Interaction Sites of Ribosome-Bound Eukaryotic Elongation Factor 2 in 18S and 28S rRNA†

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ABSTRACT: The involvement of ribosomal RNA in the binding of eukaryotic elongation factor eEF-2 to the ribosome was investigated. eEF-2 was complexed to empty reassociated 80S ribosomes in the presence of the nonhydrolyzable GTP analogue Guo*PP*[CH_2]*P*. The formed complex was treated with dimethyl sulfate, 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate, and micrococcus nuclease to allow specific modification at single-stranded regions of the rRNAs. The sites of modification were localized by primer extension using complementary deoxynucleotide primers and reverse transcriptase. The modification pattern was compared to that obtained from 80S ribosomes lacking bound eEF-2. Binding of the factor to the ribosome resulted in the protection of specific sites in both 18S and 28S rRNA, while the reactivity of 5.8S rRNA was unchanged. In 18S rRNA, the affected nucleotides were localized to the 5′- and 3′-domains, and in 28S rRNA the protected nucleotides were seen in domains II, IV, and V. The α-sarcin/ricin loop in domain VI of 28S rRNA was inaccessible for chemical modification even in the absence of bound eEF-2. However, the bound factor protected A^{4256}, located in the α-sarcin/ricin loop, from ricin-induced depurination.

During protein synthesis the ribosome reciprocates between a pre- and a posttranslocation conformation. The pretranslocation form of the ribosome has affinity for elongation factor eEF-2,[1] whereas the posttranslocation form binds eEF-1α [for a review, see Nygård and Nilsson (1990)]. The two elongation factors bind to the ribosome at partially overlapping sites located at the ribosomal interface (Nolan *et al.*, 1974; Uchiumi & Ogata, 1986; Uchiumi *et al.*, 1986; Nygård *et al.*, 1987). The interface is particularly rich in RNA (Kühlbrandt & Unwin, 1982), and sequences of both major RNA species are juxtaposed in this region (Mitchell *et al.*, 1992). The rRNA is considered to play a direct role in the ribosomal function during the translation process [for a review, see Noller (1991)]. Cross-linking experiments as well as chemical footprinting studies performed in prokaryotes have shown that EF-Tu and EF-G, homologous to eEF-1α and eEF-2, respectively, interact with two specific sequences in 23S rRNA (Sköld, 1983; Moazed *et al.*, 1988). Although the eukaryotic elongation factors have affinity for RNA (Ovchinikov *et al.*, 1978), no direct evidence for an involvement of the major ribosomal RNA species in the binding of elongation factors to the ribosome has been reported. Nevertheless, indirect evidence suggests that eEF-2 interacts with 28S rRNA (Uchiumi *et al.*, 1991; Nilsson & Nygård, 1986).

In this report we have studied the possible interaction of ribosome-bound eEF-2 with 18S, 5.8S, and 28S rRNA using chemical and enzymatic footprinting of the rRNA. The results show that eEF-2 bound to the ribosome in the presence of the nonhydrolyzable GTP analogue Guo*PP*[CH_2]-*P* specifically protects bases in both 18S and 28S rRNA from chemical and enzymatic modification. The affected sites are found in phylogenetically conserved regions of both rRNA species.

MATERIALS AND METHODS

(a) Chemicals. Dimethyl sulfate and CMCT were from Aldrich Chemie (Germany). Micrococcus nuclease, Guo*PP*[CH_2]*P*, and deoxy and dideoxy nucleotides were from Boehringer Mannheim (Germany). [γ-^{32}P]ATP and T4 polynucleotide kinase were from Amersham International (U.K.). Superscript reverse transcriptase was from Life Technologies, Inc. eEF-2 was prepared as previously described (Nilsson & Nygård, 1984). Initiator tRNA, [^{3}H]-Met-$tRNA_f$, and [^{14}C]Phe-tRNA were prepared as described (Nygård & Hultin, 1976). eEF-1α was a gift from Dr. R. Amons, University of Leiden, The Netherlands. DNA primers were synthesized according to Caruthers *et al.* (1992). The target sequences used for primer annealing were those previously described by Holmberg *et al.* (1994a).

(b) Preparation of Ribosomal Subunits. Ribosomes from mouse Ehrlich ascites cells were prepared as described by Sundkvist and Staehelin (1975). The isolated ribosomes were dissociated into 40S and 60S subunits, and the derived subunits were isolated as described by Nygård and Nika (1982). The isolated subunits were pelleted by centrifugation and suspended in 0.25 M sucrose, 70 mM KCl, 30 mM Hepes/KOH, pH 7.6, 2 mM $Mg(CH_3COO)_2$, and 5 mM β-mercaptoethanol to a concentration of 6 μM. The subunits were frozen and stored at −80 °C until used.

The ribosomes were treated with ricin according to Nilsson and Nygård (1986) where indicated.

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[1] Abbreviations: CMCT, 1-cyclohexyl-3-(morpholinoethyl)carbodiimide metho-*p*-toluene sulfonate; DMS, dimethyl sulfate; eEF-1α and eEF-2, eukaryotic elongation factors; EF-Tu and EF-G, prokaryotic protein synthesis elongation factors; Guo*PP*[CH_2]*P*, guanosine 5′-[β,γ-methylene]triphosphate; MNase, micrococcus nuclease.

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(c) Analysis of the Functional Activity of the Isolated Subunits. EF-1α- and poly(U)-dependent binding of [^{14}C]-Phe-tRNA to 80S ribosomes and poly(U)-directed synthesis of polyphenylalanine were determined as described by Janssen *et al.* (1990). Translation of globin mRNA was as previously described (Nygård & Westermann, 1982). Binding of initiator tRNA, Met-tRNA$_f$, to 80S ribosomes was according to Nygård and Hultin (1977).

(d) Formation of Ribosomal Complexes Containing eEF-2. Complete 80S ribosomes were formed from isolated subunits by mixing 120 pmol of 40S and 120 pmol of 60S in a buffer containing 0.1 M sucrose, 7.5 mM $MgCl_2$, 1 mM dithiothreitol, 42 mM Hepes/KOH, pH 7.6, and 150 mM KCl. After incubation for 10 min at 37 °C, eEF-2 and Guo*PP*[CH_2]*P* at final concentrations of 1 μM and 0.5 mM, respectively, were added and the incubation was continued for 10 min at 37 °C (Nygård & Nilsson, 1989). Control samples without eEF-2 were incubated in parallel.

For analysis of the efficiency of eEF-2 binding to the ribosome, ribosomal complexes, formed as described above but containing ^{125}I-labeled eEF-2, were analyzed by gradient centrifugation as previously described (Nygård & Nilsson, 1984).

eEF-2- and ribosome-dependent GTP hydrolase activity was determined as previously described (Nygård & Nilsson, 1989).

(e) Modification of rRNA. Modification of rRNA was as previously described (Holmberg *et al.*, 1992). The modifying reagent was added to the samples containing assembled control 80S ribosomes or 80S ribosomes in a 1:1 complex with eEF-2·Guo*PP*[CH_2]*P*. The reagents DMS and CMCT were added at a final concentration of 20 or 90 μM for DMS and 20 or 100 mM for CMCT. Enzymatic cleavage with MNase was performed at 0.2 or 0.9 μM of the enzyme (Holmberg *et al.*, 1992). Control samples were incubated without modifying reagents but were otherwise treated identically.

The chemical reagents specifically modify single-strand bases in the rRNA. DMS modifies adenines and cytosines, while CMCT modifies uridines and guanines (Ehresmann *et al.*, 1987). Additionally, at pH 7.6 CMCT modified single-strand cytosines. MNase is single strand specific and preferentially cleaves the phosphate backbone at the 5′-side of adenine or uridine (Cuatrecasas *et al.*, 1967).

(f) RNA Extraction and Identification of the Modification Sites. The RNA was extracted with phenol at pH 9 (Brawerman *et al.*, 1972). The extracted material was precipitated by ethanol, collected by centrifugation, and dissolved in H_2O. The RNA concentrations of the samples were adjusted to 1 pmol/μL, and the material was stored in small aliquotes at −80 °C.

End-labeling of the cDNA primers, primer extension, dideoxy sequencing, gel electrophoresis, and autoradiography were as previously described (Holmberg *et al.*, 1992, 1994a). The autoradiograms were quantified using a computer-assisted image analysis system.

RESULTS

For analysis of the interaction between rRNA and eukaryotic elongation factor eEF-2 on the ribosome it was essential to ensure that the 80S ribosomes used in the study were functionally active. The 80S ribosomes formed from derived 40S and 60S subunits bound 0.8 Phe-tRNA per ribosome in response to poly(U) and eEF-1α and 0.7 Met-tRNA$_f$ per ribosome in the presence of globin mRNA and initiation factors from rabbit reticulocytes. Furthermore the 80S ribosomes translated both poly(U) and globin mRNA (Nygård & Westermann, 1982).

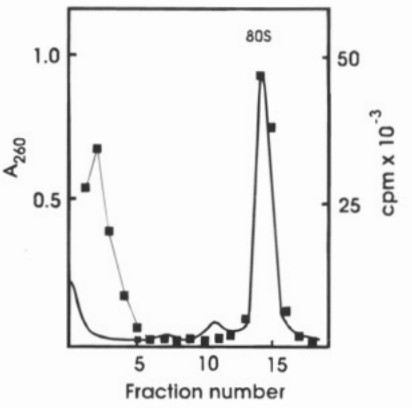

FIGURE 1: Interaction of eEF-2 with reassociated Ehrlich ascites cell 80S ribosomes. Purified eEF-2 (1 μM), labeled with ^{125}I (specific activity, 4500 cpm/pmol) was complexed to reassociated 80S ribosomes (0.5 μM) in the presence of the nonhydrolyzable GTP analogue Guo*PP*[CH_2]*P* (0.5 mM). (—) Absorbency at 260 nm; (■) radioactivity. Sedimentation is from left to right.

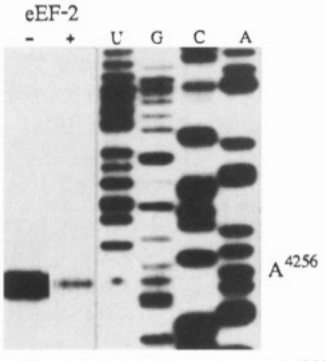

FIGURE 2: Ricin-dependent depurination of A^{4256} in the absence (−) and in the presence (+) of eEF-2. Empty 80S ribosomes or 80S ribosomes complexed to eEF-2 as described in Materials and Methods were incubated with 12 pmol of ricin for 10 min at 37 °C. Lanes U, G, C, and A are sequencing lanes.

The capability of the ribosomes to bind eEF-2 in a functionally relevant complex was of particular concern for this study. As seen in Figure 1, the 80S ribosomes were able to bind 0.85 eEF-2 per 80S ribosome in the presence of purified eEF-2 and the nonhydrolyzable GTP analogue Guo*PP*[CH_2]*P*. This is consistent with our previous observations (Nygård & Nilsson, 1984). Interaction of eEF-2 with the ribosome resulted in an activation of the ribosome and factor-dependent GTP hydrolase (Nygård & Nilsson, 1989). The functional relevance of the formed eEF-2·ribosome complex was further studied using the *N*-glycosidase ricin (Endo *et al.*, 1987). Ricin catalyzed a quantitative depurination of A^{4256} (Figure 2) located in the so-called α-sarcin/ricin loop in domain VI of 28S rRNA. The depurination resulted in a total loss of the eEF-2 binding capacity of the 80S ribosomes (Nilsson & Nygård, 1986). However, after formation of the eEF-2·Guo*PP*[CH_2]*P*·80S complex the nucleotide A^{4256} was completely protected from the catalytic action of ricin (Figure, 2), suggesting that eEF-2 formed a functionally homogeneous complex with the ribosomes.

We have studied the possible interaction between eEF-2 and rRNA using chemical (DMS and CMCT) and enzymatic (MNase) footprinting. The eEF-2-dependent changes in the

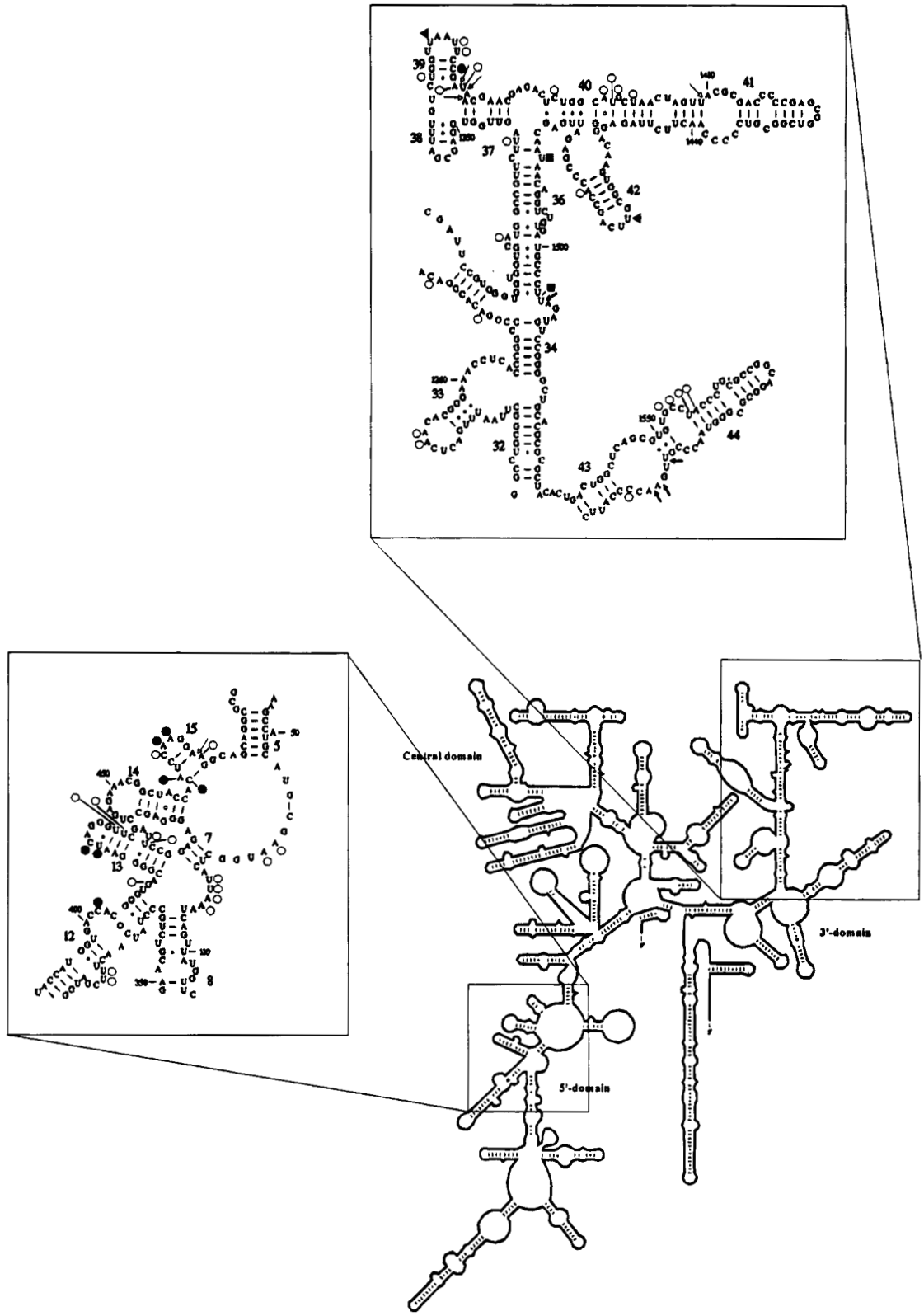

FIGURE 3: Secondary structure model of 18S rRNA (Gutell *et al.*, 1993), modified by Holmberg *et al.* (1994a), summarizing the eEF-2-dependent changes in the modification pattern of the rRNA and showing bases whose reactivities were reduced by 40−60% (▲) or by more than 60% (●) after formation of the eEF-2·Guo*PP*[CH_2]*P*·80S ribosome complex, bases totally protected from chemical modification (■) in the eEF-2·ribosome complex, phosphodiester bonds protected against MNase cleavage (solid arrows), reactive bases not affected by the formation of the eEF-2·ribosome complex (○), and phosphodiester bonds not affected by the complex (open arrows).

chemical and enzymatic modification patterns of the rRNAs were analyzed by primer extension. Binding of eEF-2 to the ribosome altered the accessibility of 18S and 28S rRNA for chemical and enzymatic modification, while the modification pattern of 5.8S rRNA was unaffected.

(I) 18S rRNA

We have found that approximately 10% of the bases in 18S rRNA are accessible to modification by DMS and CMCT within reassociated 80S ribosomes. Formation of

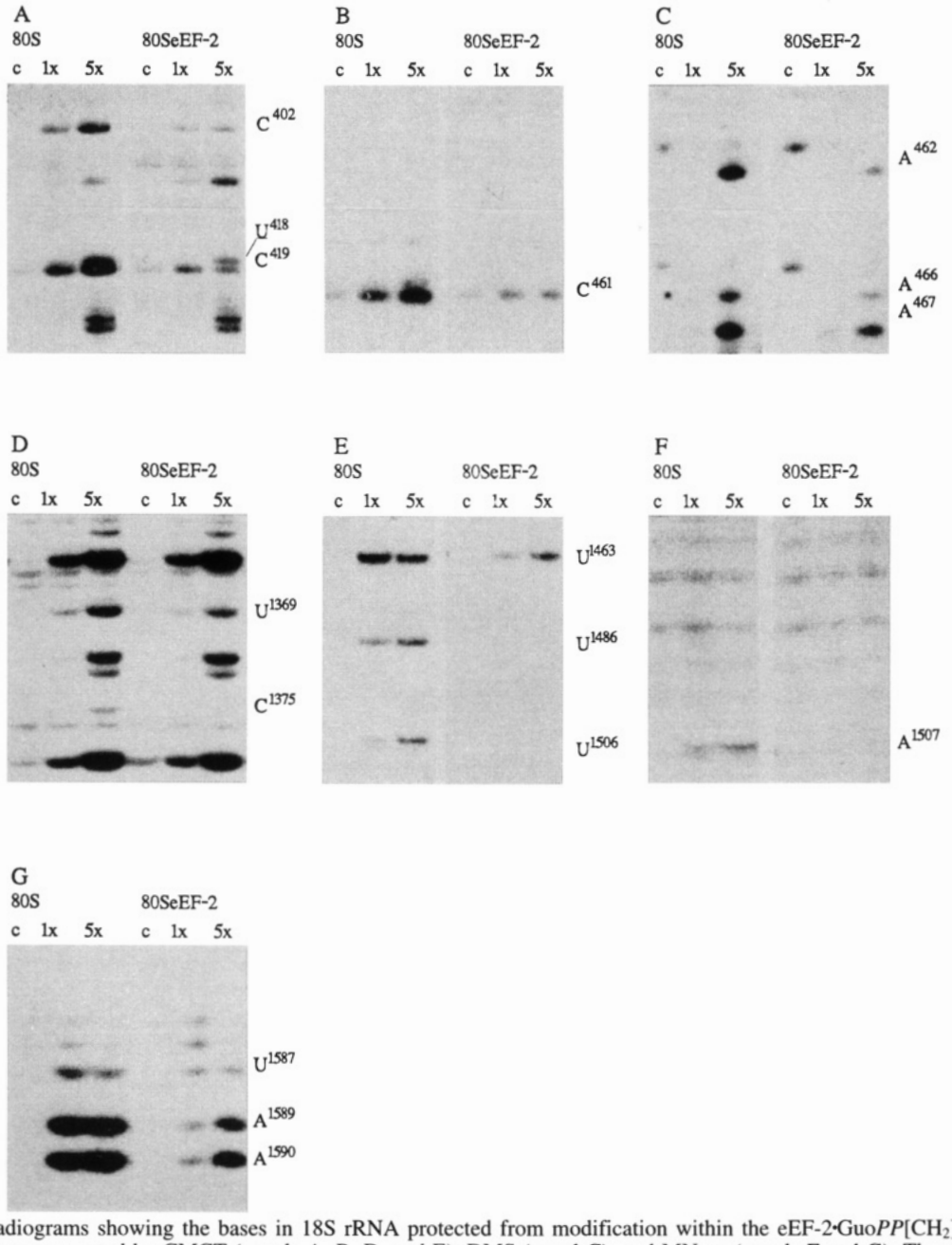

FIGURE 4: Autoradiograms showing the bases in 18S rRNA protected from modification within the eEF-2•Guo*PP*[CH_2]*P*•80S complex: modification patterns generated by CMCT (panels A, B, D, and E), DMS (panel C), and MNase (panels F and G). The concentrations of modifying reagents used were 20 and 100 mM for CMCT, 20 and 90 μM for DMS, and 0.2 and 0.9 μM for MNase, corresponding to 1× and 5×, respectively. Control samples (c) were incubated without modifying reagent.

the eEF-2•Guo*PP*[CH_2]*P*•80S complex specifically reduced the chemical reactivity of 11 bases in 18S rRNA. The affected nucleotides were found in the 5′- and 3′-domains, while the reactivity of the central domain was unaffected by the eEF-2-ribosome interaction. The results are summarized in the secondary structure model for mouse Ehrlich ascites 18S rRNA shown in Figure 3.

(a) The 5′-Domain. In the 5′-domain, eEF-2-dependent reactivity changes were seen in hairpins 12−15 (Figure 3). eEF-2 protected C^{402} (Figure 4a), located in the internal bulge in hairpin 12, from modification. Binding of eEF-2 to the ribosome also reduced the reactivities of bases U^{418} and C^{419}, located in the apical loop of hairpin 13 (Figure 4a). No eEF-2-dependent effects on hairpin 14 could be observed, as this hairpin was inaccessible for DMS and CMCT modification in both empty and eEF-2-containing 80S ribosomes. However, extensive eEF-2-dependent protection was seen at the adjacent side of hairpin 15. Here, C^{461} and A^{462}, located in the stem, and A^{466} and A^{467}, positioned in the apical loop, showed reduced exposure to chemical modification as a result of the eEF-2−ribosome interaction (Figure 4b,c). Hairpin 15 contained two additional reactive sites, one in the apical loop and one at the opposite side of the stem, which were not affected by the factor−ribosome interaction. The latter side of hairpin 15 contained the only cleavage site (between A^{470} and A^{471}) for MNase found in hairpins 12−15 (Figure 3). The accessibility of this site was not influenced by formation of the eEF-2•ribosome complex.

(b) The 3′-Domain. Binding of eEF-2 to the ribosome reduced the chemical reactivity of bases in helix 36 and in hairpins 39, 42, and 43 (Figure 3). The apical loop of helix 42 contained one base (U^{1463}) that was exposed to chemical modification. The reactivity of this base was decreased after formation of the eEF-2•ribosome complex (Figure 4e). In

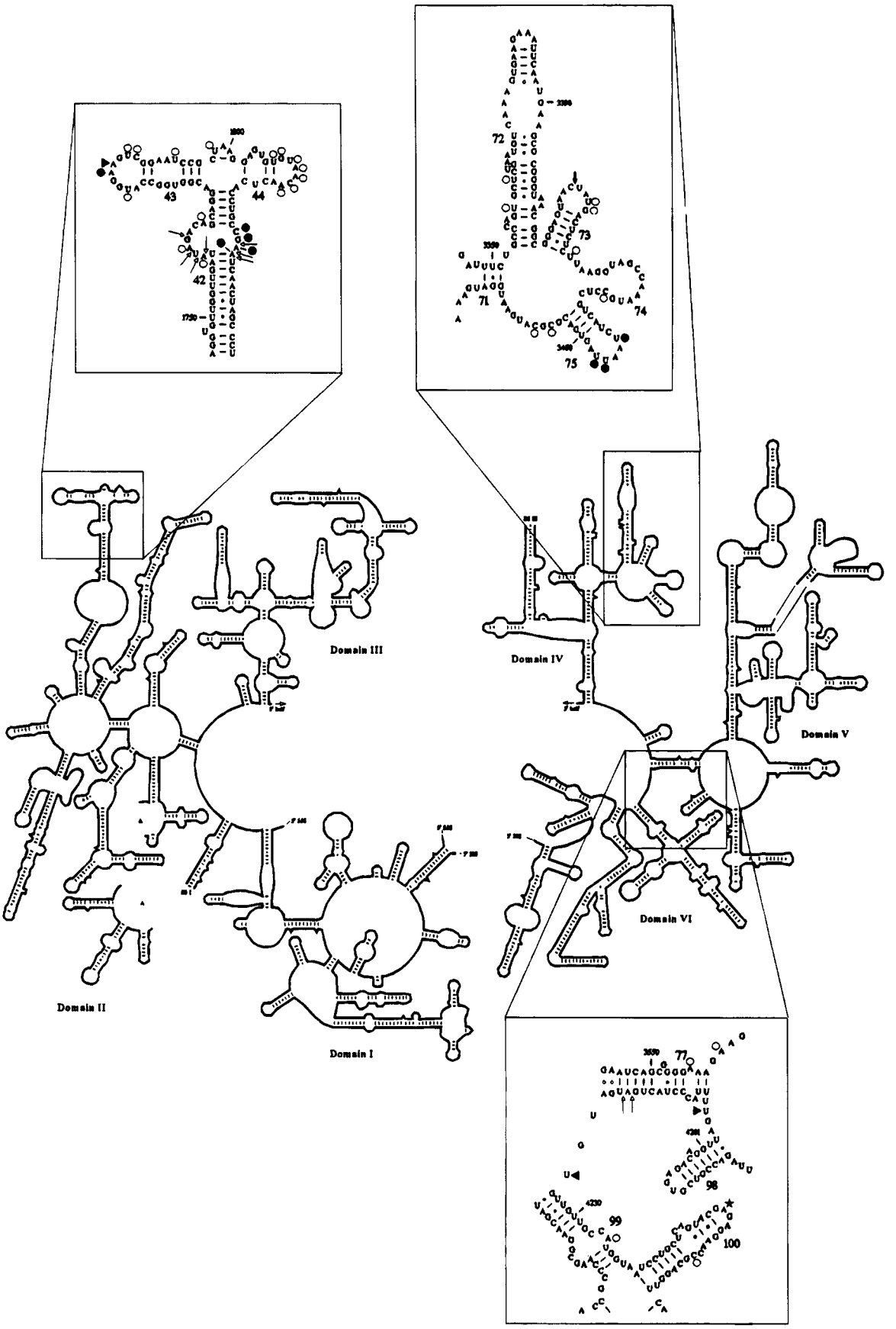

FIGURE 5: Secondary structure model of 28S rRNA (Gutell *et al.* 1993) as modified by Holmberg *et al.*, (1994a), showing the localization of the bases whose reactivities were affected by the formation of the eEF-2·ribosome complex. Bases exhibiting eEF-2-dependent protection against ricin-catalyzed depurination (★). The other accessibility changes are symbolized as in Figure 3.

empty 80S ribosomes helix 36 contained four chemically reactive bases. Two of these bases, U^{1486} and U^{1506}, located at the same side of the helix became completely protected from modification in the ribosome·eEF-2 complex (Figure 4e), while the reactivity of the two sites in the opposite strand was unaffected. Interestingly, binding of eEF-2 to the

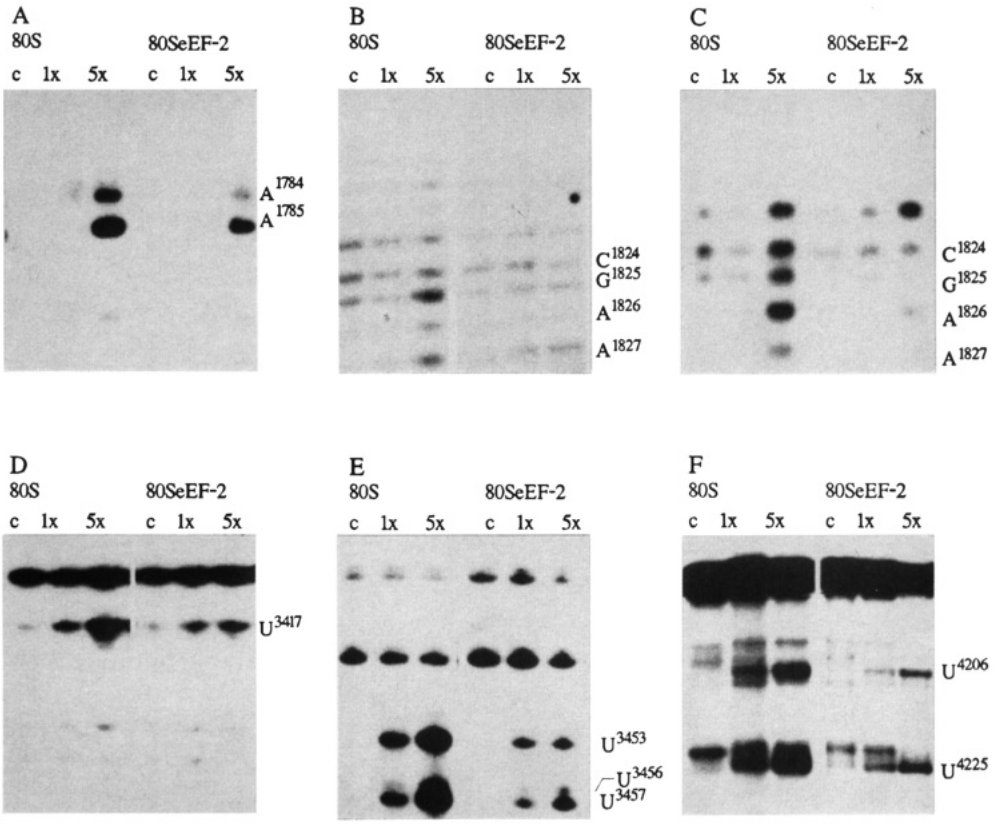

FIGURE 6: Autoradiograms showing the effect of eEF-2 on the reactivity of bases in 28S rRNA: chemical and enzymatic modification patterns generated by DMS (panels A and C), CMCT (panels B, E and F), and MNase (panel D). Refer to Figure 4 for details.

ribosome also gave a complete protection of the phosphodiester bond between U^{1506} and A^{1507} from MNase digestion (Figure 4f).

In empty reassociated 80S ribosomes hairpin 39 was modified by DMS and CMCT at seven positions. After formation of the eEF-2·ribosome complex, the reactivity of nucleotide U^{1369}, located in the apical loop, was slightly reduced, while C^{1375}, located in the stem, showed substantially reduced reactivity (Figure 4d). The other reactive bases in the hairpin were still accessible for modification in the eEF-2·ribosome complex. Analysis of the MNase sensitivity showed that hairpin 39 was not accessible for cleavage. However, the adjacent interhelical sequence contained three consecutive phosphodiester bonds in the sequence A^{1377}-UAA^{1380} that were efficiently cleaved by the enzyme in both empty and eEF-2-containing ribosomes.

eEF-2-dependent protection of the phosphate backbone of the rRNA was detected in helix 43, however, where the three phosphodiester bonds (between U^{1586} and U^{1587} and in the sequence $G^{1588}AA^{1590}$) in the internal loop of helix 43 were considerably less exposed to MNase cleavage after formation of the eEF-2·Guo*PP*[CH_2]*P*·80S complex (Figure 4g). This part of the helix was not accessible for chemical modification (Figure 3).

(II) 28S rRNA

The chemical modification experiments show that approximately 10% of the bases in 5.8S/28S rRNA are reactive to DMS and CMCT in reassociated 80S ribosomes. Formation of the 80S·eEF-2·Guo*PP*[CH_2]*P* complex was associated with distinct changes in the chemical modification patterns of domains II, IV, and V, while the other three domains and 5.8S rRNA were unaffected. All affected sites showed decreased reactivity in the factor−ribosome complex. The results are summarized in the secondary structure model for 28S rRNA shown in Figure 5.

(a) Domain II. Binding of eEF-2 to the ribosome partly protected helix 42 and the apical loop of hairpin 43 from chemical modification (Figure 5). The apical loop of hairpin 43 contained five bases that were available for chemical modification in empty reassociated 80S ribosomes. Two of these, A^{1784} and A^{1785}, became protected or slightly protected, respectively, from modification by eEF-2 (Figure 6a). Similarly, binding of eEF-2 to the ribosome resulted in a general protection of the side of the internal bulge in helix 42 that contained bases $C^{1824}GAA^{1827}$ (Figure 6b,c), while the three exposed bases located at the opposite side of the bulge were still available for modification in the eEF-2·ribosome complex.

An analysis of the MNase sensitivity of this part of the 28S rRNA showed that hairpins 43 and 44 were totally inaccessible to the enzyme, while the internal bulge in helix 42 contained four very accessible sites (in the sequence U^{1758}-AUA^{1761} and between nucleotides G^{1762} and A^{1763}) and three partly accessible phosphodiester bonds in the opposite side of the bulge (in the sequence $G^{1825}AAU^{1828}$) (Figure 5). None of these sites showed altered exposure to MNase after formation of the eEF-2·ribosome complex.

(b) Domain IV. In control 80S ribosomes the single-stranded regions of the helix loop arrangement containing hairpins 73−75 displayed a total of seven reactive bases (Figure 5). Three of these sites were located in the apical loop of hairpin 75. Formation of the eEF-2·Guo*PP*[CH_2]-*P*·80S complex drastically reduced the chemical reactivity of this hairpin as seen by the protection of nucleotides U^{3453}, U^{3456}, and U^{3457} (Figure 6e).

An analysis of the MNase sensitivity of the same region of the 28S rRNA showed that the sequence contained one

site that was sensitive to enzymatic cleavage. The site was positioned in the apical loop of helix 73 and involved the phosphodiester bond between bases C^{3416} and U^{3417}. After formation of the eEF-2·Guo*PP*[CH_2]*P*·ribosome complex the site was protected from MNase cleavage (Figure 6d).

(c) Domain V. The central ring of domain V was moderately available for chemical modification in the empty reassociated 80S ribosomes. After formation of the eEF-2−80S complex the only accessible nucleotide in the 3′-end of the ring, U^{4206}, became slightly protected from chemical modification (Figure 6f). Furthermore, U^{4225}, in the interhelical region between domains V and VI, became partly protected from modification after factor−ribosome interaction (Figure 6f). In the 80S ribosomes this nucleotide was the only modified base in the sequence.

Analysis of the MNase sensitivity of the eEF-2-affected region of domain V revealed two partly accessible phosphodiester bonds in the sequence $G^{4218}AU^{4220}$ located in helix 77 (Figure 5). The accessibility of these sites was not affected by the formation of the eEF-2·ribosome complex.

DISCUSSION

Chemical footprinting experiments performed in prokaryotes have shown that ribosomal RNA is directly involved in the interaction between the ribosome and the elongation factors (Moazed *et al.*, 1988). The footprinting data presented here show that specific sequences of the eukaryotic rRNAs are also protected against chemical and enzymatic modification by ribosome-bound eEF-2. However, the eEF-2-induced footprinting pattern was only partly identical with that reported for the prokaryotic homologue EF-G. First, eEF-2 protected sequences in both 18S and 28S rRNA, whereas EF-G only protects bases in 23S rRNA (Moazed *et al.*, 1988). Second, the sequences protected by eEF-2 in 28S rRNA were only partly homologous to those protected by EF-G.

Differences between the elongation factor binding sites on prokaryotic and eukaryotic ribosomes have also been reported using other experimental techniques. Chemical cross-linking performed in prokaryotes shows that 23S rRNA is located in close proximity to EF-G (Sköld, 1983), whereas attempts to cross-link eEF-2 to 18S or 28S rRNA in eukaryotes using the same bifunctional reagent have been unsuccessful (Nygård & Nilsson, 1987). However, eEF-2 can be cross-linked to 5S rRNA, and ribosome-bound factor also gives specific chemical and enzymatic footprints on 5S rRNA (Holmberg *et al.*, 1992).

The eEF-2-dependent footprints observed in 18S and 28S rRNA could result from a direct interaction of the factor with specific RNA sequences and/or conformational changes in the ribosomal RNA induced by the factor−ribosome interaction. The chemical and enzymatic footprinting method cannot distinguish between these two possibilities. Binding of eEF-2 to the ribosome only affected the reactivity of a few nucleotides, located in sequences that are part of putative functional domains in the ribosome (cf. below). The only exception to this rule was the eEF-2-dependent protection seen in hairpins 12−15 in 18S rRNA. However, the reactivity of the homologous region in prokaryotic 16S rRNA is affected by initiation factor 2, although the observed footprints were suggested to be unspecific (Wakao *et al.*, 1990).

(a) Involvement of 18S rRNA in the Binding of eEF-2 to the Ribosome. Several lines of evidence indicate that the elongation factors interact with the small ribosomal subunit. Elongation factor G was found to give unspecific cross-links to 16S rRNA (Sköld, 1983). Furthermore, cross-linking experiments have shown that ribosome-bound eEF-2/EF-G interacts with proteins from both the large and the small subunits (Sköld, 1982; Uchiumi *et al.*, 1986; Nygård *et al.*, 1987). In eukaryotes high-affinity interaction between eEF-2 and the ribosome requires both ribosomal subunits, as does efficient eEF-2- and ribosome-dependent hydrolysis of GTP (Nygård & Nilsson, 1989). Removal of base A^{4256}, located in the apical loop of hairpin 100 in 28S rRNA, by treatment with the *N*-glycosidase ricin (Endo *et al.*, 1987) has a conspicuous effect on the eEF-2-dependent GTP hydrolysis but only in the presence of the small ribosomal subunit (Nygård & Nilsson, 1989). Similarly, mutations in the homologous loop in prokaryotic 23S rRNA (G^{2661}) only affect the elongation process if combined with restrictive mutations in ribosomal protein S12 (Tapprich & Dahlberg, 1990), a protein that interacts with the conserved 530-loop in 16S rRNA (Stern *et al.*, 1989). Mutation of G^{530} (homologous to mouse G^{627}) affects A-site-related functions such as the ability of the ribosome to interact with EF-Tu during binding of aminoacyl-tRNA to the A-site (Santer *et al.*, 1993; Powers & Noller 1993). Thus, the data suggest that elements on both subunits act in concert to define the ribosomal domain responsible for elongation factor interaction.

(b) Helices 42−44 in 28S rRNA. Helices 43 and 44 are structurally and functionally conserved (Raué *et al.*, 1990), and the yeast and *E. coli* rRNA structures are mutually exchangeable without loss of ribosomal function (Raué *et al.*, 1990; Thompson *et al.*, 1993). The available experimental data suggest that this region of the 23/28S rRNA is involved in binding the translocation factors eEF-2/EF-G.

Interaction of eEF-2 with the 80S ribosome resulted in a protection of nucleotides in helices 42 and 43. Footprinting experiments performed in prokaryotes have also demonstrated an EF-G-dependent protection of the loop homologous to the apical loop of helix 43 (Moazed *et al.*, 1988). Furthermore, a human antibody, specific for helices 43 and 44, inhibits binding of both eEF-1α and eEF-2 to 80S ribosomes (Uchiumi *et al.*, 1991). In prokaryotes, a direct interaction between EF-G and the rRNA has been shown by cross-linking (Sköld, 1983). Moreover, in *E. coli*, helices 42−44 are involved in binding ribosomal protein L11 and the pentameric complex L10(L7/L12)$_4$ (Egebjerg *et al.*, 1990). Protein L12 has also been directly cross-linked to EF-G (Sköld, 1982). Prokaryotic ribosomal proteins L11 and L12 are homologous to mammalian ribosomal proteins L12 and P2, respectively (Suzuki *et al.*, 1990; Wool *et al.*, 1991), two proteins that have been directly cross-linked to eukaryotic elongation factor eEF-2 (Uchiumi *et al.*, 1986).

(c) The α-Sarcin/Ricin Loop. Domain VI contains the so-called α-sarcin/ricin loop in hairpin 100 (Endo et al., 1987). This loop contains the target nucleotide, A^{4256}, for the *N*-glycosidase ricin (Endo & Wool, 1982). Removal of this base reduces the ribosomal affinity for eEF-2 and blocks the elongation cycle (Olsnes & Pihl, 1972; Sperti *et al.*, 1973; Nilsson & Nygård; 1986). Furthermore, binding of eEF-2 to the ribosome inhibited the ricin-catalyzed depurination of A^{4256} (Figure 2), thereby preventing ricin-induced inactivation of the ribosome (Fernandez-Puentes *et al.*, 1976). Similarly, the homologous loop in prokaryotic ribosomes is cleaved by the nuclease α-sarcin (Schindler & Davies, 1977). In

this case modification of the rRNA inhibits the function of both EF-Tu and EF-G (Hausner *et al.*, 1987). These data strongly support the view that this part of the 23S/28S rRNA is directly involved in binding the elongation factors. In this study we could not observe any eEF-2-specific chemical footprints in this part of the rRNA. This was due to the fact the α-sarcin/ricin loop was completely inaccessible to chemical and MNase modification in the reassociated 80S ribosomes. The inaccessibility of the region for chemical modification in eukaryotic ribosomes is in contrast to the exposure of the homologous loop in prokaryotic ribosomes. In the latter particles several nucleotides are accessible for chemical modification, and the reactivity of the nucleotides is influenced by both EF-Tu and EF-G (Moazed *et al.*, 1988).

(d) The Ribosomal Interface Region. The ribosomal binding sites for the elongation factors are located at the interface between the two ribosomal subunits [for a review, see Nygård and Nilsson (1990)]. In prokaryotes, factor binding takes place at or near the neck region of the small ribosomal subunit and involves the L7/L12 stalk in the large ribosomal subunit (Girshovich *et al.*, 1981). The chemical and enzymatic footprinting data presented here indicate that two rRNA regions (helices 36 and 39 in 18S rRNA and hairpins 73−75 in 28S rRNA), assigned to the ribosomal interface (Herr *et al.*, 1979; Raué, 1990; Mitchell *et al.*, 1992; Holmberg *et al.*, 1994b), were affected by the ribosome-bound eEF-2.

Cross-linking experiments in prokaryotes have demonstrated that helix 36 is within the decoding center of the ribosome (Bhangu *et al.*, 1994) in close contact with the A-site codon on the mRNA (Dontsova *et al.*, 1992). Helices 36 and 39 in the major 3′-domain of 18S rRNA showed extensive protection against chemical and enzymatic modification in the eEF-2·ribosome complex. The prokaryotic homolog to helix 36 is considered to play an active role in peptide chain termination and in translocation of the tRNA from the A-site to the P-site (Anderson *et al.*, 1967; Sigmund *et al.*, 1984; Moazed & Noller, 1987; Murgola *et al.*, 1988; Tate *et al.*, 1993). Nucleotides within the helix take part in binding of spectinomycin (Sigmund *et al.*, 1984; Moazed & Noller, 1987; Makosky & Dahlberg, 1987), an antibiotic that specifically blocks the translocation process and affects the EF-G cycle (Anderson *et al.*, 1967; Bilgin *et al.*, 1990). Interestingly, the corresponding sequence in 18S rRNA was protected from modification when eEF-2 was bound to the ribosome, suggesting that the elongation factors interact directly with this part of the RNA.

The strongest eEF-2 induced protections in 28S rRNA were found in the apical loops of helices 73 and 75 in domain IV. This part of the 28S rRNA is strongly conserved (Raué *et al.*, 1988) and contains an unusually high number of modified nucleotides (Maden, 1990), suggesting that it is essential for the ribosomal function (Noller, 1984; Lane *et al.*, 1992). Domain IV seems to be the attachment point for protein L2 in prokaryotes, as a considerable part of the domain, except the helices homologous to helices 73−75, is protected from nuclease digestion by L2 (Beauclerk & Cundliffe, 1988). The *E. coli* protein L2 is homologous to protein L8 in rats (Chan & Wool, 1992), a protein that has been cross-linked to eEF-2 (Nygård *et al.*, 1987). Thus, the data indicate that both rRNA and protein components of domain IV are affected by interaction of eEF-2 with the ribosome.

The apical loop of hairpin 73 contained one MNase-sensitive site that was protected from cleavage in the eEF-2·ribosome complex. A corresponding nuclease-sensitive site is found in the prokaryotic ribosome. Cleavage at this site is inhibited after interaction of EF-G with the ribosome (Bochkareva & Girshovich, 1984). Furthermore, the nuclease sensitivity of the prokaryotic site is dependent on the pentameric ribosome−protein complex L10/(L7/L12)$_4$, and particles depleted of these proteins do not display any nuclease-sensitive site in the region (Bochkareva & Girshovich, 1984). Interestingly, specific footprinting data indicate that the pentameric complex binds to the region containing helix 42 (Egebjerg *et al.*, 1990). Thus, the available data suggest that these two parts of domains II and IV are within a defined area of the ribosome. The suggested spatial arrangement is also supported by our observation that eEF-2 gives specific footprints both in helix 42 and in hairpins 73 and 75.

(e) The A- and P-Sites. Many of the rRNA sequences affected by ribosomal binding of eEF-2 overlap with or are adjacent to sequences implicated in subunit association and in ribosomal A- and P-site related functions. The prokaryotic homologue to helix 36 in 16S rRNA is cross-linked to the mRNA codon positioned in the ribosomal A-site (Dontsova *et al.*, 1992). Furthermore, the prokaryotic sequences homologous to the internal bulge in helix 42 and to the apical loop in helix 43 in domain II of 23S rRNA are within the binding site of A-site-bound peptidyl-tRNA (Moazed & Noller, 1989). A-site-bound peptidyl-tRNA also gives specific footprints at U^{2609}, located in the so-called peptidyltransferase ring (Moazed & Noller, 1989). This base is homologous to U^{4206} in eukaryotic 28S rRNA, a base that was protected from modification in the eEF-2·ribosome complex. Thus, the footprinting data indicate that binding of eEF-2 to the ribosome mainly affects the reactivity of putative A-site-located nucleotides. However, previous studies of the protein components of the ribosomal factor binding site have shown that the factor preferentially cross-links to proteins described as potential candidates for the ribosomal P-site (Nygård & Nilsson, 1990, and references therein). The contradictory results could have several explanations. First, the size and shape of the ligands, such as tRNA and factors, will presumably allow contact with a relatively large part of the ribosomal surface, thereby allowing affinity labeling, footprinting, and cross-linking at multiple sites. Second, it is unlikely that eEF-2/EF-G binds to the actual A- or P-sites of the ribosome, as these sites are supposed to be occupied by tRNA in the translocating ribosome. Third, the occurrence of many overlapping footprints, generated by various ribosomal ligands, may reflect a particular ribosomal status/conformation rather than a direct ligand−RNA interaction.

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