ribosomes containing unmethylated RNA was the same as for natural 30S or 70S ribosomes. Both low Mg^{2+} concentration and SDS–sucrose gradient analyses (Ciesiolka et al., 1985) were used, since this technique reduces the background of noncovalently bound tRNA. In all samples, cross-linking was dependent on the presence of poly(U_2,G), was only to 16S RNA, and was not found when 30S subunits were omitted, in agreement with earlier studies (Schwartz & Ofengand, 1978; Ofengand & Liou, 1981; Ofengand et al., 1988). When the cross-linking yields from both 30S subunit and 16S RNA analyses were quantitated, the two sets of values were in good agreement, indicating that most or all of the cross-linking was to 16S RNA. For isolated and synthetic 30S, the values agreed with the filter assays of Table VI. However, the values for A1400 and G1400 dropped markedly, to only 3% of 30S for A1400 and 1.5% for G1400. Either the filter

assay grossly overestimated the cross-linking yield at low levels of cross-linking or the cross-link was highly labile.

Although further study was hampered by the low amount of cross-linked 16S RNA–tRNA from A1400 and G1400, we could show, by 254-nm-induced photohydrolysis (Fisher & Johns, 1976), that the cross-links were not of the cyclobutane dimer or oxetane class. By the same procedure, the photolabile nature of the C1400, C1400.1, and U1400.1 mutants was confirmed. The A1400 and G1400 cross-links are not likely to be derived via a free radical pathway such as that described by Cabrera-Juarez and Setlow (1977) for bipyrimidine and purine adducts since the cross-linking reaction was carried out in the presence of millimolar quantities of the free radical scavenger dithiothreitol.

An attempt was made to locate the site of cross-linking in A1400 and G1400 by reverse transcription arrest analysis (Nurse et al., 1987; Denman et al., 1988a). While the site of cross-linking in the synthetic C1400 RNA could be confirmed at position 1400, no detectable cross-link arrest could be seen between residues 1240 and 1458 in either A1400 or G1400. Apparently, these low-yield cross-links are distant from position 1400 in the primary structure.

Site of Cross-Linking to the Insertion Mutants C1400.1 and U1400.1. The rationale behind the construction of these two mutants was to examine the result of adding a third C residue to the two already present, C1399 and C1400, or of inserting a potentially more reactive U residue. Two possible results were anticipated. First, steric constraints introduced by the additional base could disrupt P site binding and/or cross-linking entirely. Since deleting residue C1400 markedly reduced P site binding (Ofengand et al., 1988; Denman et al., 1988b), the effect of extra residues was of interest. Second, small changes in the local structure of 16S rRNA caused by the insertion might alter the site of cross-linking relative to the 1400 position. Specifically, the question was whether the extra nucleotide would bulge out on the 5′ or 3′ side of the cross-link. If on the 5′ side, then the base 5′ to G1401, that is, C1400.1 or U1400.1, would be cross-linked. If on the 3′ side, then C1400 would presumably be the cross-linked residue. Table VI shows that the cross-linking yield of both insertion mutants was nearly the same as isolated 30S subunits. However, the lower observed P site binding values indicate that the additional nucleotide inserted between G1401 and C1400 had some effect on the structure of the P site. It was still possible that a shift in the site of cross-linking could have occurred since the base 5′ to C1400, 1399, and both insertion mutants were also pyrimidines. Reverse transcription arrest analysis showed clearly that transcription stopped at G1401 in all three samples (Figure 6). Thus, the adjacent base, C1400 in synthetic 30S, and C or U1400.1 in the insertion mutants were the bases cross-linked. The different intensities of arrest are due to the different amounts of tRNA bound (Table VI) as the same total amount of rRNA was applied to each lane. The minor band at position 1400 is due to the failure of reverse transcriptase to completely halt one base before the cross-linked residue (Denman et al., 1988a).

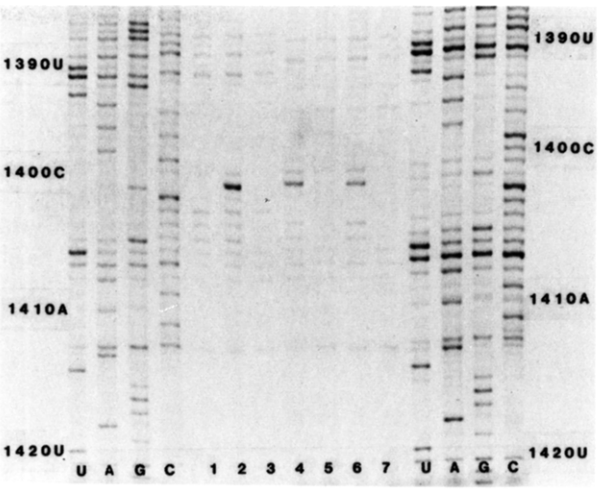

FIGURE 6: Cross-link site identification in the insertion mutants C1400.1 and U1400.1. P site binding, cross-linking, phenol extraction of total rRNA, and primer extension arrest analysis were performed as described under Experimental Procedures. Lanes U, A, G, and C (left), sequencing of synthetic 16S rRNA; lanes U, A, G, and C (right), sequencing of C1400.1 16S rRNA. Lane 1, cross-linking of 50S control in the presence of poly(U_2,G). Lanes 2 and 3, cross-linking of synthetic 30S in the presence and absence of poly(U_2,G), respectively. Lanes 4 and 5, cross-linking of U1400.1 in the presence and absence of poly(U_2,G), respectively. Lanes 6 and 7, cross-linking of C1400.1 in the presence and absence of poly(U_2,G), respectively.

Discussion

Reconstitution. The in vitro reconstitution of a functional 30S ribosome was first achieved 20 years ago by using isolated natural 16S RNA and a complete set of 21 purified 30S proteins (Traub & Nomura, 1968). In this work, we have shown that the conditions established with natural 16S RNA did not yield 30S particles when synthetic 16S RNA, which differs only in having 3 extra bases at the 5′ end and lacking all 13 methyl groups, was used. Instead, an approximately 25S particle was found (Figure 1). Increased ionic strength and temperature were required to achieve assembly of the synthetic RNA into a 30S particle which possessed the EM morphology of a true 30S ribosome (Krzyzosiak et al., 1987). Possibly, the lack of methyl groups is responsible for the requirement for higher temperature and a higher salt concentration. For example, the methylated residues might act to reduce the activation energy for correct folding under physiological conditions and/or help to direct folding along a productive, as opposed to a dead-end, path by creating a new lowest energy state at critical branch points of the assembly pathway. Although it is believed that methylation of 16S RNA occurs late in the process of assembly (Feunteun et al., 1974; Dahlberg et al., 1975), little or nothing is known about the detailed temporal or sequential nature or the substrate specificity of the methylation reactions except that the four methyl groups incorporated as $m_2^6A_{1518}$ $m_2^6A_{1519}$ require a 30S particle as substrate (Poldermans et al., 1979). We are attempting to study this question by use of the reconstitution system described in this work.

Reconstitution is not perfect. With most mutants, there is a fraction which sediments faster than 30S as well as a substantial amount of slower sedimenting material. This was especially prominent in the right-hand set of panels of Figure 2 for SYN (C1400), A1400, and G1400. Note that the slight change in conditions (Table II) which produced these results (compare left and right panels of Figure 2) markedly improved assembly of G1400. The effect was not due to the presence of RNasin or spermidine as similar results were obtained with condition C of Table II when the RNA and TP30 concentrations were as in condition D (data not shown). The peak position of the deletion mutants appears to be shifted from that of the marker 30S in Figure 3, but additional experiments show that this is due to the variability of the reconstitution process. In other reconstitutions, the two peaks are virtually coincident. Comparison of Figures 2 and 3 shows several examples of variability in reconstitution. We do not know if this is due to variation in the RNA, TP30, or exact reconstitution conditions. On the other hand, in such a complex system, perhaps

the most surprising feature is that reconstitution could be obtained at all.

Protein Content. The main conclusion to be drawn from the protein analysis of the reconstituted wild-type and mutant ribosomes is that no protein is clearly lacking in any case. Thus, even those mutants with severely deficient functional activities (Denman et al., 1988b) still have a full complement of associated 30S proteins. It was important to show this directly since sucrose gradient analysis alone is not sufficient to detect a single missing protein, as was shown for ribosomes lacking S3 (Ramakrishnan et al., 1986), and in other single or multiple protein-deficient reconstituted ribosomes (Nomura et al., 1969; Mizushima & Nomura, 1970; Buck, 1988; M. Buck, T. Olah, C. Weitzmann, and B. S. Cooperman, unpublished results).

Unit stoichiometry was not always obtained. S2 was present in reduced amounts in Δ1400, Δ1402, and 3′(+7), but no other protein was outside the ±30% range more than once for any of the samples, except for S7 in both U1400.1 and 3′(+7). The values for S20 were consistently high in all of the preparations. Since the values in Table IV were taken as a ratio of S20 in the reconstituted particle to S20 in isolated 30S, anything that reduced the S20 content of 30S such as partitioning between 30S and 50S (Hardy, 1975) would have the apparent effect of increasing the S20 values in Table IV.

During this work, we observed that proteins S15 and S18 sometimes exchanged positions and sometimes eluted together. Protein S13 occasionally eluted with S8, or with S3, and sometimes in between the two proteins. S5 and S9 also occasionally eluted together and other times were separated. The same behavior was found for S6 and S7. In these cases, the ability to recall a complete chromatographic profile at any desired wavelength from the stored data set was of major importance in correctly assigning proteins to the various peaks.

70S Formation. The primary purpose of this analysis was to determine if 70S formation could occur under the conditions used for functional assays (Denman et al., 1988b), rather than to assess the relative strength of the association constant for 70S formation among the various mutants. Consequently, the 70S formation protocol mimicked the functional assay conditions as far as was practical. For system A, the differences were the use of uncharged tRNA in place of AcVal-tRNA, and the use of a slightly lower 50S concentration, 60 nM instead of 100 nM. At most, these differences would be expected to decrease the stability of the 70S complex so that the values obtained are minimal ones. For system B, Val-tRNA and Phe-tRNA were replaced by uncharged tRNA, elongation factors and the GTP-generating system were omitted, and the 30S concentration was slightly higher, 67 nM instead of 40 nM. Except for the ribosome concentration which was kept the same as system A for ease of comparison between the two systems, the differences should, if anything, diminish association. Moreover, the utility of the centrifugation assay itself is limited in that it is a nonequilibrium separation procedure which depends, at least in part, on a slow rate of dissociation under the conditions used. Nevertheless, as Table V shows, association was quite comparable for both systems, and the synthetic 30S was about 75% as able to form 70S as natural 30S. All of the mutants except for Δ1401 were about equally active, despite their large differences in functional ability (Denman et al., 1988b), and even Δ1401 could be induced to form a high level of 70S by increasing the 50S concentration to levels used in the P site assay.

Cross-Linking. Near-UV-induced cross-linking (310–330 nm) to prokaryotic 70S and eukaryotic 80S ribosomes (Ofengand et al., 1979, 1982; Ciesiolka et al., 1985; Nurse et al., 1987) is a codon-dependent process (Ofengand & Liou, 1981) which occurs exclusively to tRNA bound to the P site, irrespective of whether or not the A site is occupied (Ofengand et al., 1986). All of the determinants necessary for proper cross-link formation reside on the 30S subunit (Denman et al., 1988a). The cross-linked species formed is a pyrimidine–pyrimidine cyclobutane dimer (Ofengand & Liou, 1980) between the 5′-anticodon base of the tRNA and C1400 of 16S rRNA or its eukaryotic equivalent (Prince et al., 1982; Ehresmann et al., 1984; Ehresmann & Ofengand, 1984; Ciesiolka et al., 1985; Nurse et al., 1987).

Since UV-induced cross-linking to the ribosomal P site is sensitive to minor structural perturbations of the tRNA–mRNA–ribosome complex (Ofengand & Liou, 1981; Ofengand et al., 1988), the cross-linking behavior of the mutants described in this work was examined. Synthetic 30S subunits which contained the phylogenetically conserved C1400 residue but lacked all methylated residues including m^4Cm_{1402} were nearly as active as isolated 30S subunits in both the rate and yield of cross-linking, and this was also true for all those mutants with sufficient levels of P site binding to permit analysis. By this test, therefore, the mutations around the C1400 residue did not perturb the anticodon loop binding portion of the P site even when deletions and insertions were created near the cross-link site. However, since P site binding of tRNA was decreased in some of the mutants, some other part of the P site must be affected.

Previously, reverse transcription arrest located the site of cross-linking in the synthetic C1400-containing 30S subunit to the expected C1400 residue (Ofengand et al., 1988). The two insertion mutants, on the other hand, no longer cross-linked to C1400, but instead the newly inserted base 5′ to G1401 was recognized rather than the one 3′ to C1399. This finding has two important implications for the structure of the P site in this region. First, it demonstrates a certain degree of flexibility for the single-stranded region spanning residues 1394–1400. Therefore, one cannot explain the observed phylogenetic conservation of both length and sequence solely as a need to maintain a correct P site structure for the anticodon loop of tRNA. Second, the fact that insertion of a base 5′ to G1401 resulted in a shift of the site of cross-linking to the inserted base suggests that G1401 may be a key positional reference point for P site binding of the anticodon loop. This conclusion is supported by the fact that G1401 is the one base of the entire 1397–1404 segment whose deletion totally inactivated the ribosome (Denman et al., 1988b).

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