Conformational Analysis of *Escherichia coli* 30S Ribosomes Containing the Single-Base Mutations G530U, U1498G, G1401C, and C1501G and the Double-Base Mutation G1401C/C1501G†

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ABSTRACT: Biochemical and genetic studies have pointed out the importance of several sites in 16S ribosomal RNA of *Escherichia coli* in the decoding process. These sites consist of the core of the decoding center (1400/1500 region) and two other segments (530 and 1050/1200 regions). To detect a possible structural link between these functionally related regions, we analyzed their sensitivity to conformational changes induced by mutations which are located in each of these regions and are known to affect the decoding process. The conformations of five segments of 16S rRNA (1−106, 406−569, 780−978, 997−1247, and 1334−1519) were analyzed by chemical probing of 30S ribosomes containing the following mutations: G530U, U1498G, G1401C, C1501G, and G1401C/C1501G. Ribosomes reconstituted with natural wild-type 16S RNA showed only minor conformational differences with respect to ribosomes isolated from cells. When 16S RNA made *in vitro* replaced natural 16S RNA, a slightly looser conformation of the central core region was found. Mutant ribosomes made by reconstitution with mutant 16S RNA made *in vitro* showed conformational effects which were in all cases localized to the region of secondary structure surrounding the site of mutation. Although the core of the decoding center (1400/1500 region) and the two other sites (530 and 1050/1200 regions) participating in the decoding function have been functionally linked, our data indicate that they are structurally independent. They also provide evidence for an unusual structure of the 1400/1500 decoding center, possibly involving noncanonical interactions. Furthermore, the absence of any conformational effect induced by the G530U mutation except at the site of mutation itself points to its direct, as opposed to indirect, involvement in the decoding function of the ribosome.

It is now a well-accepted fact that ribosomal RNA plays an important functional role in the ribosome [reviewed in Nierhaus et al. (1992) and Matheson et al. (1995)]. Major support for this view has come from the generation, by site-directed mutagenesis, of single-base changes in both the small subunit rRNA and the large subunit rRNA which affect ribosomal function *in vitro* and *in vivo* [reviewed in Triman (1996a,b)]. In one of our laboratories (J. Ofengand), sites in the highly sequence-conserved segments 518−533, 1394−1408, and 1492−1505 in *Escherichia coli* small subunit RNA which are known to be involved in decoding have been studied (Ofengand et al., 1993). Mutations at many of these sites, made in the "synthetic" ribosome system (Krzyzosiak et al., 1987; Denman et al., 1989a), have strong functional consequences (Denman et al., 1989b; Cunningham et al., 1990, 1992a,b, 1993; Santer et al., 1993; Ringquist et al., 1993).

Implicit in the conclusions drawn from the studies cited above was the assumption that (a) the conformation of the RNA in the wild-type synthetic ribosome was close to that in natural 30S particles and (b) the single-site mutations did not perturb the RNA conformation except in the immediate vicinity of the mutant base. In this work, we have tested these assumptions by chemical probing of the accessibility of individual nucleotides in both natural and synthetic 30S subunits. We have also studied five mutant particles, three of which (G530U, U1498G, and G1401C/C1501G) were chosen because they exhibit a common functional phenotype despite the distinctly different locations of their mutations (Cunningham et al., 1992b, 1993; Santer et al., 1993). Specifically, we asked whether these three mutants would show some similarities in the pattern of reactivity of their 16S RNA to chemical probes since all three have an apparently common functional defect. We were also interested in whether mutation at one site would induce a conformational change at the other sites in some characteristic or specific manner, since this would be indicative of a tertiary structural linkage between the different regions involved in the decoding function. Besides these sites, regions 1−105 and 790−965 (including the central pseudoknot) were analyzed since due to their central location in the 30S subunit they may be critical to the conformation of 16S RNA. Region 1050/1200 in helix 34 was also investigated because both biochemical (Dontsova et al., 1992)

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and genetic (Moine & Dahlberg, 1994) data have shown it to be involved in the decoding function.

MATERIALS AND METHODS

Preparation of Ribosomes. Transcription of wild-type and mutant *E. coli* 16S RNA genes and assembly of the RNA into 30S particles were carried out as described previously (Cunningham et al., 1992b, 1993; Santer et al., 1993). Isolation of 30S ribosomes and natural 16S RNA and reconstitution of the RNA with a total 30S *E. coli* protein mixture (TP30) were also carried out as described (Cunningham et al., 1992b).

5′-Labeled Primers. Deoxyoligonucleotides were prepared as described previously (Bakin & Ofengand, 1993). 5′-^{32}P labeling at high specific activity was performed in 12 μL reaction mixtures containing 50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 10 mM mercaptoethanol, 2 μM ATP (Amersham, 3 μCi/pmol), 30 μM deoxyoligomer, and 2500 units/mL polynucleotide kinase (New England Biolabs) at 4 °C for 17 h. Reactions were stopped with 6 μL of 8 M urea containing 0.007% xylene cyanol and 0.007% bromophenol blue and loaded onto a 10% polyacrylamide gel in 7 M urea. After location by autoradiography, the labeled oligomers were eluted from the excised gel pieces by heating at 95 °C for 3 min in 200 μL of sterile water, followed by 12−22 h at room temperature. The eluate was recovered, and the gel pieces were washed in 50 μL of water. The total elution efficiency was 85%. The pooled eluates were precipitated with $^1/_{10}$ of a volume of 3 M NaOAc (pH 5.8) and 3 volumes of EtOH at −70 °C for 5 min followed by centrifugation at 13 000 rpm and 4 °C for 30 min to recover the oligomers (95%). After washing twice with 80% EtOH, the samples were dissolved in water to $1.5-3 \times 10^5$ dpm/μL (specific activity, ca. 2×10^5 dpm/pmol) and stored at −20 °C.

Probing Strategy. Probing conditions, adapted from Baudin et al. (1987) and Stern et al. (1988), were chosen so we could maintain conditions as close as possible to the conditions under which the mutant ribosomes were assayed (Cunningham et al., 1992b). Seven different primers were used to examine specific areas of the 16S rRNA molecule. For regions 1−106 and 406−569, primers 110−124 and 604−624 were used, respectively. For region 780−1247, primers 986−1000, 1147−1167, and 1260−1277 were used, and for region 1334−1519, the primers were 1435−1453 and 1526−1542.

Reaction with Kethoxal. Fifty nanomolar 30S in 55 mM Hepes (pH 7.5), 18 mM $Mg(OAc)_2$, 90 mM NH_4Cl, 3 mM DTT, and 2% ethanol was reacted with 3 mg/mL kethoxal for 5 and 10 min at 37 °C. The control reaction was performed without kethoxal for 10 min at 37 °C. Reactions were stopped with 0.27 volume of stop mix [1.14 M NaOAc, 0.12 M sodium borate, and 0.5 mg/mL total *E. coli* tRNA (pH 6)] and subunits precipitated with 3 volumes of ethanol for 10−20 min at −80 °C. After centrifugation for 30 min at 12 000 rpm and 4 °C, the pellets were resuspended in 100 μL of MB buffer [9 mM Tris-HCl (pH 7.5), 85 mM LiCl, 0.9 mM EDTA, 0.4% SDS, and 30 mM sodium borate (pH 7.0)] to a concentration of 70−75 nM RNA and extracted once with 50 μL of phenol/chloroform. The aqueous phase was removed and the phenol layer washed once with 50 μL of MB buffer. The aqueous phases were pooled and precipitated with 3 volumes of ethanol as above. Pellets were washed twice with 300 μL of 80% ethanol, centrifuged as above, and dried for 5 min *in vacuo*. Pellets were dissolved in 30 mM sodium borate at pH 7.0 to a concentration of 300 nM RNA and stored at −20 °C.

Reaction with 1-Cyclohexyl-3-(2-morpholinoethyl)carbodiimide Metho-p-toluenesulfonate (CMCT). Fifty nanomolar 30S was reacted with 21 mg/mL CMCT in 70 mM sodium borate, 100 mM NH_4Cl, 20 mM $Mg(OAc)_2$, and 3 mM DTT at a final pH of 8.14 for 3 and 6 min at 37 °C. The control reaction mixture was incubated for 6 min without CMCT. Ribosomes were added to the reaction buffer just before a 2 min incubation at 37 °C prior to CMCT addition. Reactions were stopped by the addition of 0.2 volume of 2 M NaOAc (pH 4.7), 0.5 mg/mL total tRNA, and 3 volumes of ethanol. Isolation of the total RNA was performed as described above except that borate was omitted from the MB buffer, and the final solution of the RNA was in water.

Reaction with Dimethyl Sulfate (DMS). Fifty nanomolar 30S in 55 mM Hepes (pH 7.5), 18 mM $Mg(OAc)_2$, 100 mM NH_4Cl, and 3 mM DTT was incubated with 0.0056 volume of a 1:1 (v/v) mixture of DMS and ethanol for 5 and 10 min at 37 °C. Controls were incubated for 10 min without DMS. Reactions (in 150 μL) were stopped by the addition of 25 μL of 2 M NaOAc (pH 4.7), 0.5 mg/mL total tRNA, and 3 volumes of ethanol. RNA isolation was as described above for CMCT.

Primer Hybridization and Extension with Reverse Transcriptase. One picomole of 16S RNA was hybridized with 0.5 pmol of 5′-labeled primer (about 100 000 dpm) in 6 μL of HB buffer [50 mM Hepes (pH 6.8), 100 mM KCl, and 0.1 mM EDTA]. Sodium borate (15 mM) was added for kethoxal-treated samples. Samples were heated for 3 min at 70 °C and 5 min at 37 °C, and then reactions were quenched at 0 °C for 10 min. Extension was performed at 37 °C for 30 min with 2 units of reverse transcriptase (Life Science, Inc.) and each deoxynucleotide at 0.3 mM, in a 15 μL volume containing 50 mM Tris-HCl (pH 8.5), 6 mM $MgCl_2$, 80 mM KCl, 20 mM Hepes (pH 7.0), and 0.04 mM EDTA. For kethoxal-treated samples, 6 mM borate (pH 7.0) was also present. Reactions were stopped with 50 μL of 0.3 M NaOAc (pH 5.8) containing 2 μg of total tRNA and precipitated with 3 volumes of ethanol for 20 min at −80 °C. Pellets obtained after centrifugation for 20 min at 12 000 rpm were washed twice with 80% ethanol and dried for 5 min. Pellets were then resuspended in 8 μL of formamide containing 0.03% xylene cyanol and 0.03% bromophenol blue. Dideoxy-sequencing reactions were performed under the same conditions as above with 1 pmol of 16S RNA and the appropriate ddNTP at 3 μM as described by Sanger et al. (1977).

RESULTS

Chemical Probing Results. The conformations of wild-type and mutated 16S rRNA within the 30S subunit were analyzed by primer extension with reverse transcriptase after chemical modification of the molecules at 37 °C with DMS, kethoxal, or CMCT. Segments 1−106, 406−569, 780−978, 997−1247, and 1334−1519 were probed using a set of seven primers. Altogether, 59% of the 16S rRNA molecule was analyzed. The mutants analyzed were G530U, U1498G, G1401C, C1501G, and G1401C/C1501G. Along with the mutant ribosomes, wild-type 16S rRNA was analyzed in

three different contexts, namely 30S subunits isolated from cells ("isolated"), 30S subunits reconstituted with natural 16S rRNA and total proteins from 30S (TP30) ("reconstituted"), and 30S subunits reconstituted with an *in vitro* transcript of 16S rRNA and TP30 ("synthetic") (Kryzosiak et al., 1987; Denman et al., 1989a). The results are presented in Figure 1. Note that region 1435−1542 could not be investigated in isolated and reconstituted 30S due to the presence of the methylated nucleotides m^2G1516, $m^6{}_2$A1518, and $m^6{}_2$A1519 in natural 16S rRNA which strongly inhibited reverse transcriptase extension.

Conformation of Natural 16S rRNA within Isolated or Reconstituted Ribosomes. Comparison of 16S rRNA reactivity within isolated (i) 30S subunits and reconstituted (r) 30S showed only a few differences in their chemical reactivity which were confined to the 1390−1420 region (Figure 1b, K). In this region, all G residues probed with kethoxal showed a slightly higher degree of reactivity in isolated 30S compared to that in reconstituted ribosomes, and G1405 was clearly more reactive. The reactivities of the other residues in this region were unchanged. Moreover, no changes at all were detected in the other regions probed (Figure 1a,b and data not shown). These results suggest that any conformational changes which result from reconstitution are most likely subtle. A previous study using a similar probing strategy did not find any structural difference between native and reconstituted 30S subunits (Ericson et al., 1989).

Conformation of Synthetic 16S rRNA within Reconstituted Ribosomes. The reactivity of residues in synthetic (s) 30S subunits (examples are shown in Figure 1) is summarized in Figure 2 (filled and open circles). This figure also summarizes the differences in reactivity between synthetic and isolated 30S (open and filled arrowheads) which were more pronounced than those between reconstituted and isolated 30S (see above). The most significant changes in synthetic RNA when it is compared to the isolated species are increased reactivities to the various chemicals (filled arrows) found in the vicinity of positions 15−30, 555−565, 915−920, 1080−1095, 1360−1365, and 1375−1395. These regions correspond primarily to loops or branch points in the sequence which, except for 1080−1095, are mainly concentrated in the core of the 16S rRNA molecule (Figure 2). Interestingly, the 915−920 region encompasses the central pseudoknot of the 16S rRNA, which has been shown to be essential (Brink et al., 1993). A few modest reactivity decreases were observed, the most noticeable being located near the 5′-end. These observations suggest the existence of a looser, more flexible conformation of the core region in molecules reconstituted with synthetic 16S rRNA compared to those of isolated or reconstituted particles. There were also numerous spontaneous stops of reverse transcriptase extension (x) which were not found in isolated 30S. Since the stops are not observed (or present to a much weaker extent) in the sequences performed on synthetic RNA, they most likely originated from breakages induced upon incubation of the ribosomes in the modification buffer. A higher sensitivity to spontaneous cleavages supports the proposal that 16S rRNA in synthetic ribosome has a looser conformation as deduced from the reactivity pattern obtained with chemical probes.

Because mutant ribosomes were reconstituted using synthetic rRNA, the structural effects of the mutations discussed below were compared to that of RNA isolated from ribosomes reconstituted with wild-type synthetic rRNA.

Conformation of Mutant 16S rRNA within the Reconstituted Synthetic 30S Subunit. (A) Mutant U530. In the wild-type molecule (s), G530 showed a hyper-reactivity toward kethoxal and a strong reactivity toward CMCT (Figure 1a). In the mutant 30S ribosome, where G had been replaced by U, the mutated nucleotide was still highly reactive toward CMCT but, of course, the kethoxal reactivity disappeared (Figure 1a). The reactivity of the adjacent G529 also essentially disappeared. However, as the apparent reactivity of G529 in wild-type ribosomes is probably due to stuttering at the G530 stop (Nurse et al., 1987; Denman et al., 1988; Ericson & Wollenzien, 1988; Moazed & Noller, 1990), it is not surprising that both stops disappeared together. Thus, it is unclear if the reactivity observed at G529 in mutant G530U is different from that in the wild type. The reactivity of the adjacent U531 remained unchanged. The very strong reactivity of residues G530 and U531 and the moderate reactivity of A532 demonstrate their accessibility to the solvent (Figure 3). On the contrary, the low level of reactivity of the rest of the 530 loop denotes its weak exposure to solvent and/or its involvement in an organized conformation. These data are thus in good agreement with the existence of a pseudoknot involving residues 505−507 and 524−526 and two internal base pairs G521·C528 and C522·G527 (Woese & Gutell, 1989; Powers & Noller, 1991; Gutell, 1993; Gutell et al., 1994). No changes were detected elsewhere in the RNA, indicating that mutation at 530 had essentially no effect on the other tested regions.

(B) Mutant G1498. In the wild-type sequence, residue U1498 was highly reactive to CMCT (Figure 1c) but the mutant U1498G was notably unreactive. This means that the N_3 of U1498 is exposed, but the N_1 of G1498 is blocked from reaction with CMCT. However, as a modest reaction with kethoxal, which also reacts with the N_1 of G, was observed, it appears that the N_1 of G1498 was not completely blocked. The smaller size and/or polarity difference between kethoxal and CMCT may account for the differential reactivity. The kethoxal reactivities of the adjacent G1497 and G1494 residues were also reduced in the mutant molecule, as was the CMCT reactivity of U1495, whereas A1499 became slightly more DMS reactive. A remarkable effect of the mutation was observed on the opposite strand of helix 44 in the 1400 region where G1405 displayed an enhanced reactivity toward kethoxal (Figure 1b). The rest of the probed molecule was found to be unchanged. The increases and decreases of reactivity of the various nucleotides relative to the wild-type reactivity pattern are summarized in Figure 4. The results suggest either subtle conformational rearrangements or some displacement of an equilibrium between several conformers. The observed reduction of reactivity induced by the U to G change would suggest a strengthening of interactions between the 1400/1500 regions, possibly due to an additional base pairing between G1498 and C1403 (Cunningham et al., 1993), and the resultant stabilization of the adjacent G1497·C1404 base pair. However, this stabilizing effect is accompanied by the unexpected increase of reactivity of G1405, suggesting that stable C1404·G1497 and C1407·G1494 pairs are formed to the detriment of the G1405·C1496 pair. In other words, strengthening of base pairing in one location appears to be compensated for by some relaxation of pairing elsewhere.

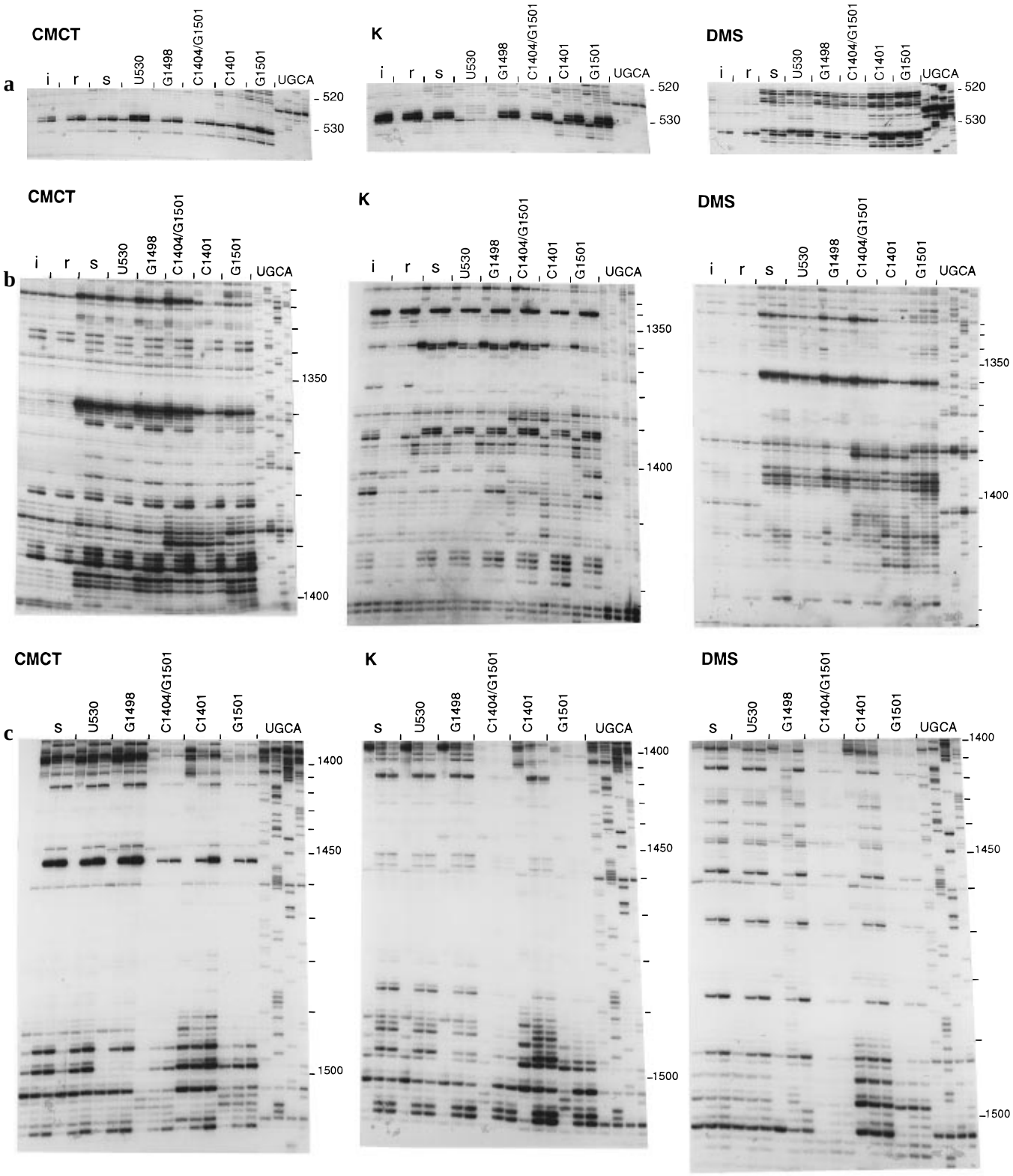

FIGURE 1: Autoradiographs showing results of 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate (CMCT), kethoxal (K), and dimethyl sulfate (DMS) probing of 16S rRNA in 30S subunits. 16S rRNA was analyzed from 30S subunits isolated from cells (i), from particles reconstituted with TP30 and natural 16S RNA (r), or from particles reconstituted with an *in vitro* transcript of wild-type 16S RNA (s) or with transcripts of the different mutant 16S RNAs (U530, G1498, C1401/G1501, C1401, and G1501). The first lane of each RNA species is a control lane without any chemical treatment. The second and third lanes are RNAs treated with the indicated reagent for the two different incubation times used with each reagent as described in Materials and Methods. Dideoxy sequencing lanes (UGCA) are shown at the right-hand side of each autoradiograph. Wild-type RNA was isolated from synthetic subunits for sequencing except for the DMS part of panel c in which was used mutant G1498: (a) region 530, (b) region 1400, and (c) region 1500. Note that mutations C1501G and G1401C/C1501G reduced the reverse transcription rate of primer 1525−1542 but not extension of the other primers used (c), probably due to less efficient hybridization. Note that this has direct consequences on the interpretation of the intensity of modifications; gel scanning was necessary to obtain data displayed in Figure 4 for G1501 and C1401/G1501 mutants. The 1500 region has two short sequences flanking the 1501 residue which can base pair to the same region as primer 1525−1542. With the mutation of C1501 to G, the segments 1498−1505 and 1535−1542 can base pair (allowing one A·C pair), making possible competition with the primer.

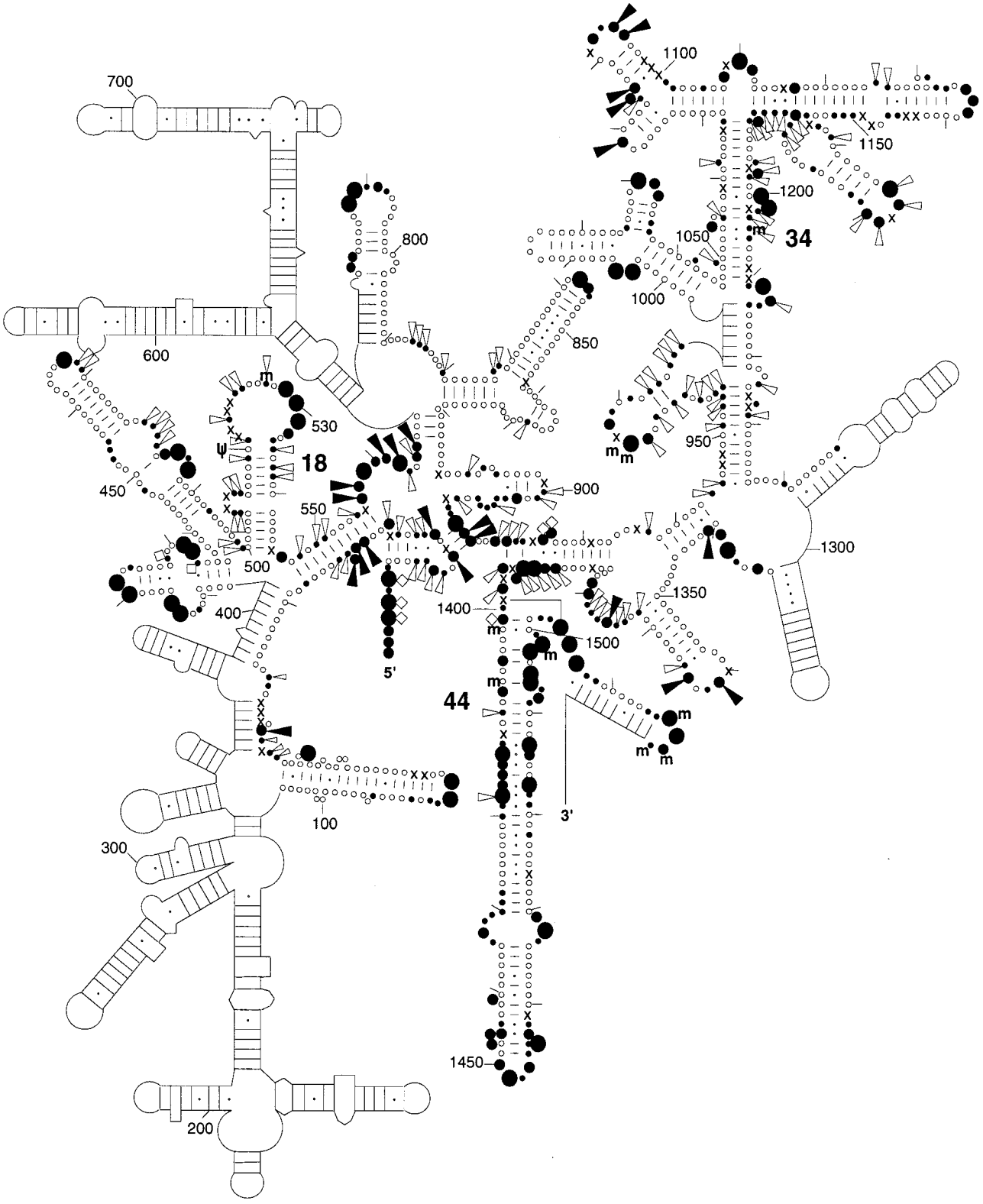

FIGURE 2: Reactivity of 16S RNA nucleotides in synthetic reconstituted 30S subunits toward chemical probes. Degrees of reactivity were defined as follows: (•) weakly reactive, (●) moderately reactive, (⬤) strongly reactive, (○) unreactive, and (×) spontaneous cleavage or pause of reverse transcriptase. Comparison of the reactivity with that of isolated 30S is shown as follows: (open arrowhead) enhancement of reactivity by 1 degree (e.g. from weak to medium reactivity) in synthetic (s) and isolated 30S (i), (filled arrowhead) enhancement of reactivity by 2 degrees (i.e. from weak to strong reactivity) in synthetic (s) and isolated 30S (i), and (◇) decrease of reactivity by 1 degree in synthetic (s) and isolated 30S (i). m is a methylated base, and Ψ is pseudo-uridine. Segments shown as line drawings were not examined. Helices of interest are numbered according to Brimacombe (1991).

(C) Mutant C1401. Mutation of G1401 to C induced the appearance of numerous reactivity changes (mostly enhancements) in the 1400 and 1500 regions, as well as in the more remote 1360 and 1375 regions. The extensive increase of reactivity, extending over 25 nucleotides on the 3′-side of helix 44 (from U1481 to U1506) and on the 5′-side of helix 44 (from A1410 to G1419), clearly demonstrates a general destabilizing effect of the G1401C mutation in this area.

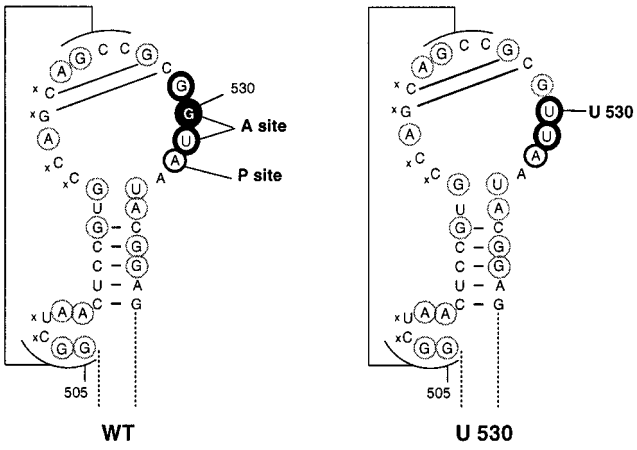

FIGURE 3: Reactivity of nucleotides of the 530 region in mutant U530 and comparison with the wild type (wt): (light circle) weak reactivity, (circle) moderate reactivity, (dark circle) strong reactivity, (●) hyper-reactivity, (×) spontaneous cleavage or pause of reverse transcriptase, (no symbol) nonreactivity.

Remarkably, this mutation also induced a decrease in reactivity in the more distant 1365 and 1375 regions; a point decrease was also observed at position U1341 (Figure 1b, CMCT and K) which became similar to the reactivity found in isolated subunits. A1398, G1405, G1486, and G1487 also showed a reduced reactivity. Despite the fact that numerous reactivities are hidden by strong stops of reverse transcription between 1400 and 1410 (Figure 1b), which already suggests that the conformation is affected by the G1401C mutation, the reactivity data clearly indicate that the single G1401C mutation induced extensive rearrangements (mainly destabilization) over a remarkably wide area on both sides of helix 44 and in the 1375 and 1360 regions (Figure 4). These results stress the role of G1401 in strengthening the conformation of helix 44, for example, by base pairing with C1501 (Cunningham et al., 1992b). The influence of the 1400/1500 region on the conformation of other regions of the 3′-end domain is also shown in the long-range effects on the 1365 and 1375 areas. Perturbations induced by the G1401C mutation might be explained by the potential for new base pairs in the mutant rRNA. For example, the stretch of five C residues between 1399 and 1404 could conceivably base pair with the run of four G residues between 1486 and 1489.

(D) Mutant G1501. The mutation C1501G also caused numerous increases of reactivity in the 1400 and 1500 region (both upstream and downstream in helix 44). In this mutant, there were also multiple stops of reverse transcriptase, although to a lesser extent than in mutant G1401C (Figure 1b,c). While C1501 was not reactive in the wild-type molecule, the mutant G residue became highly reactive, as well as the surrounding residues between G1497 and U1506. Significant changes induced by the C1501G mutation were also observed on the other side of the helix between nucleotides 1390 and 1410. All tested nucleotides between 1393 and 1405 became hyper-reactive. In addition, high-intensity, modification-independent stops of reverse transcription were observed at all C residues between 1390 and 1410. Note that the reactivity of nucleotides 1361−1365 and 1341 were similar to the ones found in isolated (i) subunits. Interestingly, both C1501G and G1401C mutations resulted in a destabilization of the 1400/1500 region and had some effect on the 1360 region. However, their effects could not be superimposed, suggesting that each of these positions is critical for maintaining the correct local and global conformation of the region. These effects support the postulated role of the proposed G1401·C1501 base pair in strengthening the overall conformation of the 1400/1500 region through tertiary interactions.

(E) Mutant C1401/G1501. The double mutant G1401C/C1501G, in contrast to the single mutants G1401C and C1501G , showed fewer reactivity changes when it was compared to wild type. Reactivity decreases at A1398 and G1405 together with the enhancement of multiple reverse transcriptase stops at the C residues were observed in the 1400 region (Figure 1b,c). On the other side of the helix, G1501 became hyper-reactive toward kethoxal (whereas its reactivity toward CMCT was weak) and positions G1504, G1505, and U1506 also became hyper-reactive. In parallel, some very strong pauses of reverse transcriptase appeared at positions 1502 and 1503. No changes were detected in the 1360 region or elsewhere in the probed regions. Comparing the reactivity pattern of G1401C/C1501G with those of the individual G1401C and C1501G mutants (Figure 4) strongly suggests that most of the conformational constraints lost in the single mutants were recovered at least in part in the double mutant.

Although the double mutant G1401C/C1501G partially compensated for the destabilizing effect of the single mutations, suggesting that base pairing between 1401 and 1501 was re-established, important differences remained, compared to the wild type (Figure 4). These differences imply that the double mutant was not able to completely restore the wild-type conformation. For example, although the main conformational constraints seem to have been restored, the hyper-reactivity of G1501 to kethoxal does not favor its involvement in a Watson−Crick interaction with C1401.

DISCUSSION

Influence of the Reconstitution Process on the Conformation of 16S RNA. The mutant ribosomes used in this study were produced by reconstitution using *in vitro*-transcribed RNA and 30S total protein (Krzyzosiak et al., 1987; Denman et al., 1989a). Initially, the influence of the reconstitution process itself on the 16S RNA conformation was determined by comparing the conformation of natural or *in vitro*-transcribed 16S RNA to the conformation of RNA in isolated 30S subunits. The data showed that the reconstitution process itself had very little effect on the conformation of 16S rRNA within 30S subunits when natural 16S rRNA was used. Only a few minor reactivity differences were detected between the 16S rRNA regions probed whether ribosomes were isolated from cells or reconstituted with natural 16S rRNA and 30S ribosomal proteins. This result indicates that the conformational changes due to reconstitution are subtle. This is consistent with the original studies (Held et al., 1973) which showed that reconstituted 30S had the same sedimentation profile and polypeptide-synthesizing activity as isolated ones, and more recent studies showing that even the partial reactions of protein synthesis were unaffected (Denman et al., 1989b; Cunningham et al., 1990, 1991). These data support the proposal that ribosome assembly is primarily dictated by its own constituents, and that isolated components of the ribosome have retained the ability to fold back to their native shape upon denaturation/renaturation.

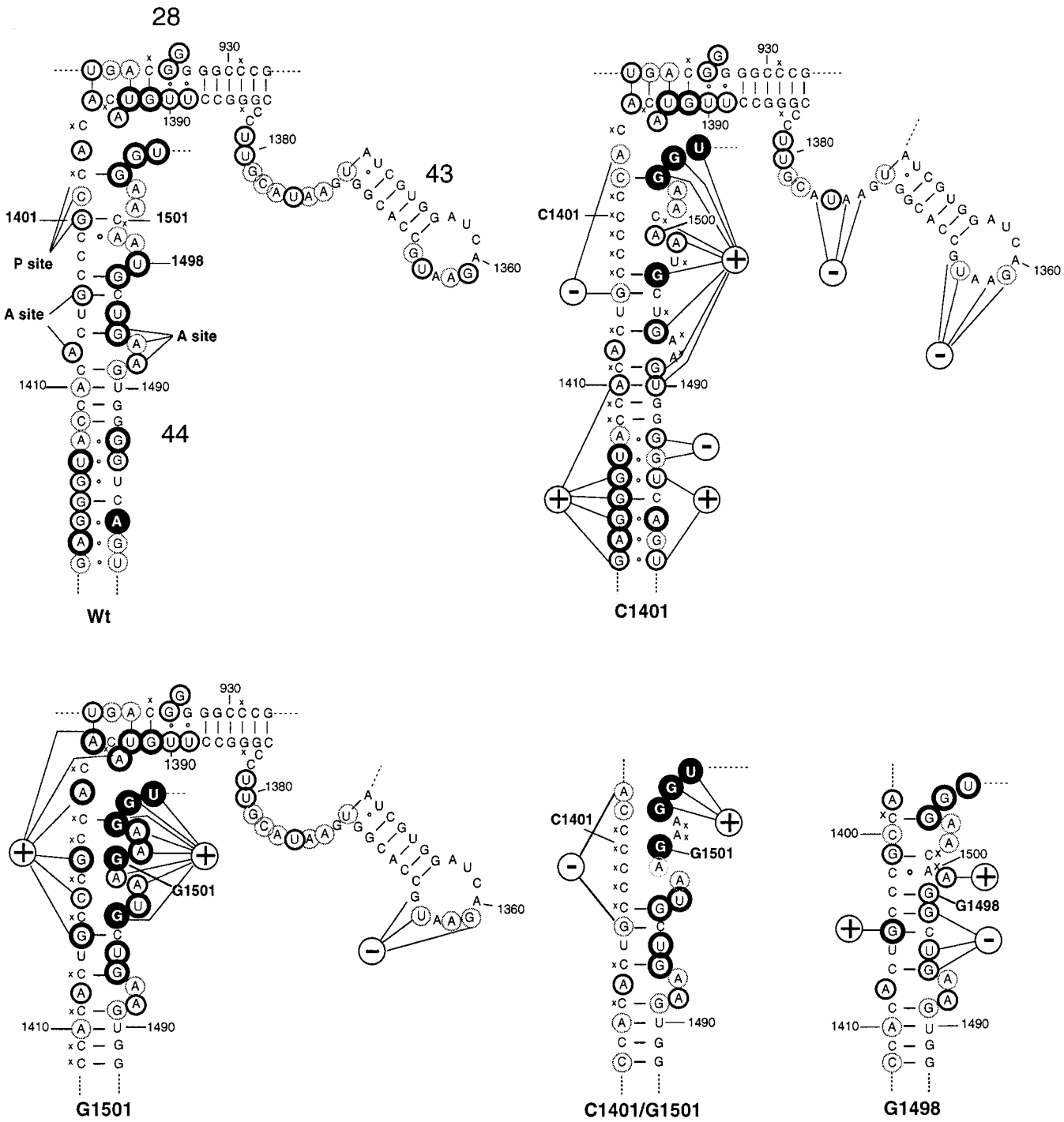

FIGURE 4: Reactivity of nucleotides of the 1400/1500 region in ribosomes reconstituted with mutant C1401, G1501, C1401/G1501, and G1498 rRNAs and comparison with the wild type (wt). Symbols are the same as those used in Figure 3. (circled plus) enhancement of reactivity in mutants and wt and (circled minus) decrease of reactivity in mutant and wt. A site and P site designate nucleotides protected by bound tRNA in the respective ribosomal binding site on 70S ribosomes (Moazed & Noller, 1990). Boldface numbers denote the mutated residues. Helices are numbered according to Brimacombe (1991) in wt.

On the other hand, ribosomes reconstituted with *in vitro*-transcribed 16S rRNA showed some noticeable increases in reactivity. These were located mainly in the core region and at certain helix junctions of the 16S rRNA. Apart from these specific reactivity differences, the rest of the tested regions were unaffected. One site showing a significant increase of reactivity in synthetic *versus* natural 16S RNA was located at the central pseudoknot (17−19/916−918). The increased reactivity in the synthetic ribosomes (Figure 2) suggests that the structure of the central pseudoknot is less stable than that in natural RNA. The central pseudoknot has been shown to be necessary for efficient translation (Brink et al., 1993; Pinard et al., 1995). Although essential functions are maintained in 30S subunits reconstituted with *in vitro*-transcribed RNA (Denman et al., 1989b; Cunningham et al., 1991), these ribosomes have a reduced activity as tested in *in vitro* translation assays compared to ribosomes isolated from cells. Our data demonstrate that synthetic 16S RNA folds into an overall conformation similar to that of natural RNA but displays a more relaxed conformation in its central core, including the central pseudoknot. Another consequence of the looser conformation is an increased frequency of

spontaneous cleavages in restricted areas of the RNA. The more relaxed structure of 16S RNA in synthetic 30S, as well as the increased rate of breakages, could be responsible for their decreased translation efficiency. It may also account for the increased size heterogeneity observed for particles reconstituted with synthetic instead of natural 16S RNA (Denman et al., 1989a; Cunningham et al., 1990, 1992a, 1993).

One possible explanation for these differences could be linked to the absence of modified bases in the *in vitro*-transcribed 16S rRNA. Indeed, several functional properties of *in vitro*-transcribed 16S RNA have already been attributed to the lack of modification (Cunningham et al., 1991; Ringquist et al., 1993). Several other studies have clearly shown the structural role of base and sugar modifications in stabilizing certain conformations, such as those responsible for the thermostability of tRNAs (Yokoyama et al., 1987; Derrick & Horowitz, 1993). Moreover, it has not yet been possible to obtain functional 50S subunits completely lacking modification (Green & Noller, 1996). Nevertheless, the importance of modified bases in 16S ribosomal RNA is still unclear. Ribosomes lacking the two characteristic m_2^6A residues can still function, albeit with slightly reduced activity in initiation of polypeptide synthesis and in maintenance of the fidelity of translation (van Knippenberg, 1986), and mutants lacking the single pseudo-uridine at position 516 (Bakin et al., 1994) are viable (L.-H. Niu and J. Ofengand, unpublished results). It should also be noted that, while some sites of modification do occur where structural relaxation is observed, there are a number of sites of relaxation without modification and some modifications where there are no relaxations (Figure 2).

Structural Relationship between the Regions of 16S RNA Involved in the Decoding Function. Many lines of evidence (including cross-linking of C1400 to the anticodon of tRNA, tRNA protection of specific bases from chemical probes, mutagenesis and their effects *in vitro* and *in vivo*, and cross-linking to mRNA positioned in the decoding site) have designated the 1400/1500 region as being at the core of the decoding center of the 30S subunit [reviewed in Zimmermann (1996)]. Two other regions, the 530 and the 1050/1200 regions, have also been proposed as essential components involved in the decoding function of the 30S subunit [reviewed in O'Connor et al. (1995)] because certain mutations in these regions are lethal *in vivo* and affect the fidelity of translation in a conditional expression system to the same extent as mutations made in the decoding center. Furthermore, the close proximity of these three regions (1400/1500, 530, and 1050/1200) was strongly suggested by their simultaneous cross-link to nucleotides of mRNA positioned in the decoding center (Dontsova et al., 1992; Rinke-Appel et al., 1994). Our data show that mutations introduced into the decoding center (G1401C and C1501G) perturbed the structure of the 1400/1500 region, with effects extending into the 1360 region, but did not affect the conformation of the other two regions, or of the rest of the molecule which was probed (60% of the total). Therefore, the decoding center 1400/1500 region, although functionally related and potentially close to 530 and 1050/1200 regions, appears to be structurally independent from the rest of the 16S rRNA. These data are consistent with the observations of Purohit and Stern (1994), who showed that the 1400/1500 decoding segment, when isolated from the rest of the molecule, can bind antibiotic, tRNA anticodon stem−loop analogues and poly(U) with similar protection patterns as in the intact 16S RNA. Thus, both sets of observations support the conclusion that the decoding center forms an independent domain within the ribosome, with an intrinsic conformation.

Structural Aspects of the 1400/1500 Region. Comparative sequence analysis (Gutell et al., 1985; Gutell & Woese, 1990), cross-linking studies (Döring et al., 1992), and site-directed mutagenesis experiments (Cunningham et al., 1992b) all indicate that interactions must take place between the 1400 and 1500 regions. Specifically, the residues at 1401 and 1501 have been proposed to base pair with one another. We observed that the disruption of the putative base pairing between positions 1401 and 1501 in mutants G1401C or C1501G resulted in an enhanced chemical reactivity in both the 1400 and 1500 regions. Conversely, the double mutant G1401C/C1501G restored to a large degree the structural constraints lost with the single mutations. These data confirm the existence of a structural link between bases 1401 and 1501 and suggest that the 1401·1501 base pair plays a critical role in the overall structure of the 1400/1500 region. The exact nature of the interaction between 1401 and 1501 remains unclear, however, as the compensatory effect of the double mutant was only partial. Incomplete restoration was also found previously in the functional studies. Whereas 30S subunits containing G1401C or C1501G were completely inactive, most functions [assembly with 50S subunits, tRNA binding in A and P sites, formation of 30S initiation complexes and reaction with puromycin, and poly(U)-directed polyphenylalanine synthesis] were all restored in the double mutant G1401C/C1501G. The only function lost was the ability to form the initial peptide bond in an fMet-tRNA-dependent protein-synthesizing system (Cunningham et al., 1992b). Since a standard Watson−Crick G·C pair can usually be inverted without a structural defect, this partial compensatory effect, found both in functional studies and in this work, by conformational probing suggests the existence of a noncanonical interaction between G1401 and C1501. Moreover, the hyper-reactivity of G1501 to kethoxal excludes a Watson−Crick interaction in the double mutant G1401C/C1501G. One possibility which would still permit kethoxal reactivity with G1501 is a noncanonical *trans* interaction involving $G(N_3)\cdot C(N_4)$ and $G(N_2)\cdot C(N_3)$ (Hou et al., 1993). This would leave $G(N_1)$ and one hydrogen atom of the N_2 amino group still available for kethoxal modification. Another difference between the wild type and the G1401C/C1501G mutant is the fact that the mutant G1501 has six potential C residues to pair with on the 3′-side of helix 44 whereas the wild-type C1501 can only partner with G1401. This could allow the formation of multiple conformational isomers in the double mutant. Alternatively, the 1401−1501 interaction might be a triple-base interaction involving another nucleotide in the immediate vicinity which cannot be formed correctly in the double mutant.

The existence of a dynamic interaction between the 1400 and 1500 regions is suggested by the effect of mutant U1498G. This mutant was created to stabilize the interaction between residues 1403 and 1498, and our data show that it does. Surprisingly, although U1498G has the same functional behavior as G1401C/C1501G, its reactivity pattern is clearly distinguishable from that of the G1401C/C1501G mutant. In both mutants, the loss of function may be due to perturbation of a dynamic equilibrium involving the interac-

tion of the 1400 and 1500 regions. For example, the equilibrium itself, or one specific conformer required for a specific function, could be perturbed by the stabilization of an inactive conformation, leading to a specific inactivation of the decoding center. A similar effect was recently described within a segment of RNA corresponding to the A site of 16S RNA where the binding of paromomycin (an antibiotic known to perturb the decoding process) was shown to stabilize a given conformation (Fourmy et al., 1996).

The 530 Region. Our results showed that the defect in the activity of the ribosome caused by the G530 to U mutation is not due to a perturbation of the 16S RNA global conformation. The mutation of G to U at position 530 had no consequence for the conformation of 16S RNA in the 530 region or for the rest of the probed 16S RNA (Figure 3). This result is in contrast to mutations at G517 which exposed residues 530−532 (Van Ryk & Dahlberg, 1995). Conversely, destabilization of the 1400/1500 region had no effect on the reactivity of this region. Hence, the very nature of the base at position 530 is directly responsible for the effects caused by mutation at this position. The U530 mutation is responsible for a lethal phenotype *in vivo* (Powers & Noller, 1990) and induces misreading in a conditional expression system (O'Connor et al., 1992). It abolishes polypeptide synthesis when directed by a natural mRNA *in vitro*, although assembly with 50S, A and P site tRNA binding, formation of 30S initiation complexes and reaction with puromycin, and poly(U)-directed polyphenylalanine synthesis are preserved (Santer et al., 1993). Several ligands of the ribosome such as tRNA (Moazed & Noller, 1990) and IF1 (Moazed et al., 1995) have been shown to cause protections in the 530 region. Our results further stress the direct involvement of base G530 in the decoding process and support the proposal that G530 forms an essential and direct contact with one ligand or several ligands of the translation apparatus (such as tRNA and/or initiation factor I). Although the location of region 530 in the 30S subunit is still subject to considerable controversy, recent data favor its physical juxtaposition to the decoding center [reviewed in Brimacombe (1995)]. Nevertheless, our data clearly showed that this region was not influenced by a serious destabilization of the 1400/1500 region, indicating again that these regions, although functionally linked and probably topographically close, are likely to be structurally independent.

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