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Structure of Synthetic Unmethylated 16S Ribosomal RNA as Purified RNA and in Reconstituted 30S Ribosomal Subunits†

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ABSTRACT: 16S ribosomal RNA was made by in vitro transcription of a cloned gene, and its structure was compared to authentic 16S ribosomal RNA. The comparison was made by subjecting the two types of 16S rRNA to chemical reagents that react specifically with unpaired bases and determining the extent of reaction by reverse transcription and gel electrophoresis of the cDNA. In solution, the rRNAs were indistinguishable in their pattern of reactivity, except for a difference at A1408 and many differences in the region between residues 470 and 562. When the 16S rRNAs were reconstituted into 30S ribosomal subunits, these reactivity differences were absent. When the synthetic 16S rRNA was reconstituted into 30S subunits and then extracted and tested in solution, its pattern of chemical reactivity in the 470-562 region was the same as authentic 16S rRNA, but differences in the 1408-1410 region persisted. This study indicates that the synthetic 16S rRNA has a secondary structure in solution similar to a native secondary structure except in two regions, one apparently incorrectly folded during synthesis and the other in which nucleotides which are normally methylated in authentic 16S rRNA may be responsible for a structural difference.

Purified 16S rRNA in solution has been shown to have a secondary structure consistent with the structure predicted from comparative sequence analysis (Noller & Woese, 1981; Van Stolk & Noller, 1984; Moazed et al., 1986; Baudin et al., 1987). This indicates that the base-pairing arrangement necessary for its biological function is stable without the presence of the ribosomal proteins, except in several local regions. However, since the source for 16S rRNA is the ribosome or ribosomal subunits, 16S rRNA may have a specific conformation because of its prior assembly into ribosomal particles. It is known that subjecting the 16S rRNA to organic solvents or heating under conditions of low ionic conditions renders the molecule difficult to reconstitute into 30S particles (Barritault et al., 1979). This raises the possibility that the native structure of the RNA molecule is quasi-stable and, once disrupted, not easily regained during in vitro reconstitution.

A second question pertaining to the behavior of the 16S rRNA concerns the role of posttranscriptional methylations in determining its structure and functional activity. In the *Escherichia coli* 16S rRNA, there are 10 posttranscriptional methylations (Noller & Woese, 1981; Noller, 1984); these are representative of the modifications that occur in eubacterial

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small-subunit ribosomal RNA (Noller, 1984). These modifications are thought to be functionally important because the methylated nucleotides are part of highly conserved regions of the primary sequence and in regions accessible for chemical modification in isolated subunits but not in functioning ribosomes or polysomes (Brow & Noller, 1983). The fact that some rRNA and tRNA posttranscriptional modifications are conserved within the major phylogenetic groups but not between them indicates that the modification systems have arisen relatively recently in evolution to modulate the structure of the RNA (Woese et al., 1983). The functional and structural properties of two adjacent m_2^6A residues at positions 1518 and 1519 in the *E. coli* 16S rRNA have been examined. In a mutant of *E. coli* which lacks the methylase that modifies these bases [described by Helser et al. (1971, 1972)], the 30S subunits have a slightly lower affinity for initiation factor IF-3 and a decreased affinity to associate with 50S ribosomal subunits (Poldermans et al., 1979, 1980). It was found that the methylations slightly lower the thermal stability of the base-paired stem (Olsthoorn et al., 1980; Tasawa et al., 1980; Van Charldorp et al., 1981). It has not been possible to characterize other methylation sites in *E. coli* 16S rRNA to this extent as yet.

To determine whether 16S rRNA would be folded into the correct secondary structure if it was not originally assembled into a ribosomal particle during transcription and did not contain posttranscriptional modifications, we have taken advantange of an in vitro transcription system to make synthetic 16S RNA for examination. Previously, the properties of unmodified tRNA have been studied by obtaining RNA molecules synthesized by in vitro transcription with T7 RNA polymerase (Sampson & Uhlenbeck, 1988; Samuelsson et al., 1988). Unmodified versions of yeast $tRNA^{Phe}$ and *Mycoplasma mycoides* $tRNA^{Gly}$ could be acylated with their corresponding amino acids, and the kinetics of the reaction were very similar to those for the authentic tRNAs. The thermal stability of the unmodified yeast $tRNA^{Phe}$ was dependent on the solution conditions; melting profiles indicated that it has a less stable tertiary structure than native yeast $tRNA^{Phe}$ when measured in 1 mM spermine and varying magnesium concentrations (Sampson & Uhlenbeck, 1988). In experiments similar to those done for tRNA, an in vitro transcription system has been used to synthesize 16S rRNA and test it for its functional properties (Krzyzosiak et al., 1987; Melancon et al., 1987). It was possible to reconstitute 30S ribosomal subunits with the synthetic 16S rRNA, and the reconstituted subunits had the characteristic *E. coli* 30S subunit morphology when observed in the electron microscope. In addition, the subunits containing the synthetic 16S RNA were able to bind tRNA with a codon and $[Mg^{2+}]$ dependence similar to 30S subunits reconstituted with authentic 16S rRNA (Krzyzosiak et al., 1987) and were active in poly(U)-directed polyphenylalanine synthesis (Melancon et al., 1987).

In this report, we have determined whether there are any measurable structural differences between the synthetic 16S rRNA and authentic 16S rRNA as judged by the pattern of chemical reactivity to base-specific reagents. The pattern of chemical reactivity was visualized by oligonucleotide-directed reverse transcription and gel electrophoresis of the cDNA (Moazed et al., 1986). This comparison was made with purified RNA molecules, RNA reconstituted into 30S subunits, or RNA molecules that had been reconstituted and then were phenol extracted. Differences between synthetic and authentic 16S RNA in solution were detected by this technique in only two regions, and these differences were not apparent in RNAs that had been reconstituted. Furthermore, the RNA structure in one of the regions remains correct after extraction from reconstituted 30S subunits.

Materials and Methods

Construction of the Plasmid for in Vitro Transcription of 16S rRNA. The plasmid used for in vitro transcription of the 16S rRNA was made by transferring the gene for 16S rRNA from the plasmid pKK3535 (a gift from Dr. H. F. Noller) into the vector pTZ19U (USB). The 1503-nucleotide fragment corresponding to the body of the 16S rRNA was isolated by digesting pKK3535 with *Dra*I and *Bst*EII. A synthetic, double-stranded DNA fragment was constructed to include the sequence of the 16S rRNA from the *Bst*EII site at position 1504 to a *Bsu*36I site which imitates the authentic 3′-terminal sequence, and a 3′-terminal *Pst*I site. This fragment was ligated to the fragment containing the body of the 16S rRNA gene, and the product was then digested with *Pst*I. The vector was prepared by digesting pTZ19U with *Hin*dIII, with mung bean nuclease to remove the 5′-overhanging nucleotides of the *Hin*dIII site, and then with *Pst*I. This vector and the *Dra*I–*Pst*I fragment containing the 16S rRNA gene were ligated and used to transform *E. coli* HB101. The resulting plasmid, pTZ19U/16S, was verified by restriction analysis and by sequencing the regions at the 5′ and 3′ side of the insert with Sequenase (USB).

In Vitro Transcription and Characterization of the Synthetic RNA. Plasmid pTZ19U/16S was digested with *Bsu*36I and then phenol extracted. T7 RNA polymerase was purified from cells that contain a cloned copy of the polymerase gene (Davanloo et al., 1984) according to procedures provided by Dr. F. W. Studier. A typical transcription reaction (0.625 mL) contained 40 mM Tris-HCl (pH 8.0), 8 mM $MgCl_2$, 24 mM NaCl, 2 mM spermidine, 10 mM dithiothreitol, 1.25 mM each NTP, 50 μg of *Bsu*36I-digested pTZ19U/16S, and 190 μg of T7 RNA polymerase. The reaction was incubated for 4 h at 37 °C, then was treated with 10 units of RNase-free DNase (Promega), and then was phenol extracted and ethanol precipitated. The RNA pellet was washed with 70% ethanol and then was redissolved in 300 μL of Rec-20 buffer [20 mM Hepes, pH 7.5, 400 mM NH_4Cl, 20 mM $Mg(OAc)_2$, and 4 mM mercaptoethanol (Krzyzosiak et al., 1987)]. The integrity and size of the RNA transcripts were verified by agarose gel electrophoresis in TBE buffer (TBE is 89 mM Tris, 89 mM boric acid, and 10 mM EDTA). A typical yield of RNA under these conditions was 850 μg of 16S rRNA from 50 μg of DNA template. The 5′ terminus of the transcript was determined by primer extension using an oligonucleotide complementary to positions 100–117 and reverse transcriptase (Wollenzien, 1988). The transcript contains five nucleotides more than the authentic 16S rRNA at the 5′ terminus, and the sequence (5′NGGAA) is consistent with the structure of the template DNA close to the T7 promoter. The 3′ terminus of the 16S transcript was determined by 3′ end labeling with RNA ligase and [5′-^{32}P]pCp (England & Uhlenbeck, 1978) and complete digestion with RNase T1 or partial digestion with RNases T1, Phy M, U2, and *Bacillus cereus*. These reactions were electrophoresed on 20% polyacrylamide/8 M urea gels using TBE buffer and were compared to similar reactions performed with authentic 16S rRNA as standards. Greater than 80% of the 3′ termini of the synthetic 16S rRNA produced the same T1 fragment as the authentic 16S rRNA, with the remaining fraction one nucleotide longer. The correct sequence of the 3′ terminus could be read when the 16S RNA transcript was used as the substrate for RNA sequencing. The complete sequence of the 16S RNA transcript was verified by including

dideoxynucleotides in reverse transcription reactions at the time that the chemically modified samples were examined; it was found to be the same as the authentic 16S rRNA.

Reconstitution of 30S Subunits. Authentic 16S rRNA was purified by extraction of 30S subunits with phenol and 0.5% SDS in the cold using SCE buffer (SCE is 0.15 M NaCl, 0.015 M sodium citrate, and 1 mM EDTA). The RNA was ethanol precipitated and redissolved in SCE or Rec-20 buffer before use. For the purification of 30S ribosomal subunit proteins, 30S subunits were first purified on short sucrose gradients according to the procedure of Staehelin and Maglott (1971). Proteins were then isolated by acetic acid extraction (Nierhaus & Dohme, 1979). 16S RNA transcript and authentic 16S rRNA were reconstituted by using the conditions described by Krzyzosiak et al. (1987) with some changes. For reconstitution, usually 400 μg of RNA was incubated in 3-mL reactions with 4 equiv of total protein from 30S subunits. The final incubation was 10 min at 50 °C, instead of 20 min. The reaction was layered on top of 7.5–30% sucrose gradients in RB buffer [20 mM Hepes, pH 7.5, 100 mM NH_4Cl, 10 mM $Mg(OAc)_2$, and 1 mM dithiothreitol]. The fractions corresponding to 30S subunits were combined and precipitated by the addition of magnesium acetate to 20 mM and 1 volume of ethanol. The precipitate was collected by low-speed centrifugation and was redissolved in activation buffer [200 mM NH_4Cl, 20 mM $Mg(OAc)_2$, 50 mM Tris-HCl, pH 7.2, and 6 mM mercaptoethanol]. The reconstituted 30S subunits were activated by heating at 40 °C for 30 min before being used for structural experiments or activity measurements or being frozen at −70 °C for later use. The phe-tRNA^{Phe} binding assay of Zamir et al. (1971) was used to determine the ability of the subunits to bind phe-tRNA^{Phe} in the presence of poly(U).

The protein composition of the reconstituted 30S subunits was determined by dissociating 100 μg of 30S subunits at 20 μg/μL with 0.1 volume of 1 M $Mg(OAc)_2$ and 0.05 M EDTA and 2 volumes of glacial acetic acid. The supernatant was collected after centrifugation, and the dissociation step was repeated on the pellet. The supernatants were combined, were precipitated with 5 volumes of acetone, and were dissolved in 20 μL of 10 mM Bis-Tris, acetic acid, pH 4.2, 8 M urea, and 1% mercaptoethanol. The products were electrophoresed on a two-dimensional gel system in which the first dimension was 4% acrylamide/0.066% bis(acrylamide)/8 M urea/0.01 M Bis-Tris, acetic acid, pH 3.8 (Madjar et al., 1979), and the second dimension was 18% acrylamide/0.25% bis(acrylamide)/6 M urea/20 mM Bis-Tris, acetic acid, pH 3.7 (Kenney et al., 1979). The proteins were visualized by Coomassie staining. Total protein from native 30S subunits was used as the standard to compare the intensity of the individual proteins.

Chemical Probing Experiments. Three chemical reagents, dimethyl sulfate (DMS), kethoxal, and 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate (CMCT), were used. These react specifically with the four bases and can be detected by analysis with reverse transcriptase (Moazed et al., 1986). The procedures of Moazed et al. (1986) were used with some changes. Five micrograms of RNA or the equivalent amount of 30S subunits was reacted in 50-μL total volume for each reaction. The samples were heated at 42 °C for 20 min and then cooled on ice before analysis. The reagent concentrations and incubation times were the following: (1) 1 μL of 1/4× DMS in ethanol for 2 h at 4 °C; (2) 1 μL of 1/8X DMS in ethanol for 10 min at 37 °C; (3) 5 μL of 37 mg/mL kethoxal in 20% ethanol for 4 h at 4 °C; (4) 5 μL of 3.7 mg/mL kethoxal in 20% ethanol for 10 min at 37 °C; (5) 25 μL of 42 mg/mL CMCT in BMK buffer (70 mM potassium borate, pH 8.1, 0.3 M KCl, and 20 mM magnesium acetate) for 2 h at 4 °C; (6) 20 μL of 42 mg/mL CMCT in BMK buffer for 10 min. The same conditions were used for the purified RNA and the 30S subunits. After reaction, the RNA samples were precipitated with ethanol and redissolved at a final concentration of 0.1 μg/μL. The 30S subunit samples were phenol extracted, precipitated with ethanol, and redissolved at a final concentration of 0.1 μg/μL.

Primer extensions using AMV reverse transcriptase and ^{32}P-labeled oligonucleotide primers were done as previously described (Wollenzien, 1988). In the present experiments, 0.1 μg of RNA template, 1 pmol of ^{32}P-labeled oligonucleotide primer, and 2.2 units of AMV reverse transcriptase (Life Sciences) were used in each 20-μL reaction. The oligonucleotides used in different reactions and their sites of complementarity were the following: 16S-Four [nucleotides (nt) 100–117], 16S-23A (nt 260–277), 16S-511 (nt 511–533), 16S-Three (nt 559–575), 16S-20 (nt 686–702), 16S-24 (nt 1016–1032), 16S-27A (nt 1256–1273), 16S-One (nt 1453–1466), 16S-6E (nt 1509–1530), Primer-030 (nt 1527–1542). The reverse transcription reactions were ethanol precipitated and redissolved in 4 μL of water, and 1 μL was electrophoresed on 8% polyacrylamide or 5% polyacrylamide sequencing gels.

RNA Structure Prediction. The program PCFOLD (Zuker & Sankoff, 1986) was used on an IBM AT computer to predict secondary structures containing the minimum free energy for selected RNA regions.

Results

Structural Analysis of Synthetic 16S rRNA and Authentic 16S rRNA in Solution. Synthetic 16S rRNA transcripts, obtained from in vitro transcription of the *Bsu*36I-digested pTZ19U/16S DNA, and authentic 16S rRNA were redissolved in Rec-20 buffer and renatured by heating at 42 °C for 20 min. They were then incubated with chemical reagents that react with bases at hydrogen-bonding positions. Bases are not reactive at these positions if they are in canonical base pairs (Noller et al., 1987). The reagents used were DMS for adenosine and cytosine, kethoxal for guanosine, and CMCT for uridine (Moazed et al., 1986). The pattern of reactivity was determined by reverse transcription reactions initiated by DNA oligonucleotide primers. Because the reverse transcriptase is not able to incorporate a nucleotide onto a chemically modified base, terminations occur one nucleotide before the site of modification. In these experiments, a set of 10 oligonucleotide primers was used to examine the synthetic and authentic 16S RNAs. For each chemical probe, reactions were done at 4 and 37 °C in order to determine if there were differences in thermal stability between the synthetic and authentic 16S RNA molecules. cDNA made from synthetic and authentic rRNA modified under identical conditions was electrophoresed in adjacent lanes of the gel; the autoradiogram was then examined for differences between adjacent lanes. The synthetic 16S RNA was sequenced at the same time to ensure that there were no sequence changes that occurred during the subcloning procedure that would generate apparent chemical reactivities at positions where they were not expected. In the figures of the gel patterns, the reactivity of a nucleotide is indicated at the band where the termination occurs—one step on the 3′ side of the actual sequence position.

Patterns of chemical reactivity in three regions of the 16S rRNA are shown in Figures 1–3. The region from position 1434 to 1357 (Figure 1) contains two nucleotides that are posttranscriptionally methylated, C1402 at positions 4 and 2′

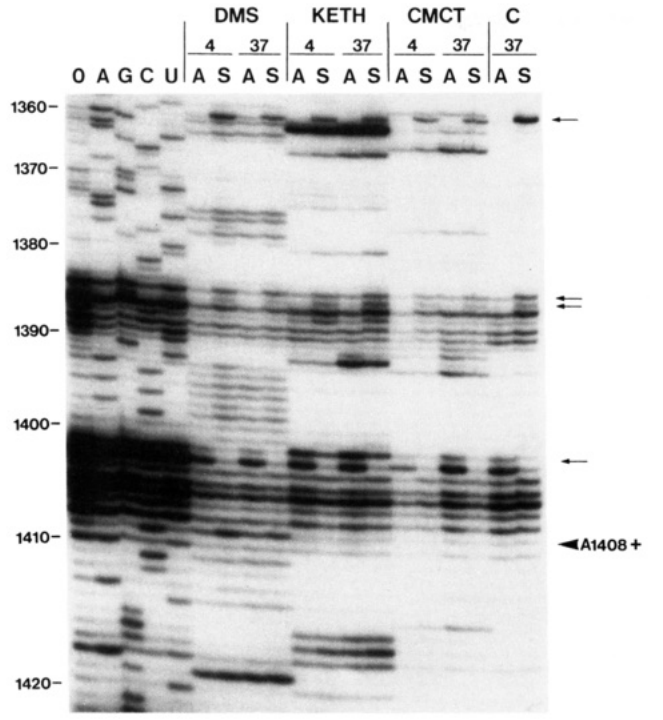

FIGURE 1: Identification of chemically modified sites in purified synthetic 16S RNA and authentic 16S rRNA. RNA molecules were reacted with dimethyl sulfate (DMS), kethoxal (KETH), or 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate (CMCT) at 4 or 37 °C. The oligonucleotide primer 16S-One (complementary to nucleotides 1453–1466) was used to initiate reverse transcription at position 1452. The segment from 1420 to 1370 is shown in the figure. Each comparison is shown as a pair of lanes containing the authentic 16S rRNA (A) and the synthetic 16S RNA (S). The lanes marked 0, A, G, C, and U contain a control reverse transcription sample and samples terminated with dideoxynucleotides to indicate the nucleotide sequence of the synthetic 16S RNA. The arrowhead indicates residue A1408 in the synthetic RNA that has a greater chemical reactivity in this region. The arrows indicate positions where there are differences in degradation.

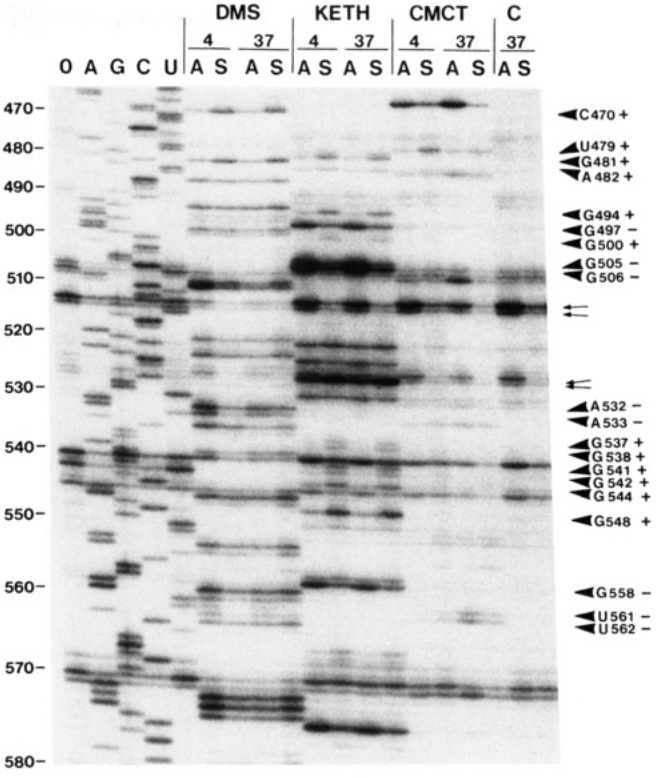

FIGURE 2: Pattern of chemical reactivity and degradation in the region from 570 to 470. The oligonucleotide primer 16S-20 that hybridizes from positions 686 to 702 was used to initiate reverse transcription through this region of the 16S RNA. Lane notations are as described in the legend to Figure 1. The arrowheads indicate differences in the pattern of chemical reactivity; (+) and (–) indicate an increased or decreased reactivity of synthetic 16S RNA compared to authentic 16S rRNA. The arrows indicate differences in degradation between the two RNA samples.

and C1407 at position 5. A reverse transcriptase hesitation point was observed at one nucleotide 3′ to C1402 in authentic 16S rRNA. There was a small difference in chemical reactivity at residue A1408. In two out of four independent experiments, A1408 in the synthetic 16S RNA showed a slightly greater degree of chemical reaction with DMS. This position may be affected by the absence of methylation at C1407. There were a number of other positions where there were band intensity differences between the synthetic and authentic samples; however, these differences were also present in the control lanes. There were no other reproducible chemical reactivity differences in this part of the molecule. The chemical reactivity of a series of residues in this region cannot be compared because of the high background of cDNA termination sites that routinely occur in this part of the 16S RNA in both samples. These presumably arise because the RNA is partially degraded at these positions.

One region where differences in chemical reactivity and in the pattern of degradation were observed in repeated independent experiments is from nucleotide 470 to 562 (Figure 2). Synthetic 16S RNA exhibited reduced reactivity at G558, U561, and U562, increased reactivity at a series of residues from G537 to G548, decreased reactivity at residues A532 and A533, and at G505 and G506, and increased reactivity of several residues from 470 to 500. In some experiments, there was significant degradation of the synthetic 16S rRNA from residue 536 to 546 that prevented comparison to the authentic 16S rRNA. Both the synthetic 16S rRNA and authentic 16S rRNA have the expected pattern of chemical reactivity before and after this region. The altered reactivity in this region of the synthetic 16S rRNA may be due to the absence of methylation at the N7 position of G527, or, alternatively, it may be due to an incorrect folding that is confined to this region. This question will be investigated in a following section.

In the 3′ major RNA domain (nucleotides 920–1380), most synthetic RNA samples exhibited a pattern of degradation that was not observed in the authentic 16S rRNA. Part of this region is shown in Figure 3. Degradation was apparent in all of the lanes containing synthetic 16S RNA, including the control sample that had not been incubated with any chemical reagent (lane C, samples S). Most of this degradation in the synthetic 16S rRNA occurred during its synthesis since it was also present in samples that were not activated at 42 °C or subjected to the conditions used for chemical modification (results not shown). We have not detected chemical reactivity differences in the 3′ major domain.

There were no other reproducible differences in chemical reactivity in other regions of the 16S RNA. The 3′-terminal region was examined with two oligonucleotide primers that initiate reverse transcription either at position 1526 or at position 1508, in anticipation of differences due to the absence of methylations at A1518 and A1519 in the synthetic 16S RNA. No differences were detected (data not shown). The patterns of chemical reactivity in this region in both of the RNAs were consistent with the expected secondary structure.

Reconstitution of Synthetic and Authentic 16S rRNA. In order to compare synthetic 16S rRNA and authentic 16S rRNA in 30S subunits, the RNAs were reconstituted with proteins from 30S subunits. Synthetic 16S RNA reconstituted

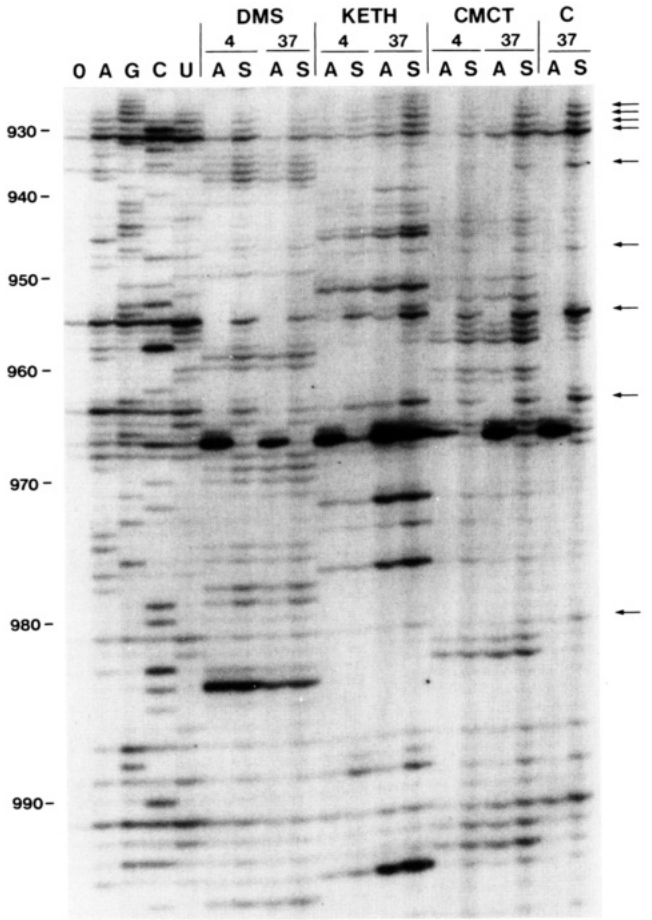

FIGURE 3: Pattern of chemical reactivity and degradation in the region from 980 to 930. The oligonucleotide primer 16S-24 that hybridizes to positions 1016–1032 was used to prime reverse transcription through this region. The same pattern of chemical reactions and control lanes is shown in this figure as in Figure 1. The arrows indicate differences in the degradation pattern.

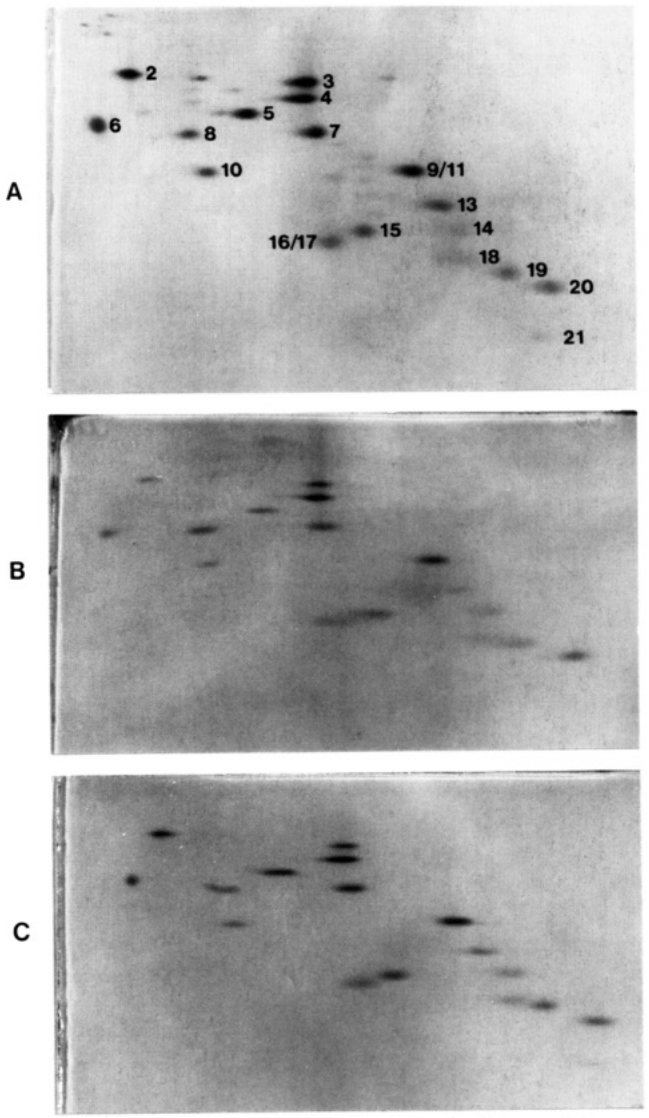

FIGURE 4: Two-dimensional gel electrophoresis of the proteins extracted from native 30S subunits and subunits reconstituted with authentic and synthetic 16S RNA. (A) Pattern of proteins from native 30S subunits. The identity of the spots is written according to the assignments of Madjar et al. (1979) and Kenny et al. (1979). Note that S1 is normally not present on this gel system, and in this preparation of 30S proteins, S12 was not present. The remaining 19 proteins are present as 17 identifiable spots. (B) Proteins from 30S subunits reconstituted from synthetic 16S RNA. (C) Proteins from 30S subunits reconstituted from authentic 16S rRNA.

44% as efficiently as the authentic 16S rRNA. Subunits were purified on sucrose gradients; those reconstituted with synthetic 16S rRNA had a normal sedimentation profile compared to native 30S subunits and 30S subunits reconstituted with authentic 16S rRNA (data not shown). In their ability to bind tRNA, subunits containing synthetic 16S RNA had 100 ± 14% (three independent samples) of the activity of native subunits that had been prepared in parallel on sucrose gradients under the same conditions. The actual values of tRNA binding were 0.21 mol of tRNA/mol of 30S subunit for each sample.

To determine if the RNAs were totally reconstituted, proteins from the reconstituted subunits were isolated and examined. Figure 4 shows the pattern of two-dimensional gel electrophoresis of proteins from native 30S subunits, and 30S subunits reconstituted with synthetic and with authentic 16S rRNA. Protein S1 does not enter this gel system, so no comparison could be made for it. In the experiments shown, protein S12 was not present in the protein preparation from the starting subunits (panel A), so it was not present in any of the samples. However, S12 has been present in protein preparations made subsequent to the experiments shown here; in reconstitutions, S12 is present in both the authentic and synthetic samples (results not shown). In the 30S subunits reconstituted from synthetic RNA, 16 of 17 spots were present at the expected intensities compared to the other samples. As protein pairs S9/S11 and S16/S17 are not resolved in this gel system, it was not possible to judge individually if all four were present. However, the intensity of the combined spots was the same in all three samples. One difference was seen: protein S21 was reduced in amount in the subunits reconstituted from synthetic 16S RNA. This protein was seen as a faint band in the samples from the native subunits (panel A) and reconstituted 30S subunits made with authentic 16S rRNA (panel C) but was not detectable in the proteins isolated from reconstituted 30S subunits made with the synthetic 16S RNA (panel B).

Chemical Reactivity in Reconstituted 30S Subunits. Chemical probing experiments were conducted on reconstituted 30S subunits to determine if there were any structural differences in the synthetic 16S rRNA and authentic 16S rRNA. For these experiments, native 30S subunits were used as an additional control. The pattern of reactivity in the region of the 16S rRNA from position 590 to 480 of all three RNA samples is shown in Figure 5. There was significant reduction in the reactivity for all three samples compared to the pattern

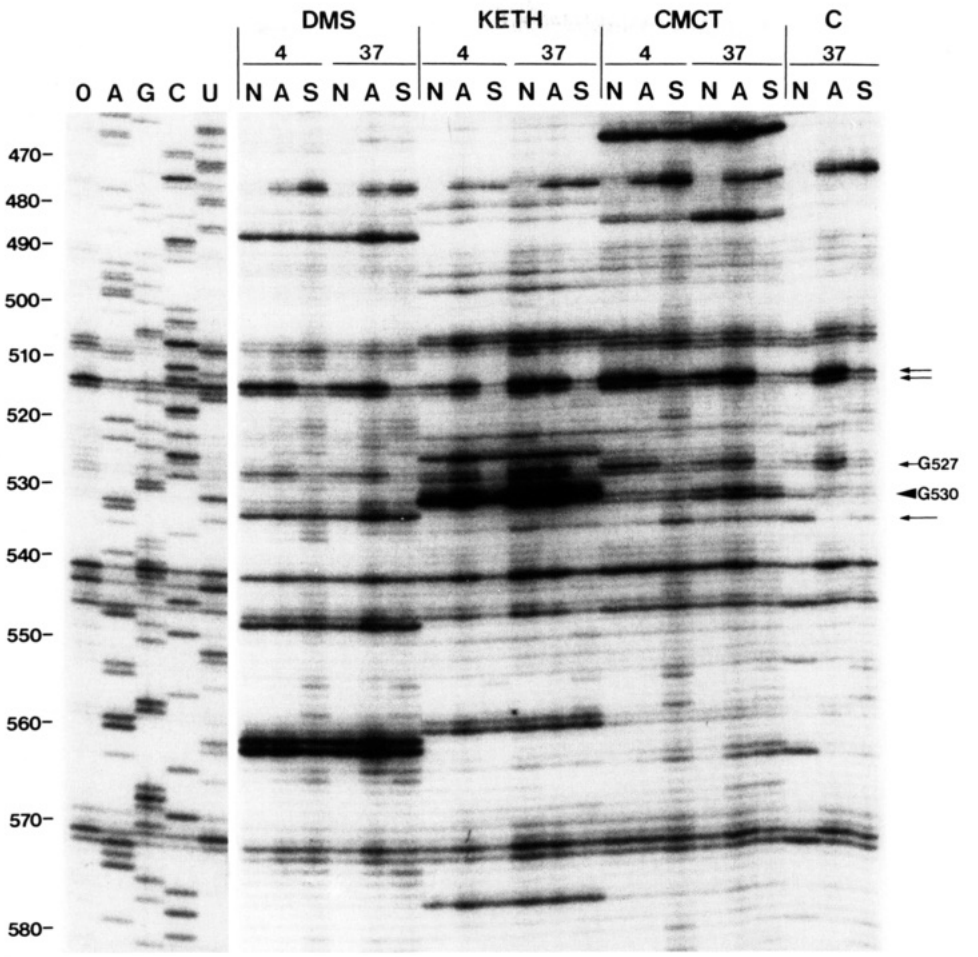

FIGURE 5: Pattern of chemical reactivity for 16S RNA probed in native and reconstituted 30S subunits. The oligonucleotide primer 16S-20, complementary to nucleotides 686–702, was used to initiate reverse transcription through the region from 570 to 470. Each set of reactions consists of three samples: native 30S subunits (N), subunits reconstituted with authentic 16S rRNA (A), and subunits reconsituted with synthetic 16S RNA (S). Lane notations are as described in Figure 1. Arrows indicate the differences in the pattern of degradation. In this figure, the arrowhead indicates G530, which becomes hyperreactive in the 30S subunit.

that occurs in purified RNA, which is characteristic for the 16S rRNA examined in the 30S subunit (Moazed et al., 1986). Reactivity differences between synthetic and authentic RNA that were observed in solution were not detected when these RNAs were reconstituted.

Another characteristic of the pattern of chemical modification in the 30S subunit is the appearance of a number of hypermodified residues (Moazed et al., 1986). The pattern of hypermodified bases in both the synthetic and authentic 16S RNA was the same. Notably G967, which is normally m_2G, was hypermodified with kethoxal when it was tested in reconstituted 30S subunits (data not shown). This indicates an exceptional accessibility to the N1 and N2 positions of G967. However, other residues that are otherwise methylated in the authentic 16S rRNA (i.e., A1518 and A1519) were not hypermodified (data not shown).

Chemical Reactivity on Reextracted Synthetic 16S rRNA after It Has Been Reconstituted. An experiment was done to determine whether the differences in the chemical reactivity of purified synthetic 16S rRNA in the region from 470 to 562 and at position 1408 were due to the absence of methylations on the RNA or were due to incorrect folding of the RNA during in vitro transcription. Synthetic 16S rRNA was first reconstituted and then phenol extracted under the same conditions used to prepare authentic 16S rRNA. The RNA in solution was then subjected to chemical modification. We reasoned that if the synthetic 16S rRNA inherently had a different structure due to the absence of methylation, the chemical reactivity difference would return after the RNA had been extracted from the subunit. If the chemical reactivity difference was due to incomplete or incorrect folding during synthesis, this defect might be corrected during the in vitro reconstitution and would not be displayed in the subsequent chemical modification experiments.

The pattern of reactivity in the region from 460 to 570 is shown in Figure 6 and for the region surrounding 1408 in Figure 7. Chemical reactivity differences between the synthetic and authentic 16S rRNA in the 460–570 region are now absent; some small differences in the intensity of bands occur, but these are at positions where there are differences in the control lanes. We conclude, therefore, that the 16S rRNA that was successfully reconstituted has a native secondary structure and this is stable when the RNA is extracted. On the other hand, a chemical reactivity difference between synthetic and authentic 16S rRNA is still seen at the position corresponding to A1408 in these samples. In addition, in this sample, there is a difference in reactivity between the two RNAs at position 1410. This indicates an inherent structural difference between the two RNAs in solution in this region.

Discussion

The structures of synthetic and authentic 16S rRNAs were analyzed by chemical modification of purified RNA in solution and in reconstituted 30S subunits. The patterns of reactivity we see throughout the 16S rRNA are similar to those reported by Moazed et al. (1986) except for differences in intensity that

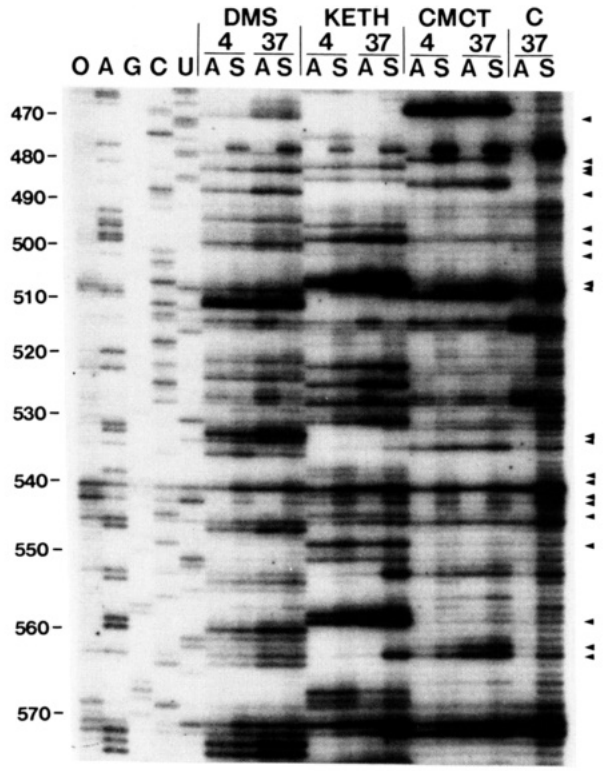

FIGURE 6: Pattern of chemical reactivity for 16S RNA in solution after extraction from 30S subunits. The oligonucleotide primer 16S-20 was used to examine the region from nucleotides 570 to 470 as indicated on the left. The lanes are indicated with the same notation as before. The arrows on the right indicate positions that showed altered reactivity in the synthetic RNA compared to the authentic RNA. There are a large number of steps at which the synthetic RNA shows greater hydrolysis due to its reconstitution and reextraction before testing.

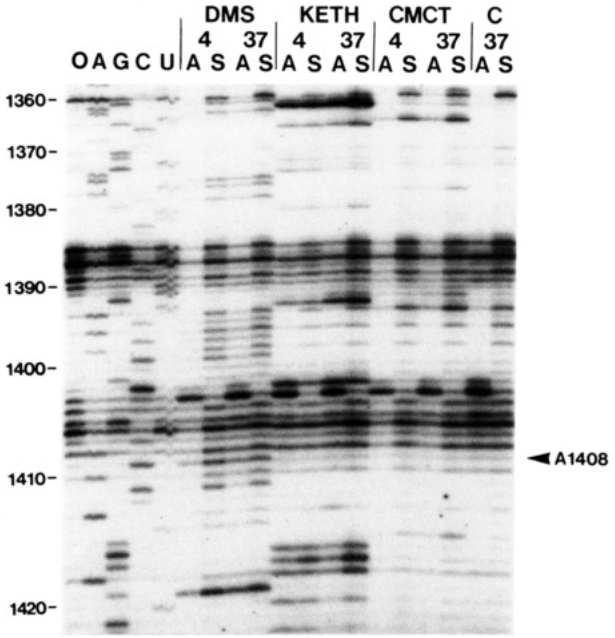

FIGURE 7: Pattern of chemical reactivity for 16S RNA in solution after extraction from 30S subunits in the region from position 1420 to 1360. Position A1408 (indicated on the right) shows a different chemical reactivity that cannot be accounted for by differences present in the control lanes. There is also a reactivity difference that corresponds to position 1410 in this sample.

are probably due to a difference in the overall extent of the chemical reactions. In our experiments, 4 and 37 °C were used to assess possible thermal stability differences in the RNA. Some changes in reactivity were seen, and these are more pronounced than the changes seen by Moazed et al. (1986) in which 16 and 30 °C were used as the standard temperatures. In these experiments, corresponding synthetic and authentic RNA samples were always run in adjacent lanes; this allows us to rapidly identify differences in the samples without having to score the reactivity of every single nucleotide.

In solution, these RNAs exhibited similar chemical reactivities, except in two restricted regions. Both of these regions normally contain methylated nucleotides, and this raised the possibility that the differences were due to the absence of methylation in the synthetic RNA. These differences are summarized in Figure 8. One of the differences occurred at position A1408, where there was an increase in DMS reactivity in the synthetic 16S RNA. This residue has not been proposed for any specific base pair as yet. The chemical reactivity difference was not detected in 30S subunits, but it was detected again when the synthetic 16S rRNA was reextracted from 30S subunits. This indicates that the difference in reactivity may be due to some change in the RNA structure surrounding C1407 which is normally m^5C in authentic 16S rRNA. Although this region is functionally very important, 30S subunits containing synthetic 16S rRNA have been shown to have normal activity in a number of different assays (Krzyzosiak et al., 1987; Melancon et al., 1987). This indicates that the 30S subunit is able to accommodate the absence of methylations in this region of the RNA and form an active functional configuration.

The greatest number of chemical reactivity differences were seen in a region extending from position 470 to position 562. The differences we observed included changes both in the pattern of chemical reactivity and in the pattern of degradation of the RNA. The pattern of chemical reactivity in the synthetic 16S rRNA in this region has been examined in RNA in solution, in reconstituted subunits, and in RNA in solution after the RNA had been reisolated from reconstituted subunits. By this comparison, we were able to separate the effects of possible incorrect folding in this region during in vitro transcription and the absence of methylation that would normally occur at G527. Changes in reactivity in solution in the synthetic RNA compared to the authentic RNA are shown in Figure 8. These differences are not present in reconstituted subunits nor are they present in the RNA when it is reextracted from 30S subunits. The pattern does not fit the expected reactivities that should result from the native secondary structure (Figure 8A) or a general decrease in the stability of the native secondary structure. A number of G residues between positions 537 and 548 that should be in stable base pairs are more reactive in the synthetic RNA than in the authentic RNA, and several residues (G505, G506, G558, U561, U562) that should be in single-stranded regions in the native structure are less reactive in the synthetic RNA compared to the authentic RNA. This indicates that this section of the synthetic 16S rRNA transcript before it is reconstituted is probably folded in an alternate secondary structure in at least part of the synthetic 16S rRNA.

A prediction for the structure of the sequence from 502 to 550 that contains the minimum free energy indicates that the most stable structure should contain the interactions (500–504)·(541–545), (511–517)·(534–540), (517–519)·(529–531), and (521–522)·(527–528). The most recent version of the structure of 16S rRNA derived from comparative sequence analysis (Dams et al., 1988) indicates that the first three of these base-pairing interactions should be present in the region from 518 to 533 in the functional ribosome. This is the sec-

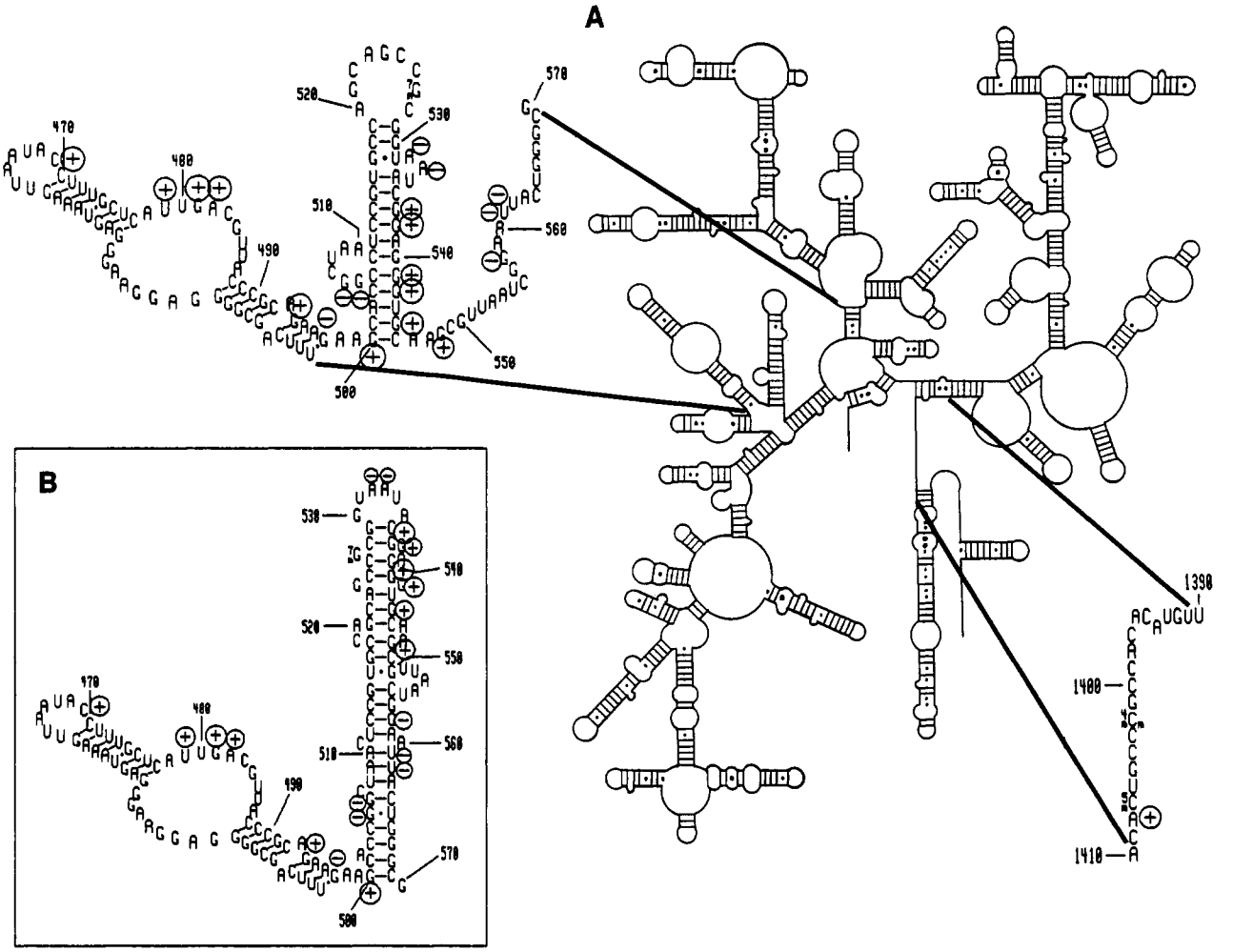

FIGURE 8: Summary of the differences in the patterns of chemical reactivity between purified synthetic 16S RNA and authentic 16S RNA. (A) The schematic diagram of the secondary structure for 16S rRNA proposed by Dams et al. (1988) is shown. Chemical reactivity differences in the synthetic 16S RNA are shown on the expanded regions as (+) or (−) to indicate positions that are more or less reactive than in the authentic 16S rRNA. These differences occur in the purified RNA but not in reconstituted 30S subunits. The differences in the 470–562 region are not present in synthetic 16S RNA in solution if it has previously been reconstituted. Methylations that occur at positions G527, C1402, and C1407 in authentic 16S rRNA are indicated. (B) An alternate secondary structure for the region 500–570 that accommodates some of the chemical reactivity changes.

ondary structure shown in Figure 8A. When a larger section of the RNA sequence from 430 to 570 was folded to produce a theoretical minimum free energy, in the interval from 500 to 570, a structure that is an alternative to the native secondary structure was predicted. This structure, shown in panel B in Figure 8, contains base pairs between the 500–515 region and the 536–569 region at its bottom and between the 516–529 region and the 536–550 region at its top.

There is a correspondence between the pattern of chemical reactivity in the synthetic 16S rRNA (before it is reconstituted) and the alternate secondary structure shown in Figure 8B. The bottom part contains the nucleotides G505, G506, G558, U561, and U562 which are all less reactive in the synthetic 16S RNA; they would be in single-stranded regions in the native secondary structure but are in base pairs in this alternate structure. The top part contains the nucleotides G537, G538, G541, G542, G544, and G548 which are all more reactive in the synthetic 16S RNA; they would be in stable base-paired regions in the native structure but are in a region of interrupted base pairs or are in single-stranded positions in the alternate secondary structure. The alternate structure in the interval 500–570 accounts for half of the chemical reactivity differences that are seen. It does not account for decreases in reactivity of A532 and A533 which are in single-stranded regions in both models and for the increase in reactivity of G500 which is base paired in both models, and it does not account for a number of differences in the adjacent base-paired region from 437 to 497. It may be possible that differences in the accessibility of the A residues are responsible for their reactivity changes and that differences in the three-dimensional compactness of the region are responsible for the remaining differences. In addition, if the alternate structure were present, it would leave two distant parts of the 16S RNA [(27–37) and (881–886)] in different structures than they are in the native secondary structure; we did not record reproducible chemical reactivity differences in these regions of the RNA. Nevertheless, it is likely that at least part of the synthetic 16S rRNA adopts this alternate conformation in this region from 500 to 570. We are presently investigating this possibility by altering the nucleotide sequence in this region of the 16S rRNA to determine if we can prevent the aberrant chemical reactivity pattern and improve the ability to reconstitute the synthetic 16S rRNA.

In addition to these chemical reactivity differences in the synthetic 16S rRNA, in the 3′ major RNA domain there usually were differences in the patterns of degradation. This degradation probably occurred during the in vitro transcription reaction because the extent of degradation varied with each synthetic RNA preparation.

Reconstitution of the synthetic 16S RNA into 30S subunits

changes the structure of the 16S RNA such that the pattern of chemical reactivity is the same as the pattern detected in 30S subunits containing authentic 16S rRNA. The protein composition of the reconstituted 30S subunits was the same for the two types of 16S RNA, except for the absence of protein S21 in the subunits made with the synthetic RNA. Protein S21, in combination with proteins S4, S5, S6, S7, S8, S11, S12, S15, S16, S17, S18, and S20, has been found to alter the pattern of chemical reactivity only at G800 (Noller et al., 1987; Stern et al., 1988). We did not observe this alteration, however, in our analysis a different combination of proteins was present. Also, we did not observe any difference in the pattern of chemical modification between native 30S subunits and either of the reconstituted samples. Some differences might be expected between these samples because neither of the reconstituted samples contained S12. S12 by itself modifies the reactivity of the 16S rRNA at several positions in the regions 881–917 and 1392–1395 (Stern et al., 1988). Apparently, as our measurements were made with almost all of the other ribosomal proteins in place, the presence or absence of S12 did not alter the reactivity at these locations.

The synthetic 16S rRNA reconstituted into 30S subunits exhibited the same pattern of hypermodified sites as the authentic 16S rRNA. This indicates that the overall structure of the synthetic and authentic 16S RNA is the same in the reconstituted 30S subunits. Residue G967, which is normally m_2G, was hypermodified by kethoxal. However, this was the only residue which reacted in this manner. Therefore, it is not possible to make any general conclusions about the accessibility of bases in the 30S subunit and the likelihood that these bases will be methylated.

We have not detected any difference in the structure of the synthetic 16S RNA in the region surrounding positions 1518 and 1519. These residues in authentic 16S rRNA are both m_2^6A. Van Charldorp et al. (1981) previously characterized a 49-nucleotide fragment from the 3′ end of 16S rRNA that did not contain these modifications. They observed a 2 °C increase in its temperature of melting over that of an identical fragment containing the methylations. The melting temperatures in 0.2 M NaCl were 75 and 73 °C for the unmethylated and methylated fragments, respectively. It is difficult to compare the chemical probing experiment to the melting experiment because the nature of the measurement is different and the ionic conditions are different. However, we can conclude that the chemical probing experiment will not be as sensitive as the T_m measurement in determining small stability differences. On the other hand, the chemical probing experiment has the advantage of reporting the property of each nucleotide within a large RNA, so it is of great utility in determining structural rearrangements that might be difficult to detect by spectroscopic methods.

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