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In Vitro Synthesis of 16S Ribosomal RNA Containing Single Base Changes and Assembly into a Functional 30S Ribosome

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ABSTRACT: Functional 30S ribosomes were reconstructed from total *Escherichia coli* 30S ribosomal proteins and 16S ribosomal RNA synthesized in vitro by T_7 RNA polymerase. Up to 700 mol of RNA/mol of template could be obtained. The transcript lacked all ten normally modified bases and had three additional 5′ G residues, an A → G change at position 2, and, in 22% of the molecules, one or two extra 3′ residues. The synthetic 16S RNA could be assembled into a particle that cosedimented with authentic 30S and was indistinguishable from 30S by electron microscopy. When supplemented with the 50S subunit, the particles bound tRNA to the 70S P site in a codon- and Mg^{2+}-dependent manner. The specific binding activity was 94% that of particles reconstituted with natural rRNA and 52% that of native 30S. Cross-linking to P site bound tRNA was also preserved. Changing C-1400, the residue known to be close to the anticodon of P site bound tRNA, to A had little effect on reconstitution, but the C → G substitution caused a marked inhibition of assembly. tRNA could bind to both reconstituted mutants, but cross-linking was greatly reduced. These results show that none of the modified bases of 16S RNA are essential for P site binding and that position 1400 may be more important for ribosome assembly than for tRNA binding. Base-specific in vitro mutagenesis can now be used to explore in detail the functional properties of individual residues in ribosomal RNA.

The ribosome is a complex subcellular organelle consisting of numerous proteins, two large rRNAs, and one small rRNA divided between two unequally sized subunits. The primary structure of the three RNAs and 52 proteins of the *Escherichia coli* ribosome is known (Wittman, 1982), and detailed models for the secondary structure of the RNAs (Gutell et al., 1985; Brimacombe & Stiege, 1985) and some of the proteins have been proposed (Wittman, 1982; Liljas, 1982). The location of many of the proteins on and in the ribosome has been determined (Stöffler & Stöffler-Meilicke, 1984; Lake, 1985; Ramakrishnan et al., 1984) as well as the topographical location of certain of the RNA residues (Brimacombe & Stiege, 1985). The tertiary structure of the RNA within the ribosome is also beginning to be understood (Noller & Lake, 1984; Brimacombe & Stiege, 1985; Ofengand et al., 1985; Expert-Bezancon & Wollenzien, 1985; Hui & Cantor, 1985; Atmadja et al., 1986; Gutell et al., 1986; Brimacombe et al.,

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1986). Despite this considerable progress, a great deal remains unknown. In particular, the significance of modified nucleotides located at precise positions within the RNA sequence and of sequence-conserved stretches of nucleotides located in single-stranded regions of the RNA remains largely undetermined.

One of the conserved sequences in 16S RNA has attracted particular interest because of the observation that the 5′ anticodon base of P site bound tRNA is juxtaposed to a residue at its center (Ofengand et al., 1979; Ehresmann & Ofengand, 1984). This residue, C-1400 in *E. coli*, and the eight nucleotides to either side have their counterparts in virtually all small subunit rRNA sequences known (Gutell et al., 1985). Cross-linking from the 5′ anticodon base of tRNA to C-1400 or its equivalent has also been observed across species lines (Ehresmann & Ofengand, 1984; Ciesiolka et al., 1985). Despite the striking conservation of both sequence and ability to cross-link to tRNA, no functional role is known for this region. It is single stranded in all secondary structure models, and no ribosomal proteins have yet been shown to interact with it.

In order to understand the functional and structural role that is apparently played by C-1400 and its surrounding nucleotides, we have introduced single base changes in this region of 16S RNA using a system that will allow the eventual study of each of the partial reactions of protein synthesis in which the ribosome is engaged. Site-directed mutagenesis of 16S rRNA has been reported previously by Dahlberg and his colleagues (Stark et al., 1982, 1984; Zwieb & Dahlberg, 1984; Jemiolo et al., 1985; Zwieb et al., 1986; Steen et al., 1986), by Meier et al. (1986), and by Gregory and Zimmermann (1986), although so far all except four (Zwieb & Dahlberg, 1984; Jemiolo et al., 1985; Gregory & Zimmermann, 1986; Meier et al., 1986) have been deletions of various sizes. A difficulty with the approach used by these workers is that in vitro assays of ribosome function are limited by the presence of an unknown fraction of host cell ribosomes in the preparations from mutant plasmid infected cells.

To avoid this drawback, we sought a way to make 100% mutant ribosomes so that the classical quantitative assays of A and P site binding, translocation, and peptidyl transferase activity could be applied. This was accomplished by synthesis of mutant 16S RNA in vitro and reconstitution with added ribosomal protein to yield a particle possessing both 30S morphology and 30S function. The results also demonstrate unequivocally that none of the base modifications in 16S RNA are essential for assembly or for tRNA binding.

Experimental Procedures

Buffers. RA buffer is 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), pH 7.5, 100 mM NH_4Cl, 2 mM $Mg(OAc)_2$, and 1 mM DTT. RB buffer is RA buffer adjusted to 10 mM $Mg(OAc)_2$. RC buffer is 20 mM Hepes, pH 7.5, 60 mM NH_4Cl, 10 mM $Mg(OAc)_2$, and 6 mM mercaptoethanol. RD buffer is 20 mM Hepes, pH 7.5, 100 mM NH_4Cl, 20 mM $Mg(OAc)_2$, and 5 mM mercaptoethanol. RE buffer is RC buffer plus 400 mM NH_4Cl. Rec-20 buffer is 20 mM Hepes, 7.5, 400 mM NH_4Cl, 20 mM $Mg(OAc)_2$, and 4 mM mercaptoethanol. Rec-20M buffer is Rec-20 buffer adjusted to 500 mM NH_4Cl. TBE buffer is 50 mM tris(hydroxymethyl)aminomethane (Tris), 50 mM boric acid, 1 mM ethylenediaminetetraacetic acid (EDTA), and 7 M urea, pH 8.3.

Gene Constructs. E. coli GM119 (dam^-) was transformed with pKK3535 carrying the rrnB gene (Brosius et al., 1981). Cleavage of the isolated plasmid with *Bcl*I and *Bst*EII yielded the 1490-bp fragment corresponding to residues 15–1504 of 16S RNA, which was easily separated in 0.7% low melting point agarose. Synthetic oligomers were prepared on an Applied Biosystems 380B synthesizer, manually deprotected, and purified by polyacrylamide gel electrophoresis (PAGE). Pairwise annealing at 0.2 μM was done at 65 °C for 5 min in 25 mM Tris, pH 8.0, 150 mM NaCl, and 0.1 mM EDTA followed by cooling to 23 °C over a 30-min period. pUC19 (Norrander et al., 1983) was cleaved with *Kpn*I and *Xba*I and purified by 0.7% low melting point agarose gel electrophoresis. Four-way ligation of the pUC19-cleaved vector (0.05 pmol), two synthetic oligomers (0.1 pmol each), and the rrnB 1490-bp fragment (0.1 pmol) was done in 25 mM Tris, pH 7.8, 10 mM $MgCl_2$, 2 mM DTT, and 0.4 mM ATP with 3 units of T_4 DNA ligase at 12 °C for 16 h. After transformation, restriction analysis of the resulting plasmid DNA isolated from transformed single colonies showed that all five of the *Kpn*I, *Bcl*I, *Bst*EII, *Mst*II, and *Xba*I sites were present in 10 of 13 isolates tested. The DNA sequence of the synthetic fragments and all ligation junctions was confirmed by direct sequence analysis using primed double-stranded DNA dideoxy sequencing with AMV reverse transcriptase (Sanger et al., 1977). The resulting plasmid, pWK1, was digested with *Bsm*I and *Nco*I, purified by 0.7% low melting point agarose gel electrophoresis, and ligated to the annealed 44-mer–50-mer containing either A-T or G-C at position 1400 instead of C-G to generate the derived plasmids, pRD1 and pRD2, respectively (Figure 1A). DNA sequence analysis, as above, confirmed the sequences as shown.

RNA Preparations. For synthetic 16S RNA, plasmid DNAs from pWK1, pRD1, and pRD2 were linearized by cleavage with *Mst*II (0.5 unit/μg of DNA) at 37 °C for 7 h, the cut being confirmed by agarose gel electrophoresis. The linearized plasmid DNAs (2 μg/mL) were transcribed in a mixture containing 40 mM Tris, pH 8.0, 8 mM $Mg(OAc)_2$, 25 mM NaCl, 2 mM spermidine, 10 mM dithiothreitol (DTT), 400 units/mL RNasin (Promega Biotech), 0.5 mM each of ATP, CTP, UTP, and GTP, and 400 units/mL T_7 RNA polymerase at 38 °C for 5 h. DNA was removed by treatment with DNase followed by phenol/chloroform extraction (Maniatis et al., 1982). High molecular weight RNA was isolated by Sephacryl S-200 chromatography in 20 mM K_2HPO_4, pH 7.0, 100 mM NaCl, and 10 mM EDTA. Natural 16S and 23S RNA were isolated from 70S ribosomes by treatment with 1% sodium dodecyl sulfate (SDS) at 37 °C for 5 min in 20 mM Hepes, pH 7.5, 100 mM NaCl, and 2 mM EDTA followed by sucrose gradient centrifugation at 15 °C in the same buffer except 0.1% in SDS. RNA was recovered by precipitation with 67% ethanol and 0.2 M KOAc, pH 5.0, at −20 °C for 30 min. Both natural and synthetic RNAs were dialyzed vs. Rec-20 buffer and stored at −140 °C.

Sequencing Analysis. Primer-extension dideoxy sequencing (Sanger et al., 1977) used for the 5′-terminal and C-1400 internal regions was done by incubating 2.0 pmol of the appropriate primer with 2.5 pmol of natural 16S RNA or synthetic transcript in 8 μL of hybridization buffer (7.5 mM Tris·HCl, pH 7.0, 62.5 mM NaCl) for 1 min at 90 °C. After slow cooling to room temperature over a 10-min period, 2 μL of 30 mM $MgCl_2$–25 mM DTT was added, followed by the addition of 2.5 μL (25 μCi) of [α-^{32}P]dATP (400 Ci/mmol). Aliquots, 2.75 μL, of the resultant mixture were then combined with 2.5 μL of 250 μM dCTP, 250 μM dTTP, 250 μM dGTP, 50 mM NaCl, 34 mM Tris (pH 8.3), 6 mM $MgCl_2$, 5 mM DTT, and either 3.6 μM dideoxy-A, 200 μM dideoxy-T, 100

μM dideoxy-C, or 50 μM dideoxy-G. Primer extensions were carried out at 42 °C in the presence of 1 μL (1.25 units) of AMV reverse transcriptase. After 15 min, 1 μL of chase solution, 2 mM in dATP, dTTP, dCTP, and dGTP in the same buffer as the dideoxynucleotide mixtures, was added to each reaction mixture and incubation continued at 42 °C for another 15 min. Reactions were stopped by addition of 5 μL of stop solution (90% formamide, 20 mM EDTA, 0.3% xylene cyanol, and 0.3% bromophenol blue). Samples (3.0 μL) of each reaction mixture were denatured at 90 °C for 3 min, chilled to 0 °C, and immediately electrophoresed on a 10% polyacrylamide gel (0.4 mm thick) for ca. 2 h at 2000 V in TBE buffer. The gel was autoradiographed at −80 °C for 5–15 h without an intensifying screen. Enzymatic sequencing of [^{32}P]pCp-labeled oligonucleotides was performed following protocol E998 of P-L Biochemicals, an adaptation of the method of Donis-Keller (1980). Digestion with CL3 followed the same protocol. The ladder was generated by heating in 67% deionized formamide at 100 °C for 5 min. Electrophoresis was on 0.4-mm 10% polyacrylamide gels in TBE buffer.

Reconstitution. Synthetic or natural RNA was reconstituted in Rec-20M buffer at 2.8 A_{260} units/mL of RNA with 4 equiv of TP30, the complete mixture of 30S ribosomal proteins. Incubation was for 12 min sequentially at each of the following temperatures: 40, 43, 46, and 48 °C. The final incubation was at 50 °C for 20 min. At each temperature change, the sample was left in the bath as the temperature was raised over a 3-min interval and then incubated for 12 min at the new temperature. After quick cooling to 0 °C, the samples were analyzed by sucrose gradient centrifugation.

Electron Microscopy. Ribosomal subunits at an A_{260} of 0.05 in RB buffer were adsorbed to a thin (~30 Å) carbon support film floated off freshly cleaved mica. After approximately 5-min adsorption, the film with the attached ribosomal particles was transferred into a contrasting solution of 0.5% aqueous uranyl acetate and then picked up with a 400-mesh copper grid, blotted, and air-dried. The grids were examined in a JEM 1200EX electron microscope operated at 60 kV and a direct magnification of 50000×. About 300 particles from each specimen were used for a statistical evaluation of their morphology.

Materials. Plasmid pKK3535 (Brosius et al., 1981) was the gift of M. Santer, Haverford College, and pUC19 (Norrander et al., 1983) was purchased from Boehringer. *E. coli* GM119 (dam$^-$) was a gift of R. Crowl of Hoffmann-La Roche Inc. T_7 RNA polymerase and all restriction endonucleases were from New England Biolabs. T_4 RNA ligase and the RNA sequencing enzymes were from Pharmacia, except CL3 enzyme which was from BRL as was RNase-free bovine serum albumin (BSA). AMV reverse transcriptase and RNasin were from Promega Biotech. T_4 DNA ligase was from IBI. [α-^{32}P]dATP and [5′-^{32}P]pCp were from Amersham. Deoxyoligonucleotides were prepared on an Applied Biosystems 380B or 381A DNA synthesizer, deprotected, and purified by 15% polyacrylamide gel electrophoresis in TBE buffer. After gel elution, the samples were desalted by adsorption to a Waters Sep-Pak column. Elution was with 60% methanol–H_2O. [^{32}P]pCp-labeled 16S RNA was prepared by incubation of 20 pmol of RNA in 50 μL of 50 mM Tris·HCl, pH 8.0, 20 mM $Mg(OAc)_2$, 3.3 mM DTT, 25 μM ATP, 20 μg/mL BSA, and 10% v/v Me_2SO for 24 h at 4 °C with 40 pmol of [^{32}P]pCp (3 μCi/pmol) and 10 units of T_4 RNA ligase (Bruce & Uhlenbeck, 1978). The mixture was purified by electrophoresis on a 3% polyacrylamide gel run in TBE buffer. The 16S rRNA was eluted, precipitated 3 times from 0.2 M KOAc, pH 8.0, and 75% ethanol at −80 °C for 10 min, and dissolved in H_2O. [^{32}P]pCp-labeled 30S subunits (Kelly & Cox, 1981) were prepared as for 16S RNA except that 10 mM Mg^{2+}, 0.5 mM ATP, 40 pmol of 30S subunits, and 18 units of ligase were incubated in 60 μL for 2–4 h without BSA. The labeled 30S subunits were purified by sucrose gradient centrifugation in RA buffer. The pooled fractions were stored at −140 °C and used without removal of the sucrose. *E. coli* 70S ribosomes, poly(U_2,G) and Ac[^{3}H]Val-tRNA were obtained as previously described (Ofengand et al., 1979). The 50S and 30S subunits were prepared from 70S by sucrose gradient centrifugation in RA buffer or in RE buffer (Expert-Benzancon et al., 1974). The subunits were recovered by poly(ethylene glycol) precipitation (Expert-Benzancon et al., 1974), suspended in either Rec-20 buffer or RC buffer, and stored in aliquots at −140 °C. The 30S ribosomes were activated (Zamir et al., 1974) before use by heating for 30 min at 40 °C in RD buffer. Total 30S protein (TP30) was prepared from 30S subunits as described by Nierhaus and Dohme (1979) for 50S protein except that solution of the acetone precipitate was in Rec-20 buffer plus 6 M urea, addition of unbuffered Tris was omitted, the dialysis buffers were Rec-20 ± 6 M urea, and acetic acid addition was omitted. The preparation was stored in aliquots at −140 °C. One A_{230} unit of TP30 in Rec-20 was taken equal to 8 equiv units, 1 equiv unit being the amount of protein associated with 1 A_{260} unit of 16S RNA in a 30S ribosome (K. Nierhaus, personal communication).

Other Methods. Analysis of the ^{32}P content of gel slices (for Figure 3) was by Cerenkov counting with care to maintain the gel pieces in the same orientation in the counting vials. Phenol extraction of RNA from reconstituted ribosomes was done by vigorous shaking for 5 min at room temperature of particles in RD buffer containing 1% SDS with an equal volume of redistilled phenol (BRL) previously equilibrated with RD buffer adjusted to 10 mM mercaptoethanol. The phenol layer was washed with RD buffer, the aqueous phases were extracted 4 times with ether, and the RNA was precipitated with 2 volumes of ethanol at −20 °C for 30 min. Reprecipitation with ethanol from RD buffer was repeated until a stable A_{260} value was obtained. A total of 64% was recovered by this procedure. The 3′-terminal nucleotide composition was determined by digestion of 500–1500 cpm of [^{32}P]pCp-labeled 3′-terminal T_1 oligonucleotide eluted from the gel of Figure 2B together with 5–40 μg of carrier tRNA and 3 units of RNase T_2 in 6 μL of 15 mM sodium citrate, pH 5.0, and 0.9 mM EDTA for 30 min at 55 °C. After addition of a 5-μL solution of 3′-nucleotide markers, 5 μL of the mixture was applied to each of two TLC cellulose plates (Eastman) and chromatographed in solvents I and II. Solvent I, concentrated HCl–2-propanol–H_2O (17.6:68.0:14.4), separates Up and Cp from Ap + Gp. Solvent II, 0.1 M Na-PO_4, pH 6.8–solid $(NH_4)_2SO_4$–1-propanol (100:60:2, v/w/v), separates Ap and Gp from Up + Cp. The combination of both solvents allows analysis of all four nucleotides. After radioautography to confirm the separation, zones were transferred into scintillation vials and counted. The distribution is expressed as a percent of the total recovered cpm. Elution of oligonucleotides from gels was done by shaking the gel pieces with 0.5 M $NH_4(OAc)_2$, 10 mM $Mg(OAc)_2$, and 1 mM EDTA, pH 8.0, at 37 °C for 15–18 h. For elution of 16S RNA, 0.1% SDS was added to the elution buffer, and 15 h of shaking at 37 °C was used. Denaturing agarose gels (Lehrach et al., 1977) contained 6% formaldehyde, 20 mM 3-(*N*-morpholino)propanesulfonic acid, 5 mM NaOAc, 5 mM EDTA, pH 7.0, and 2% agarose. The sample, in the above running buffer plus 53% formamide,

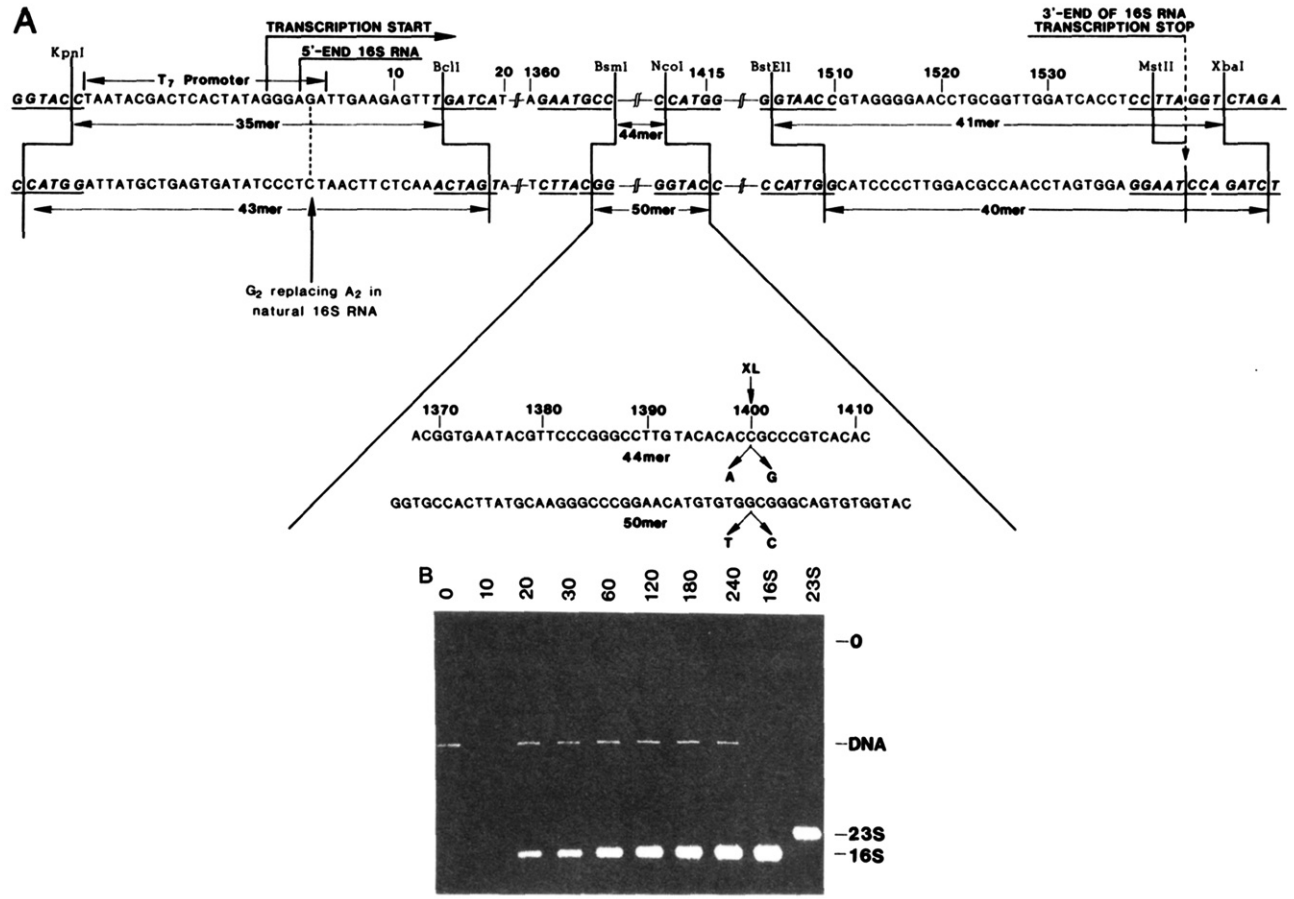

FIGURE 1: (Panel A) Structure of the reconstructed 16S RNA gene showing the synthetic ends, restriction sites used, ligation junctions, and sites of mutation. Recognition sites for the restriction endonucleases are shown in underlined italics, with the sites of cleavage as indicated. The six synthetic oligomers, denoted 35-mer to 50-mer, were placed as indicated. The 5′ end of the RNA transcript and of the natural 16S RNA differ by three G residues. In addition, A-2 of the natural RNA was replaced by G-2 in the synthetic version. The 3′ ends of the two RNAs are coterminal. The T_7 promoter indicated corresponds in sequence to the class III promoter series (Dunn & Studier, 1983). The site of cross-linking of the 5′ anticodon base of P site bound tRNA (Prince et al., 1982; Ehresmann & Ofengand, 1984) is indicated as well as the two base changes at that position. The numbers shown correspond to the positions of natural 16S RNA. (Panel B) Kinetics of transcription of plasmid DNA. Plasmid DNA from pWK1 was transcribed as described under Experimental Procedures. The reaction was stopped at the indicated times by quick freezing at −80 °C. Samples were analyzed by electrophoresis on 0.7% agarose run in 89 mM Tris–borate, pH 8.0, 2 mM EDTA, and 0.5 μg/mL ethidium bromide. Times of reaction are in minutes. For 0 time, T_7 polymerase was omitted. 16S and 23S RNA isolated from ribosomes were used as markers. Visualization was under UV light.

was heated at 65 °C for 5 min before being loaded onto the gel.

Results

Reconstruction of the 16S RNA Gene. The reconstruction scheme for the 16S RNA gene is illustrated in Figure 1A. Both the 3′- and 5′-terminal sequences of the 16S RNA gene were reconstructed by chemical synthesis. The 5′-end duplex was programmed to contain the *Kpn*I cloning site at its 5′ end followed by the sequence of the strong class III T_7 polymerase promoter Φ10 (Dunn & Studier, 1983). Transcription from the T_7 promoter starts with the 3′-terminal six nucleotides of the promoter sequence, as indicated. This results in a transcript that is three bases longer than the natural 16S RNA and with a single base pair change at the second base pair of the 16S RNA gene. The 3′-end duplex, created by chemical synthesis, contains the unique *Mst*II site, which is followed directly by the *Xba*I cloning site. Transcription of the *Mst*II-cleaved plasmid was designed to give an RNA molecule whose 3′ end would be identical with that of natural 16S RNA.

Plasmid pWK1 was prepared by ligation of the 1490-bp *Bcl*I/*Bst*EII fragment of the 16S RNA gene cut out from the plasmid pKK3535 (Brosius et al., 1981) with two synthetic duplexes (35–43-mer, 41–50-mer) and *Kpn*I/*Xba*I-digested pUC19 (Norrander et al., 1983). An interesting feature of this ligation reaction was the use of nonphosphorylated synthetic duplexes so that only a single phosphodiester bond per duplex could be formed at each ligation junction. Despite this, the structure of the overhanging ends together with little or no cross-homology between their sequences resulted in a high yield (77%) of the expected plasmid among the analyzed transformants.

The sequence of the reconstructed parts of the plasmid pWK1 was confirmed from −46 to +29 (numbering system as in Figure 1 for the natural rRNA) and from +1460 to +1552 by DNA sequence analysis (Sanger et al., 1977) (data not shown). Base changes at position 1400 from C to A or G were effected on pWK1 by insertion of the desired double-stranded oligonucleotides. Double digestion with *Bsm*I and *Nco*I was used to remove the desired segment, which was then replaced by the indicated synthetic oligonucleotides. Restriction analysis showed that over 90% of the transformants possessed both *Bsm*I and *Nco*I sites, and sequence analysis from base pairs 1330 to 1424 showed the expected base changes (data not shown).

RNA Synthesis and Characterization. The kinetics of transcription of 16S RNA from the T_7 promoter are shown in Figure 1B. Clearly, large quantities of full-length transcripts can be made in relatively short time periods. On a preparative scale, the yield after isolation has varied from 400 to 700

Table I: tRNA Binding Capacity of Reconstituted Ribosomes[a]

	A	B	C
synthetic RNA	0.49 (0.49)	0.24 (0.25)	0.05 (0.05)
natural RNA	0.57 (0.56)	0.58 (0.59)	
synthetic/natural	0.86 (0.88)	0.42 (0.42)	

[a] Fractions from the ribosome gradients of Figure 4 were pooled as indicated on the figure and recovered by precipitation with 1 volume of EtOH after raising the Mg^{2+} to 20 mM. Recoveries ranged from 70 to 90%. After suspension in RD buffer, the particles were activated by heating at 40 °C for 30 min. Nonenzymatic tRNA binding was tested by incubating 50 mM Hepes, pH 7.5, 20 mM Mg^{2+}, 100 mM NH_4Cl, 20 μg/mL poly(U_2,G), 106 nM 50S, and 66–90 nM 30S equivalents at 37 °C for 10 min, followed by addition of 90 nM Ac[^{3}H]Val-tRNA and a further 20-min incubation. tRNA binding to the reconstituted 70S ribosomes was measured by adsorption to cellulose nitrate membranes (Ofengand et al., 1979). Under these conditions, binding was proportional to the amount of ribosomes added up to at least 90 nM. A blank in which 30S particles were omitted was subtracted from all values, which were then expressed as picomoles of tRNA bound per picomole of 30S equivalents added relative to control untreated activated 30S particles as 1.00. 30S subunits isolated from the gradient shown in Figure 4C were 90–100% as active as the control 30S particles. Values in parentheses were obtained in the presence of 2 mM spermidine. Standard 30S binding capacity was 0.53 and 0.57 pmol/pmol in the absence and presence of spermidine, respectively.

mol/mol of DNA template added. It is now routine to obtain up to 140 A_{260} units of RNA from 40 μg of pWK1 plasmid in a 20-mL reaction.

RNA sequence analysis showed that initiation began as indicated in Figure 1A. As shown in Figure 2A, the three additional nucleotides were clearly evident, as was the substitution of G-2 for A-2. Since the sequence as far as residue 28 was correct, the sequence of the chemically synthesized oligomers must also have been correct and ligated correctly to the 1490-bp fragment. The nature of the 5′-terminal base could not be determined from this analysis, but since the correct base was identified by sequencing of the gene (data not shown), it is reasonable to assume that the synthetic RNA began with G and the natural RNA with A. Figure 2B shows that the substitutions made in the DNA at C-1400 were correctly transcribed into mutant RNA and that the sequence spanning the cassette insert and ligation junctions, 1360–1418, was also correct at the RNA level.

The sequence at the 3′-terminal region was verified in the following way. First, dideoxy sequencing from the 3′ end of the RNA (Figure 3A) established the correctness of the sequence from well before residue 1492 to residue 1531. A different RNA, transcribed from pWK1 linearized at the *Xba*I site, was used in this experiment in order to extend the sequencing closer to the 3′ end. The remaining part of the sequence of the RNA transcribed from the *Mst*II-linearized plasmid was studied by [^{32}P]pCp labeling of the purified RNA and complete digestion with RNase T_1 to yield the purified 3′-terminal oligonucleotide, 1530–1542. Purification of this oligomer from synthetic and control natural RNA prior to sequencing revealed the existence of additional bands 13 and 14 nucleotides long in the synthetic RNA (Figure 3B). This was not a consequence of insufficient digestion with RNase T_1 since a 10-fold increase in the amount of enzyme did not affect the distribution of ^{32}P. Nineteen percent of the molecules possessed one additional residue (band B) and 3% two additional ones (band C). The 5% band B found in the natural RNA may be due to some contaminant carried along during the isolation of the RNA from 30S ribosomes. It would not be expected to be in the in vitro synthesized RNA. Analysis of the nature of the 3′ terminus of bands A and B from the synthetic RNA and band A from the natural RNA was accomplished by complete digestion with RNase T_2 and analysis of the mononucleotides. Band A from both natural and synthetic RNA contained only A, as expected, but band B from the synthetic RNA contained 65% A, 5% G, 17% C, and 13% U (data not shown). Enzymatic sequencing of the [^{32}P]-pCp-labeled T_1 oligonucleotide from the *Mst*II-cleaved synthetic RNA verified the remainder of the sequence, from 1531 to 1541 (Figure 3C and data not shown). Parallel analysis of synthetic band B verified that it was one residue longer and that the residue must have been added to the 3′ end since the characteristic CCUCC sequence was now shifted upward by one residue.

The failure of a 10-fold excess of RNase T_1 to change the percent distribution, the presence of 3′-nucleotides other than A in 33% of band B, and, in particular, the sequencing results of Figure 3C leave no doubt that the extra nucleotide was added to the 3′ end rather than resulting from failure to cleave after G-1530 in addition to G-1529. Analysis of synthetic RNA isolated from reconstituted ribosomes (fraction A of Figure 4B) by phenol extraction of the RNA, [^{32}P]pCp labeling, and separation of the 3′-terminal T_1 oligonucleotide showed a similar distribution of normal and $N + 1$ sized 3′-terminal fragments (Figure 3B). Thus, this extra nucleotide has no influence on reconstitution ability. It is also unlikely to have any effect on P site binding activity, as ribosomes reconstituted from natural and synthetic RNA had essentially the same activity (see Tables I and II).

Even though the linearized plasmid DNA was used without purification from the *Mst*II cleavage mixture, full-length intact RNA was obtained. Agarose gel analysis as in Figure 1B and denaturing agarose gels (Lehrach, 1977) all showed only one major band of the expected size (data not shown).

30S Subunits from Synthetic RNA. After preliminary experiments in which time, temperature, buffer salts, and protein/RNA ratios were varied, a set of conditions was found that gave good reconstitution of both natural and synthetic RNA. The main differences from the conditions of Held et al. (1973) were (a) the use of a higher final temperature and an annealing program, (b) the use of a higher salt concentration, and (c) an increased protein/RNA ratio. As shown in Figure 4, reconstitution mixtures prepared in this way

Table II: Codon Dependence of Binding and P-Site Cross-Linking of tRNA to Reconstituted and Control 30S Subunits[a]

	pmol of tRNA/pmol of 30S						
	binding			cross-linking			%
ribosome	plus poly(U_2,G)	minus poly(U_2,G)	Δ	plus poly(U_2,G)	minus poly(U_2,G)	Δ	cross-linking
30S control	0.59	0.05	0.54	0.27	0.01	0.26	48
natural RNA	0.36	0.03	0.33	0.17	0.01	0.16	49
synthetic RNA	0.35	0.03	0.32	0.14	0.01	0.13	41

[a] Reconstituted and control 30S subunits (fraction A of Figure 4) were obtained and assayed for tRNA binding as in Table I but at 16 mM Mg^{2+}. P site cross-linking of AcVal-tRNA to ribosomes was done by near-UV irradiation as previously described, without the Mylar filter (Ofengand et al., 1979) for 30 and 45 min at 0 °C. The two time points, averaged for the table, differed by <5%. All values are expressed as picomoles of tRNA bound per picomole of 30S or equivalent added. Percent cross-linking is 100 × Δ cross-linking/Δ binding.

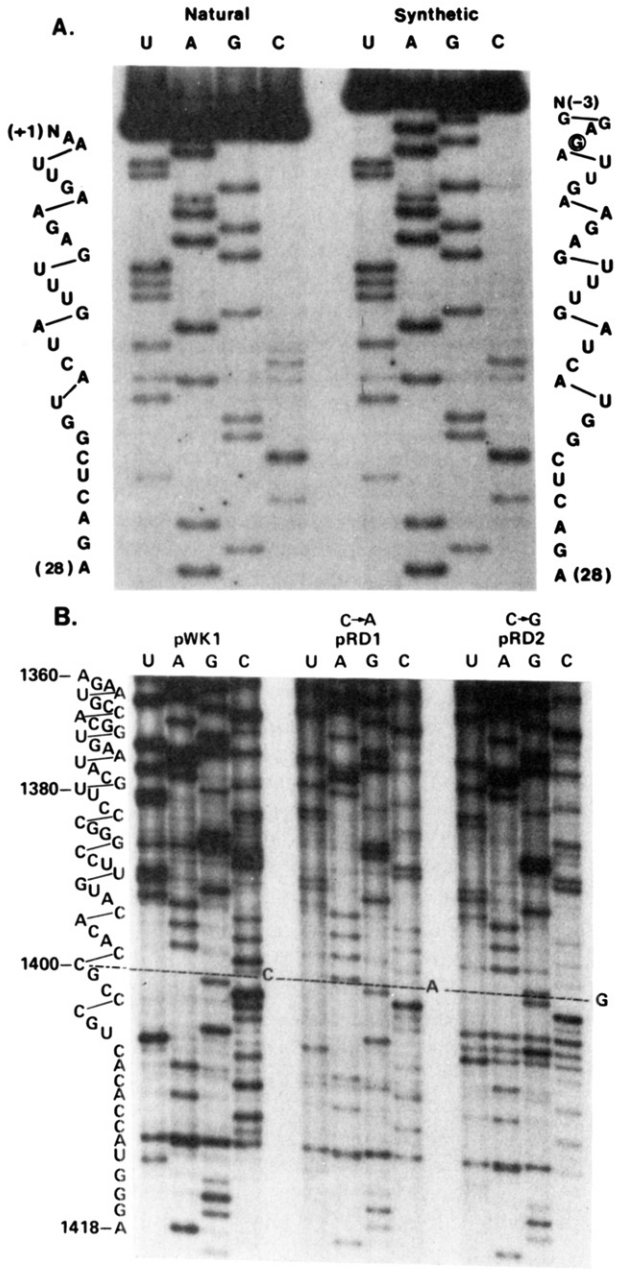

FIGURE 2: Sequence analysis of in vitro RNA transcripts from plasmids pWK1, pRD1, and pRD2. (Panel A) Sequencing of the region from the *BclI* ligation junction to the 5′ end. Primer extension from residue 47 to the 5′ end was done with a synthetic oligomer complementary to residues 48–65 and AMV reverse transcriptase. The lanes are labeled according to the RNA sequence. Natural RNA is RNA isolated from 70S ribosomes. Synthetic RNA is RNA transcribed from pWK1. The sequence is as shown. Numbers correspond to the mature 16S RNA sequence. (Panel B) Sequence analysis across the *BsmI*–*NcoI* ligation junctions. Primer extension was from residue 1430 toward the 5′ end with a synthetic oligomer complementary to residues 1431–1453. The base substitutions at position 1400 are indicated on the figure.

yielded particles whose sedimentation behavior and distribution were almost identical with those of ^{32}P-labeled 30S added as an internal control. Note, however, that with the synthetic RNA (panel B) there was a small amount of material sedimenting faster than the ^{32}P-labeled 30S control as well as material sedimenting slower. When the complete incubation and isolation procedure was applied to control 30S subunits (panel C), no differences between the absorbance profile and the ^{32}P distribution was found.

Electron microscopic examination of fraction A from panels A and B of Figure 4 after ethanol precipitation and resuspension showed that particles with *E. coli* 30S morphology had been produced (Figure 5a,b). There was no difference in the appearance of these particles at the presently achievable resolution level for stained biological specimens. Both types of reconstituted particles revealed the well-established characteristic structural features of the 30S *E. coli* subunit—the head, the cleft, and the large side protrusion, which is mainly responsible for the subunit asymmetry. However, the amount of such 30S particles in the reconstituted preparation was less, about 43% (a) and 47% (b), respectively, compared to 80–90% for normal isolated 30S subunits. In addition, about 27% of the particles from both preparations possessed the structural features of 30S but lacked certain refinements of morphology. The remaining 25–30% of the particles could not be classified. Electron microscopy of 30S particles performed both before and after ethanol precipitation did not reveal any resolvable structural differences. This is in agreement with the fact that 30S subunits isolated from gradients by ethanol precipitation retained full tRNA binding activity (data not shown).

tRNA Binding Capacity of Synthetic 30S Subunits. The tRNA binding capacity of the particles reconstituted from synthetic RNA was only 49% that of an untreated 30S standard subunit but was 88% as active as a particle reconstituted from natural RNA (Table I, fraction A). Although Held et al., (1973) obtained fully active 30S subunits by reconstitution, we have not been able to duplicate this result with our assay. As shown in Table I, addition of spermidine did not improve the relative binding capacity. Fraction B of the natural RNA reconstitution was as active as fraction A, but fraction B from the synthetic RNA gradient was only half as active as fraction A. Moreover, fraction C, which accounted for 20% of the absorbance of fractions A–C, had virtually no binding activity. This region of the gradient probably contains 16S RNA assembled with an incomplete assortment of proteins or RNA that has failed to form a complex in the correct conformation.

The Mg^{2+} and codon dependence of tRNA binding to the reconstituted natural and synthetic 30S is shown in Figure 6. There is a similar Mg^{2+} concentration dependence of tRNA binding for both types of reconstituted particles and for the standard 30S, and all three show an excellent codon dependence as well. These parameters are good evidence for tRNA binding to a normal ribosomal P site.

Additional evidence for P site binding of tRNA is given in Table II. In this experiment, the ability to form the UV light induced covalent cross-link between the 5′ anticodon base of tRNA, residue 34, and C-1400 of 16S RNA was examined. Previous work has shown that this reaction is both P site specific (Ofengand et al., 1979) and codon dependent (Ofengand & Liou, 1981). Moreover, relatively subtle alterations in the source of ribosomes, tRNA, or polynucleotide have dramatic effects on the cross-linking yield (Ofengand et al., 1979, 1982; Ofengand & Liou, 1981; Ciesiolka et al., 1985), which appears to be a measure of rather small changes in the geometry of the tRNA anticodon–mRNA–16S RNA complex in the ribosome. The experiment of Table II shows that cross-linking to particles reconstituted from natural 16S RNA was equal to that with standard 30S subunits, but the particles reconstituted from synthetic RNA were only 85% as active. This difference, although small, was reproducible. It appears that the synthetic 30S has some slight conformational differences from a normal 30S. The most likely source of the perturbation is the lack of modification of C-1402, only two bases away from the cross-linking residue C-1400, which is normally an m^4Cm residue. However, one cannot at this

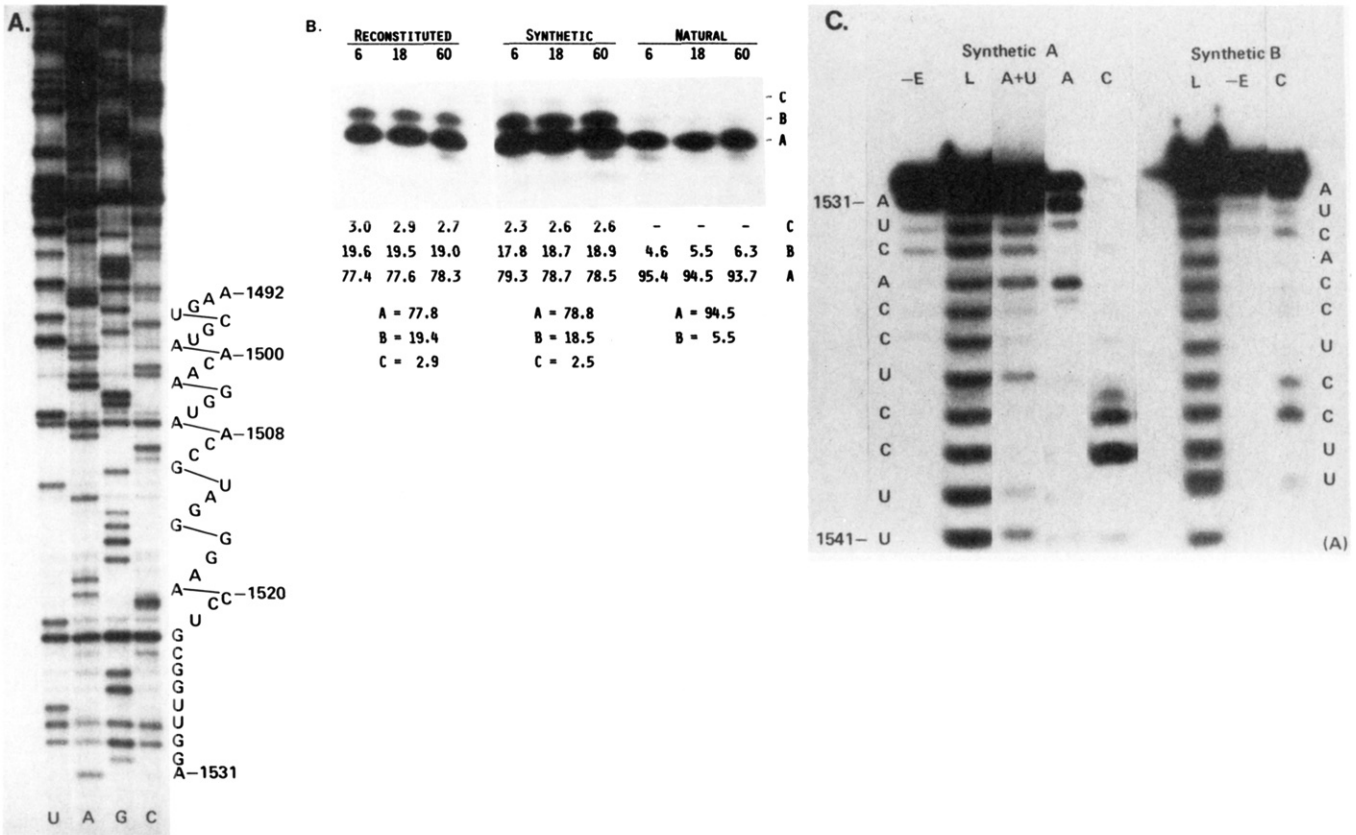

FIGURE 3: Sequencing analysis of the 3′-terminal region of the in vitro transcript from pWK1. (Panel A) Primer extension from residue 1535 was done with a synthetic oligomer complementary to residues 1536–1549 of an RNA made by using as the template pWK1 linearized at the *Xba*I site. The lanes are labeled according to the RNA sequence. Numbers correspond to the mature 16S RNA sequence. (Panel B) Size heterogeneity of the 3′-terminal T_1 oligonucleotide. [^{32}P]pCp-labeled 16S RNA (0.3–0.5 pmol) was digested with 6, 18, and 60 Sankyo units of RNase T_1/pmol of RNA in 50 mM Hepes, pH 7.5, at 55 °C for 30 min and electrophoresed on 20% polyacrylamide gels in TBE buffer. Ladder lanes (panel C) verified that bands A and B were 12 and 13 residues long, respectively. The percentage distribution of ^{32}P in the bands and the average value is shown at the bottom of the figure. Natural, RNA extracted from native ribosomes; synthetic, RNA transcribed from *Mst*II-linearized pWK1; reconstituted, synthetic RNA extracted from ribosomes (Fraction A of Figure 4B) with phenol. (Panel C) Enzymatic sequencing of oligomers A and B from panel B. Synthetic oligomers A and B were eluted from the gels of panel B and sequenced enzymatically. L, ladder; A+U, RNase Phy M; A, RNase U_2; C, RNase CL3; –E, no enzyme.

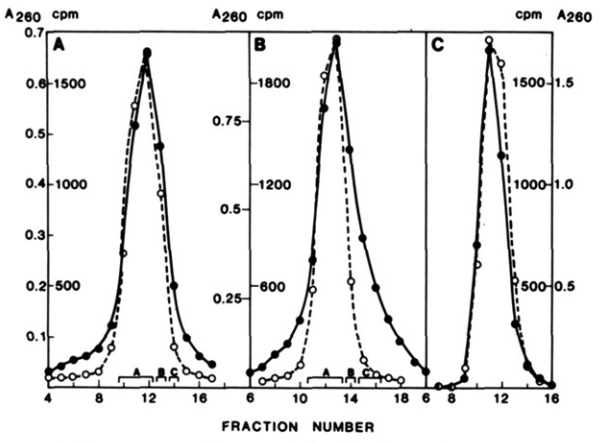

FIGURE 4: Sucrose gradient analysis of 30S particles reconstituted from TP30 and natural or synthetic 16S RNA. Reconstitution of 30S particles from natural or synthetic 16S RNA and total *E. coli* 30S proteins was done as described under Experimental Procedures. A small quantity (<0.6% of the reconstitution mixtures) of [^{32}P]-pCp-labeled 30S subunits was added to the reconstitution mixture, and the samples were centrifuged through 10–30% sucrose gradients in RB buffer. The direction of sedimentation is to the left. Panels A and B, 3.6-mL samples, SW27 rotor. Panel C, 1.2-mL sample, SW40 rotor. Filled circles, absorbance at 260 nm; open circles, ^{32}P-labeled 30S marker. Panel A, natural RNA; panel B, synthetic RNA; panel C, control activated 30S subunits incubated in Rec-20M as for reconstitution but without addition of TP30. Recovery of applied A_{260} units was 31, 49, and 68% for panels A–C, respectively. Fractions were pooled as indicated for further analysis.

Table III: Nucleoside Analysis of 16S RNA Extracted from Reconstituted 30S Subunits[a]

nucleoside	mol of nucleoside/mol of 16S ribosomal RNA				
	literature[b]	natural	natural reconstituted	synthetic	synthetic reconstituted
A	387	389	386	384	384
U	313	312	311	312	311
C	349	356	357	357	357
G	483	473	476	477	478
m^2G	3	3.1	3.1	<0.1	0.1
m^7G	1	0.8	0.9	<0.1	<0.1
$m^6{}_2A$	2	1.7	1.7	<0.1	<0.1
m^5C	2	2.2	2.0	<0.1	<0.1
m^4Cm	1	*e*	*e*	*e*	*e*
m^3U	1[c]	0.7	0.9	<0.1	<0.1
ψ	0 (2)[d]	2.0	1.3	<0.1	0.2

[a] Natural and synthetic RNA was obtained as described under Experimental Procedures. Natural and synthetic reconstituted RNA was obtained by phenol extraction of the reconstituted ribosomes (fraction A) of Figure 4A,B. Analysis was by HPLC after complete digestion to nucleosides (Gehrke et al., 1982; Gehrke & Kuo, 1987). [b] Noller (1984). [c] Hsuchen and Dubin (1980). [d] Dubin and Günalp (1967). [e] Not analyzable by this procedure.

juncture rule out conformational changes induced by long-range effects of the lack of base modifications elsewhere. The excellent codon dependence of both binding and cross-linking seen in Table II is typical for 70S ribosomes, and the cross-linking yield of about 50% is also characteristic for this system

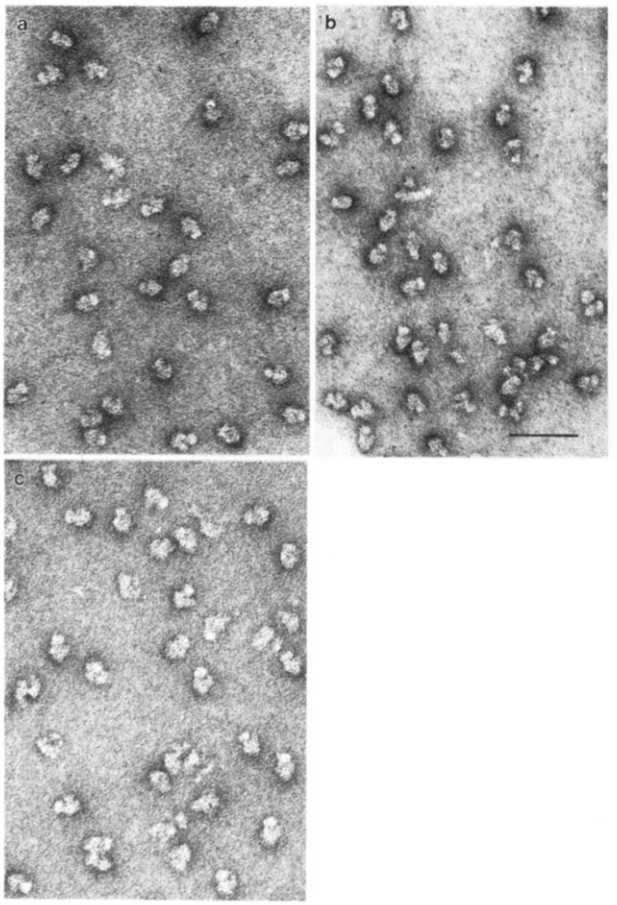

FIGURE 5: Electron micrographs of 30S ribosomal subunits obtained by reconstitution from total protein and 16S RNA: (a) extracted from 30S subunits, (b) synthesized in vitro from plasmid pWK1, and (c) synthesized from plasmid pRD1. Stained with 0.5% aqueous uranyl acetate. Bar, 50 nm.

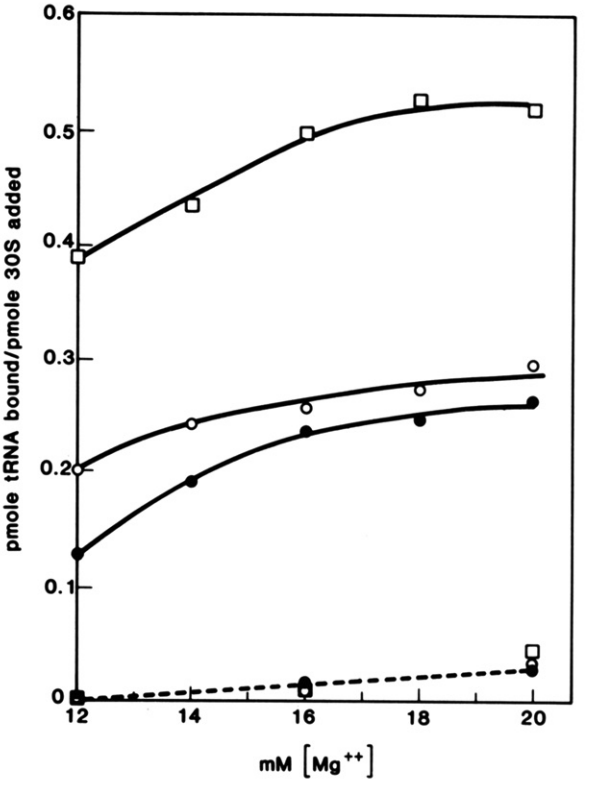

FIGURE 6: mRNA and Mg^{2+} dependence of tRNA binding to reconstituted ribosomes. The ribosomes of fraction A from the gradients of panels A and B of Figure 4 were recovered, activated, and assayed as in the legend to Table I. All values are expressed as picomoles of tRNA bound per picomole of 30S equivalent added. The ratio of natural and synthetic reconstituted 30S to control 30S was, after correction for the codon-independent binding, 0.54 and 0.47, respectively. Open squares, untreated control 30S particles; open circles, reconstituted with natural 16S RNA; filled circles, reconstituted with synthetic 16S RNA; solid line, plus poly(U_2,G); dashed line, minus poly(U_2,G).

(Ofengand et al., 1982; Ciesiolka et al., 1985).

In order to be sure that no base modification occurred during reconstitution as a result of contaminants present in the TP30 preparation, 16S RNA was extracted from the reconstituted particles of Figure 4, fraction A, and analyzed, together with some control RNAs, for normal and modified base content (Table III). Quantitation of the normal bases showed that within experimental error both natural and synthetic RNA had the same base composition, which was also in good agreement with literature values. Within the limits of this type of analysis, the results confirm that synthetic RNA is an authentic copy of 16S RNA. Five of the six known modified bases were found in the expected amounts in natural RNA, whether extracted from 30S subunits or from reconstituted particles. m^4Cm was not analyzable in this system. There were undetectable amounts of any of the modified bases in the synthetic RNA, as expected, and only small amounts of m^2G in the synthetic RNA from reconstituted ribosomes.

The surprising feature of this analysis was the presence of pseudouridine in the natural 16S RNA, at 2 mol/mol of RNA. Pseudouridine has not previously been considered to exist in *E. coli* 16S RNA (Noller, 1984) although it had been reported at a stoichiometry of 1.9 mol/mol previously (Dubin & Günalp, 1967). Pseudouridine was absent from the synthetic RNA, but a low level (10–15% of the natural RNA) was found after reconstitution. Since both natural and synthetic reconstituted ribosomes showed the same tRNA binding activity, it is not possible for this low level of pseudouridine to be involved in P site binding. Even on a molar basis, there would be insufficient ribosomes containing even one residue to account for the amount of tRNA bound.

Synthetic 30S Particles Mutant at C-1400. The purpose behind construction of plasmid pWK1 and the development of the transcription and reconstitution system described above was to allow the in vitro testing of the functional effect of single base changes in ribosomal RNA. So far, two such changes have been made. As indicated in Figure 1, the cross-linking base C-1400 was changed to A-1400 in the plasmid pRD1 and to G-1400 in the plasmid pRD2. Neither purine should be capable of the cross-linking reaction, which involves cyclobutane dimer formation (Ofengand & Liou, 1980). U-1400, as another pyrimidine, was deemed of lesser interest. Sequencing analysis by primed reverse transcription confirmed the sequence shown (Figure 2B). We did not anticipate that a single base change at position 1400 would influence assembly as no ribosomal proteins are known to interact with this region. The expectation proved correct for the A-1400 mutant (Figure 7A). The G-1400 mutant, however, assembled poorly (Figure 7B) even when 3 mM spermidine was added to the reconstitution mixture. Kakegawa et al. (1986) have observed a modest increase in functional activity of ribosomes reconstituted with spermidine. However, in our hands, essentially identical gradients were obtained with or without spermidine (data not shown). The only effect of spermidine appeared to be an improvement in the recovery of ribosomes from the gradients.

Although all of the G-1400 RNA formed particles, the position of the main fraction was indicative of a particle lacking

Table IV: Codon Dependence of Binding and Cross-Linking of tRNA to Reconstituted Mutant 30S Subunits[a]

	pmol of tRNA/pmol of 30S						
	binding			cross-linking			%
experiment	plus poly(U_2,G)	minus poly(U_2,G)	Δ	plus poly(U_2,G)	minus poly(U_2,G)	Δ	cross-linking
30S control	0.76	0.11	0.65	0.32	0.01	0.31	47
C-1400 → A-1400							
pool A	0.40	0.06	0.34	0.05	0.03	0.02	6
pool B	0.09	0.03	0.06				
C-1400 → G-1400							
pool A	0.19	0.07	0.12	0.03	0.01	0.02	(17)
pool B	0.05	0.03	0.02				

[a] Reconstituted mutant ribosomes, pooled as indicated in Figure 7, were recovered and assayed for binding and cross-linking as in Table II, except that the Mg^{2+} concentration was 18 mM. The 30S control subunits were isolated from the gradient of Figure 4C. The precentage in parentheses is subject to considerable experimental error due to the low values involved.

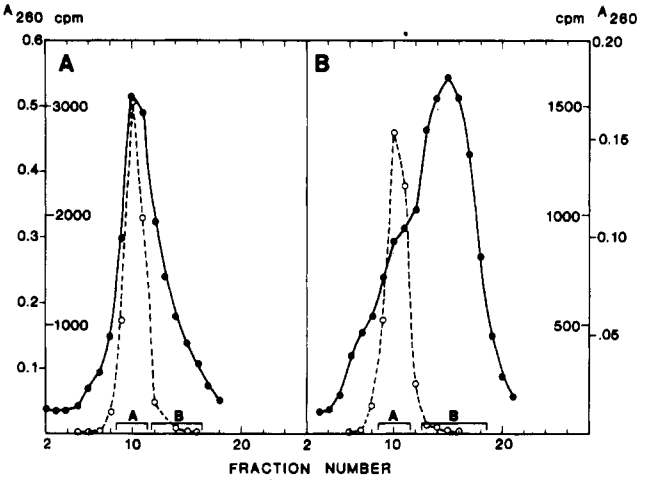

FIGURE 7: Reconstitution of mutant synthetic 16S RNA with total ribosomal proteins. Synthetic mutant RNA was obtained, reconstituted with TP30, and analyzed as in the legend to Figure 4, except that the reconstitution mixture contained 3 mM spermidine and 16 mM $Mg(OAc)_2$ (Kakegawa et al., 1986), 3.6-mL sample and SW27 rotor. Fractions were pooled as indicated for further analysis. Panel A, C-1400 → A-1400; panel B, C-1400 → G-1400. Filled circles, absorbance at 260 nm; open circles, ^{32}P-labeled 30S marker.

some ribosomal proteins. A small part, however, appeared to sediment with marker ^{32}P-labeled 30S. These two regions were fractionated as indicated on the figure. The A-1400 complex was also fractionated into two pools, as indicated. Fraction A of the A-1400 complex was examined in the electron microscope. The morphology of the 30S subunits obtained from this mutant 16S RNA was similar to that of reconstituted natural or synthetic 30S subunits (Figure 5c). The fraction (42%) of particles showing excellent morphological characteristics was about the same as in Figure 5a,b, the only difference being a small decrease, to 22%, of 30S particles lacking certain refinements of morphology and a concomitant increase, to 36%, of unclassifiable particles.

The mutant reconstituted ribosomes were then assayed for P site binding and cross-linking activity (Table IV). Pool A from the A-1400 mutant was 93% as active as the nonmutated ribosomes, when the values from Tables I and II were compared to those of Table IV. Pool A from the G-1400 mutant was one-third as active, and both pool B fractions were considerably less active. Clearly, the particles sedimenting at less than 30S lacked functional activity. Since all of the particles must be mutant, being derived by in vitro transcription from a mutant plasmid, it follows from the activity of the A-1400 pool A fraction that C-1400 is not essential for tRNA binding at the P site. The lower activity for pool A of the G-1400 mutant by comparison is most likely a reflection of its impure state.

A surprising result was the ability of the A-1400 mutant to cross-link at all, since, as noted above, A and G should not be capable of the same photoreaction as C (or U). The apparent cross-linking obtained with the G-1400 mutant probably only reflects the uncertainties inherent in the very low experimental values obtained. The most reasonable explanation for the low-level cross-linking of A-1400 is that substitution of C-1400 by A forces the cmo^5U-34 of the tRNA into closer contact with C-1399, which then cross-links when irradiated. Normally the cross-linking reaction is completely specific for C-1400 (Ehresmann & Ofengand, 1984). This hypothesis is being tested by direct determination of the site of cross-linking in this mutant.

Discussion

The stimulus for this work came from the recent report by Lowary et al. (1986), that full-length tRNA could be synthesized in vitro by T7 polymerase and that the synthetic product lacking all modified nucleotides could be aminoacylated. As we have demonstrated here, a similar situation exists for 16S rRNA, an RNA some 20 times larger. While Lowary et al. (1986) were able to make 320 mol of tRNA/mol of template, we have obtained more than twice that value for a 20-fold longer RNA. Thus, it appears now possible to make virtually any desired amount of rRNA, mutant at almost any desired position, not only for reconstitution and function experiments but for physical studies as well.

The RNA so produced appears to initiate and terminate as expected. This was directly verified by RNA sequencing of both terminal regions. The only unusual aspect was apparent stuttering at the 3′ end, resulting in the addition of an extra nucleotide in about one-fifth of the molecules. The additional nucleotide was not added randomly, there being a preference for an additional A residue, but U, C, and G were well represented also. This phenomenon of addition of an extra nucleotide during run-off transcription by T_7 polymerase has also been observed by O. Uhlenbeck (personal communication). It has also been observed with the analogous SP6 polymerase (Dreher et al., 1984).

During the course of this work, Steen et al. (1986) reported that T_7 polymerase could copy the entire rrnB operon into a 30S RNA, but they did not further characterize the RNA product other than by its susceptibility to RNAse III. An analogous in vitro synthesis by T_7 RNA polymerase of polio virus RNA containing two extra nucleotides at the 5′ end and three to seven residues at the 3′ end was also recently described (van der Werf et al., 1986). While the yields appeared reasonably good by visual inspection of the published gel photograph (no quantitative data being given), infectivity of the RNA was only 5% of the natural RNA. In our work, synthetic RNA containing three extra G residues and one base change

at the 5′ end and lacking all of the 10 modified nucleotides found in natural 16S RNA (Noller, 1984) was fully active.

The surprising feature of the modified base analysis was the presence of pseudouridine in both natural RNA and, at a low level, in synthetic RNA from reconstituted ribosomes. The pseudouridine could not come from contaminating tRNA or 23S RNA since there was a total absence of minor nucleotides characteristic of these molecules, such as ribothymidine, dihydrouridine, 4-thiouridine, 1-methylguanosine, and 6-methyladenosine. The most likely interpretation is that the previous analysis (Dubin & Günalp, 1967) was correct and two pseudouridine residues are located somewhere along the 16S RNA molecule. Since pseudouridine was absent from the synthetic RNA, its presence after reconstitution can only come from contaminating RNA in the TP30 preparation, or by enzymatic conversion of U to Ψ. We favor the latter hypothesis because of the absence of all other minor nucleotides diagnostic for tRNA, 16S RNA, or 23S RNA. In this context, it should be recalled that pseudouridine synthetase needs no cofactors (Arena et al., 1978).

It seems clear from this work that the six different modified bases of 16S RNA located at ten specific sites are not essential for ribosomal function at least as monitored by nonenzymatic but codon-dependent P site binding. The m^6_2A-1518 and -1519 residues were previously shown to be unessential for normal ribosomal function (Polderman et al., 1979, 1980), although their absence does affect translational fidelity (Van Buul et al., 1984), modifies the stability of the adjacent stem structure [Heus et al. (1983) and references cited therein], and makes the ribosome kasugamycin resistant (Helser et al., 1972). More recently, it was shown that the m^4Cm-1401 residue could be mutated to U without affecting the ability of the host cell to grow (Jemiolo et al., 1985). However, until this work, no similar information was available for any of the other modified bases. Now that the ability to reconstitute a morphologically correct and functional 30S particle lacking all modified bases exists, other ribosomal functions such as enzymatic A site binding, translocation, peptide bond formation, etc. can be tested in a direct and quantitative manner.

Our results also show that precise 5′ and 3′ ends of 16S RNA are not essential for function. Three additional G residues at the 5′ end and a change from A to G at position 2 did not block reconstitution or function. This confirms and extends previous work (Dahlberg et al., 1978; Feunteun et al., 1974), which showed that additional nucleotides at the 5′ end of 16S RNA impaired neither assembly nor, apparently, function. Mochalova et al. (1982) also showed that the 5′ end could be modified, in this case by addition of a DNP group, without impairing assembly or morphology of the 30S subunit. By contrast, in vitro assembly of particles from precursor 16S RNA, which has extra nucleotides at both 5′ and 3 ends and is submethylated, did not yield a particle capable of binding tRNA (Wireman & Sypherd, 1974). The additional residue at the 3′ end present in one-fifth of the molecules also appeared to have no negative effect. This is consistent with previous reports that addition of DNP or fluorescein groups to the 3′ end of natural 16S RNA blocked neither assembly of the ribosome nor function (Lürmann et al., 1981; Stöffler-Meilicke et al., 1981).

Reconstitution of the synthetic RNA with TP30 to give a particle that sedimented like 30S and that, according to EM, had all the morphological properties of a normal 30S required some adjustment of the standard reconstitution conditions of Held et al. (1973). When those conditions were used, the synthetic RNA formed a slower sedimenting particle, while the natural RNA control behaved like 30S. The final conditions used in this work were arrived at empirically and may not yet be fully optimized. This needs further study. Nevertheless, we could obtain a preparation in which approximately 44% of the particles had all morphological characteristics of 30S subunits, whether the RNA was extracted from native 30S subunits or was synthesized from nonmutant or the A-1400 mutant plasmid. This value fits rather well with the average value for P site binding activity relative to isolated 30S subunits of 0.53 (Table I), when one considers that 90% of a typical 30S preparation normally shows characteristic morphology.

The reconstitution studies showed that a single base change of C-1400 to G was sufficient to markedly affect the reassembly of RNA and TP30 into a 30S subunit. Surprisingly, conversion of C-1400 to A had no such effect. The failure of the G-1400 RNA to reconstitute well was seen with four independent RNA preparations, and the G-1400 RNA migrated like the A-1400 RNA on denaturing agarose gels. Thus the result is unlikely to be due to some defect in the RNA unrelated to the presence of G-1400. There must be some involvement of this nucleotide in the tertiary folding of 16S RNA, such that A is tolerated but G is not. This aspect needs further study. In particular, it would be desirable to try alternate reconstitution conditions in an attempt to obtain better reassembly with this product.

Both changes, C-1400 to A or to G, had little effect on nonenzymatic P site binding although the results with the A-1400 mutant were more clear-cut. Probably the lower values for the G-1400 mutant are due to contamination by inactive fraction B particles. In both cases, mutation markedly reduced the ability to cross-link to tRNA. The persistence of a low level of cross-linking in the A-1400 mutant may be due to cross-link formation with the 5′ adjacent base, C-1399. Normally, only C-1400 is cross-linkable, but we have previously described an example in which cleavage of yeast 18S RNA at the equivalent of U-1393 allowed a low level, about 2%, of cross-linking to the equivalent of C-1399 (Ehresmann & Ofengand, 1984). This result suggests that the 5′ anticodon base, cmo^5U-34, may be partially intercalated between C-1399 and C-1400. If so, then cross-linking to C-1399 when C-1400 is absent might be feasible.

The effect of these two mutations on all of the functions of the ribosome has not yet been tested. This work is currently in progress. Nevertheless, the close contact between tRNA and rRNA evidenced by the cross-linking of the tRNA anticodon to C-1400 was a P site specific reaction (Ofengand et al., 1979). Consequently, the fact that a change of this base to A or G has no marked effect on P site binding is of interest. Analogous results have recently been reported by Meier et al. (1986). These authors made three base changes in this region, creating the two double mutants A-1399/C-1401 and A-1399/U-1401, from the parental C-1399/G-1401. Although the assay used was considerably less quantitative than the one described in our work, neither of the mutants showed detectable changes in their ability to bind to poly(U) in a $tRNA^{Phe}$-dependent manner.

With the system described in this paper it is now possible to substitute any single base in 16S RNA and study mutant ribosome function in a direct and quantitative manner, employing the well-defined partial assays of protein synthesis. One caveat should be noted. All these studies are by necessity performed in a background of unmodified rRNA. If the combined lack of base modifications *plus* single base changes should be synergistic and not additive, then the unmodified

and nonmutated ribosome may not be an adequate control. At this stage, it is not possible to evaluate the likelihood of such a possibility.

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Structural Analysis of Covalently Labeled Estrogen Receptors by Limited Proteolysis and Monoclonal Antibody Reactivity†

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ABSTRACT: We have used limited proteolysis of affinity-labeled estrogen receptors (ER), coupled with antireceptor antibody immunoreactivity, to assess structural features of ER and the relatedness of ER from MCF-7 human breast cancer and rat uterine cells. MCF-7 ER preparations covalently labeled with [^{3}H]tamoxifen aziridine ([^{3}H]TAZ) were treated with trypsin (T), α-chymotrypsin (C), or *Staphylococcus aureus* V8 protease prior to electrophoresis on sodium dodecyl sulfate gels. Fluorography revealed a distinctive ladder of ER fragments containing TAZ for each protease generated from the M_r 66 000 ER: for T, fragments of 50K, 38K, 36K, 31K, 29K, and 28K that with longer exposure generated a 6K fragment; for C, fragments of 50K, 38K, 35K, 33K, 31K, 19K, and 18K that with longer exposure generated 14K and 6K fragments; and for V8, ca. 10 fragments between 62K and 28K. Two-dimensional gels revealed charge heterogeneity (two to three spots between p*I* 5.5 and 6.2) of the 66K ER and the T-generated 28K meroreceptor form. Immunoblot detection with the primate-specific antibody D75P3γ revealed that all immunoreactive fragments corresponded to TAZ-labeled fragments but that some small TAZ-labeled fragments (V8-generated forms <47K and T-generated forms <31K) were no longer immunoreactive. In contrast, use of the antibody H222Spγ revealed a correspondence between TAZ-labeled and immunoreactive fragments down to the smallest fragments generated, ca. 6K for T and C and 28K for V8. MCF-7 nuclear and cytosol ER showed very similar digest patterns, and there was a remarkable similarity in the TAZ-labeled and H222-immunoreactive fragments generated by proteolysis of both MCF-7 and rat uterine ER. These findings reveal great structural similarities between the human (breast cancer) and rat (uterine) ER and between nuclear and cytosol ER, indicate charge heterogeneity of ER, and allow a comparison of the immunoreactive and hormone attachment site domains of the ER. The observation that T and C generate a ca. 6K TAZ-labeled fragment that is also detectable with the H222 antibody should be of interest in studies determining the hormone binding domain of the ER and in amino acid sequencing of this region.

The estrogen receptor is an intracellular protein that appears to be responsible for mediating the biochemical and physiological effects of estrogens in estrogen target tissues and cells. While a great deal is known about the tissue distribution and intracellular dynamics of this protein (Gorski & Gannon, 1976; Katzenellenbogen, 1980; King & Greene, 1984; Welshons et al., 1984, Katzenellenbogen et al., 1985), only recently is information becoming available about the detailed biochemistry of this protein (Green, G. L., et al., 1986; Greene, S., et al., 1986).

Since the estrogen receptor is present in very low concentrations in target cells, and most estrogen-based ligands bind reversibly to the receptor, the receptor has proven difficult to purify to the extent required for detailed protein analyses. The recent availability of two reagents—a covalent labeling ligand for the receptor, tamoxifen aziridine (Katzenellenbogen et al., 1983; Katzenellenbogen & Katzenellenbogen, 1984), and monoclonal antibodies to the estrogen receptor (Greene, 1984; Greene et al., 1984)—has now made possible a detailed analysis of the structural and functional domains of this important regulatory protein.

In this paper, we report a detailed structural analysis of the estrogen receptor using proteolytic digestion and monoclonal antibodies to define the hormone binding and immunoreactive domains of this protein. For these studies, we use estrogen receptors from an estrogen-responsive human breast cancer cell line (MCF-7) and from rat uterus. Our findings provide

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