Probing the conformational changes in 5.8S, 18S and 28S rRNA upon association of derived subunits into complete 80S ribosomes

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ABSTRACT

The participation of 18S, 5.8S and 28S ribosomal RNA in subunit association was investigated by chemical modification and primer extension. Derived 40S and 60S ribosomal subunits isolated from mouse Ehrlich ascites cells were reassociated into 80S particles. These ribosomes were treated with dimethyl sulphate and 1-cyclohexyl-3-(morpholinoethyl) carbodiimide metho-p-toluene sulphonate to allow specific modification of single strand bases in the rRNAs. The modification pattern in the 80S ribosome was compared to that of the derived ribosomal subunits. Formation of complete 80S ribosomes altered the extent of modification of a limited number of bases in the rRNAs. The majority of these nucleotides were located to phylogenetically conserved regions in the rRNA but the reactivity of some bases in eukaryote specific sequences was also changed. The nucleotides affected by subunit association were clustered in the central and 3'-minor domains of 18S rRNA as well as in domains I, II, IV and V of 5.8/28S rRNA. Most of the bases became less accessible to modification in the 80S ribosome, suggesting that these bases were involved in subunit interaction. Three regions of the rRNAs, the central domain of 18S rRNA, 5.8S rRNA and domain V in 28S rRNA, contained bases that showed increased accessibility for modification after subunit association. The increased reactivity indicates that these regions undergo structural changes upon subunit association.

INTRODUCTION

The functional contribution of the ribosome in protein biosynthesis has traditionally been attributed to its proteins while the ribosomal RNAs have been considered to provide the structural framework essential for assembling the proteins into functional domains. However, today there is substantial phylogenetic, genetic and biochemical evidence for a direct functional role of ribosomal RNA in the translation process. In prokaryotes it has been shown that the rRNA is directly engaged in mRNA selection whereas its involvement in the binding of tRNA and translational factors as well as in the peptidyl-transferase function has been demonstrated by indirect techniques such as site directed mutagenesis, affinity labelling, chemical cross-linking and chemical and enzymatic footprinting [for a review see ref (1)].

The ribosomal RNA is not uniformly distributed in the ribosome. Instead the interface between the two subunits is enriched in RNA (2), suggesting that RNA also plays an active role in the interaction between the ribosomal subunits in the complete ribosome. This view is supported by structural and functional experiments (3-9). A close proximity of RNA and ribosomal proteins from the two complementary subunits has also been demonstrated by short range chemical cross-linking (10).

Structural and functional data concerning the rRNAs have almost been entirely obtained from the prokaryotic ribosome and only limited contributions have come from the more complex eukaryotic particles. So far, the possible participation of some of the rRNAs in subunit association has been investigated using chemical and enzymatic footprinting techniques (11-13). However, to our knowledge most previous studies of the involvement of eukaryotic rRNA in subunit association have been based on comparisons between the accessibilities of rRNA for modification in derived subunits and in isolated native ribosomes (12, 13). As pointed out by Noller (1) and Döring et al. (14), the native monosomes may contain extra ribosomal components that could affect the interpretation of the experimental data. Thus, this type of comparison may not be totally relevant for identifying RNA sequences of particular importance for subunit association.

In this report we have compared the chemical reactivity of 5.8S, 18S and 28S rRNA in isolated derived ribosomal subunits with that of the same rRNAs in 80S ribosomes formed by association of the derived subunits. The comparison was done using the two single-strand specific reagents dimethyl sulphate (DMS) and 1-cyclohexyl-3-(morpholinoethyl) carbodiimide metho-p-toluene sulphonate (CMCT). Subunit association resulted in the protection of specific regions in 18S and 28S rRNA from
modification while other defined regions in all three rRNA species showed increased accessibility for modification in the 80S ribosome.

MATERIALS AND METHODS

Materials

Dimethyl sulphate and CMCT were from Aldrich Chemie (Germany). Deoxy and dideoxy nucleotides were from Boehringer Mannheim (Germany). [γ-32P]ATP and T4 polynucleotide kinase were from Amersham International (UK). Superscript reverse transcriptase was from Life Technologies, Inc.. cDNA primers were synthesized as described by Caruthers et al. (15). The specific sequences used for primer annealing were as previously described (16). The cDNA primers were end-labelled using [γ-32P]ATP and purified as previously described (11). Ribosomal subunits were prepared from mouse Ehrlich ascites cells as previously described (10, 16).

Formation of 80S ribosomes from derived subunits

Complete 80S ribosomes were formed by reassocation of isolated derived ribosomal subunits (17). The subunits, 120 pmol 40S and 120 pmol 60S, were incubated in a buffer containing 0.1 M sucrose, 7.5 mM MgCl2, 1 mM dithiothreitol, 42 mM Hepes/KOH, pH 7.6 and 150 mM KCl for 10 min at 37°C and chilled on ice. For modification of individual subunits, the isolated 40S and 60S particles were incubated under identical conditions.

Modification of rRNA

Chemical modification was performed as previously described (16). The reagents DMS and CMCT were used at final concentrations of 20 or 90 μM for DMS and 20 or 100 mM for CMCT to allow statistical modification of the rRNA (18). Control samples were incubated in the absence of DMS or CMCT but were otherwise treated exactly as the modified samples.

Identification of modification sites

Ribosomal RNA was extracted from the ribosomes with phenol according to Brawerman et al. (19). The rRNA was dissolved in H2O and the concentration adjusted to 1 pmol/μl (16).

RESULTS

We have investigated the possible involvement of rRNA in ribosomal subunit joining by comparing the chemical modification patterns of 5.8S, 18S, and 28S rRNA obtained from derived subunits (16) with that from 80S ribosomes formed by association of the derived subunits. For the interpretation of the experimental data it was important to know the proportion of ribosomal subunits that participated in the 80S formation. Analysis of the subunit joining showed that more than 85% of the subunits reassociated into 80S ribosomes under the ionic conditions used (Fig. 1).

Chemical modification of 18S, 5.8S and 28S rRNA in the derived ribosomal subunits show that the three molecules are equally exposed to the single strand specific reagents DMS and CMCT, i.e. 10% of the bases are reactive (16). Upon association of the subunits into an 80S ribosome, 43 of these bases became less accessible for modification while 16 bases showed enhanced reactivity (Figs. 2 and 3). A selection of autoradiograms showing the subunit induced alterations in the reactivity of the bases in 5.8S, 18S and 28S rRNA is shown in Fig. 4.

18S rRNA

Subunit association altered the chemical modification patterns in both the central and the 3′-domains of 18S rRNA whereas the reactivity of the bases in the 5′-domain was unaffected. The central domain contained eight bases that became less exposed to modification following 80S ribosome formation (Fig. 2). Six of these bases (U1003, U1024, U1027, C1028, A1029 and A1039) were located in helix 21. As these bases were the only reactive sites found in this sequence of the RNA, formation of the 80S ribosome associated with a protection of the whole sequence. Furthermore, subunit association caused a considerable protection of the single site (U971) located in hairpin 23. This base was the only reactive base found in this part of the rRNA and was classified as hyper reactive in the derived 40S subunits, i.e. the base was fully accessible for modification at the lowest reagent concentration used (16). Similarly, the bulge in hairpin 25 contained one base (A1072) that showed reduced reactivity after subunit association. The reactivity of the adjacent modified base was however unaffected by the subunit association.

Association of the two ribosomal subunits was also accompanied by increased reactivity of four bases in the central domain (Fig. 2). One site (A888) (Fig. 4) was found in the unstructured expansion segment while the remaining bases were located in the hinge region between helices 22, 23 and 24. Two of these bases, U941 and U942, were located in single strand regions of the rRNA while the third base, A1003, was putatively engaged in a non-canonic A-A base pair. The latter base was specifically exposed to chemical modification in the complete 80S ribosome (Fig. 4), suggesting that the A-A base pair must be destabilized after subunit association. Formation of the 80S ribosomes also induced new natural stops for the reverse transcriptase in helix 21. Here, the putative helical bases G935, G937, G939 and C939 functioned as natural stops in the 80S ribosomes but not in the derived 40S subunits (Fig. 4).

Figure 1. Efficiency of 80S ribosome formation. Isolated derived 40S and 60S subunits were allowed to associate into 80S ribosomes as described in materials and methods and the extent of 80S formation was analyzed by centrifugation on 10 to 30 % (weight by volume) sucrose gradients (17). After centrifugation for 110 min at 260 000 g, the gradients were monitored at 260 nm.

Figure 2. Complete 80S ribosomes were formed by reassociation of isolated derived ribosomal subunits (17). The subunits, 120 pmol 40S and 120 pmol 60S, were incubated in a buffer containing 0.1 M sucrose, 7.5 mM MgCl2, 1 mM dithiothreitol, 42 mM Hepes/KOH, pH 7.6 and 150 mM KCl for 10 min at 37°C and chilled on ice. For modification of individual subunits, the isolated 40S and 60S particles were incubated under identical conditions.

Figure 3. Formation of 80S ribosomes from derived subunits. Complete 80S ribosomes were formed by reassociation of isolated derived ribosomal subunits (17). The subunits, 120 pmol 40S and 120 pmol 60S, were incubated in a buffer containing 0.1 M sucrose, 7.5 mM MgCl2, 1 mM dithiothreitol, 42 mM Hepes/KOH, pH 7.6 and 150 mM KCl for 10 min at 37°C and chilled on ice. For modification of individual subunits, the isolated 40S and 60S particles were incubated under identical conditions.

Figure 4. Modified bases in 18S rRNA are shown as grey-shaded black for those modified in 40S and 60S subunits but not in 80S ribosomes, black for those modified in 40S and 60S subunits and 80S ribosomes, and grey-shaded black for those modified in 80S ribosomes only.
In the 3'-domain, subunit joining affected the reactivity of bases in hairpin 47 (Fig. 2). This hairpin is considered to be part of the decoding centre of the ribosome and the prokaryotic homolog is positioned at the subunit interface (1). Association of the 40S and 60S subunits specifically protected the two neighbouring bases A<sub>1816</sub> and A<sub>1819</sub> from chemical modification (Fig. 4). Thus, the only two reactive bases found in this part of the 18S rRNA sequence were protected by the large ribosomal subunit.

28S rRNA
Subunit association altered the chemical modification pattern of all domains in 28S rRNA except for domains III and VI. Most of the affected bases became less susceptible to modification but enhanced reactivities were also observed (Fig. 3). Joining of the two subunits affected the accessibility of seven nucleotides in domain I. Five of these bases were located in the part of the domain formed by 5.8S rRNA. Here the moderately exposed

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Figure 2. Secondary structure model of mouse 18S rRNA (42) showing changes in the modification pattern induced by subunit association. Bases that showed increased reactivities by 20 to 50% (□) or by more than 50% (●) after subunit association. Bases that showed 20 to 50% (○) or more than 50% (●) protection after 80S ribosome formation.
Figure 3. Secondary structure model of mouse 28S rRNA (42, 43) showing the localization of the bases whose reactivities were affected by subunit-association. Changes in chemical accessibility are as in fig. 2. Eukaryote specific expansion segments (ES).
region containing hairpins eight and nine became further exposed after 80S ribosome formation as half of the nucleotides that were available for modification in the derived 60S subunits showed enhanced reactivity. In addition, U^{127} located in the apical loop of helix nine, specifically became available for modification in the 80S ribosome (Fig. 4). The remaining two affected bases were located in the 28S part of domain I. These two bases (U^{68} and C^{72}) showed substantially reduced reactivity after formation of the 80S ribosome.

Domain II contained two regions in which subunit association affected the chemical footprinting pattern (Fig. 3). The two regions involved the apical loop of hairpin 31 and the eukaryote specific hairpins 28.2 and 28.3. The latter hairpins contained 12 bases that were moderately to highly available for modification in the derived 60S subunits (16). Eight of these bases showed decreased reactivity in the 80S ribosome. Thus, subunit joining partly protected the apical loop of hairpin 28.2 (Fig. 4) and the entire hairpin 28.3. The second affected region in domain II, the apical loop of helix 31, contained three adjacent bases that were moderately reactive in the derived 60S subunits. One of these bases, A^{1377}, became considerably more exposed after 80S ribosome formation while the adjacent bases A^{1375} and A^{1376} became less susceptible to modification.

Domain IV is essential for the ribosomal performance during
protein synthesis and parts of helices 73 to 75 are within the binding sites for aminocyl- and peptidyl-tRNA (20, 21). The domain contains one of the most exposed sequences found in 28S rRNA (16). In the derived 60S subunits, this sequence, forming hairpins 73 to 75, contained 13 bases that were available for modification by DMS and CMCT. After subunit association the reactivity of the bases in the sequence between the apical loops of the two adjacent hairpins 73 and 74 was reduced (Figs. 3 and 4) while the accessibilities of the bases in hairpin 75 and the adjacent side of hairpin 74 were unaffected by the subunit association.

Domain V showed extensive alterations in the chemical footprinting pattern after formation of the 80S ribosome (Fig. 3). The region containing helices 87 to 93 contained 15 bases that became less exposed to modification in the 80S ribosome. All bases in hairpin 89 that were reactive in the derived 60S subunits became moderately to substantially protected in the 80S ribosome. Similarly, the apical loop of hairpin 93 became protected against modification in the 80S ribosome. Three additional single bases (G1879, U3957 and C3988) in this part of domain V were protected from modification as a result of subunit association.

In contrast to the protection seen in the previous region of domain V the part containing helices 94 to 97 showed, with one exception, increased reactivity after subunit association (Fig. 3). The single protected base (C4127) was found in the apical loop of helix 96. Bases with increased reactivity were found in the loop of helix 94 where the two adjacent uridines U4070 and U4071 became hypersensitive. Furthermore, the two bases (A4100 and U4103) found in the interhelical sequence between helices 94 and 95 became considerably more reactive after 80S ribosome formation. Similarly, the reactivity of the single accessible nucleotide in the apical loop of hairpin 97 (U4152) showed increased availability for modification after formation of the 80S ribosome. The eukaryotic 28S rRNA contains four large expansion segments that are not included in the phylogenetic secondary structure model in fig. 3. These segments contain 33% of all bases in 28S rRNA. Our analysis showed that the accessibility of only two of these bases was affected by the 80S ribosome formation. In expansion segment I, the base C585 became substantially exposed after subunit association while C4584 in expansion segment IV showed reduced reactivity in the 80S ribosome (Fig. 4).

**DISCUSSION**

The interface region of the 80S ribosome contains a high proportion of rRNA (2). Although there is no phylogenetic evidence for a direct base-pairing between the rRNAs of the two complementary subunits, accumulating data suggest that the rRNAs are involved in subunit interaction. In prokaryotes the accessibility of both 16S and 23S rRNAs for chemical and enzymatic modification is altered after joining of the two ribosomal subunits (3, 4, 6, 22) and association of the subunits is abolished by specific changes in the base composition of the rRNAs (5, 7, 8). Furthermore, chemical cross-linking experiments have shown that the 3' domain of 16S rRNA is in close vicinity of domain IV in 23S rRNA (9).

In this report we have studied the changes in chemical accessibility of the rRNAs caused by the formation of complete eukaryotic 80S ribosomes from isolated derived subunits. As previously discussed (16) these subunits are considered functional as; i) the derived subunits bind initiation factors and Met-tRNA<sub>F</sub> (23) ii) the subunits efficiently associate into 80S ribosomes iii) the re-associated ribosomes bind elongation factor 2 in the presence of GTP and GTP analogues and hydrolyse GTP in an eEF-2 dependent manner (17) iv) the 80S reassociated ribosomes translate globin mRNA in the presence of elongation and initiation factors (24).

Formation of the 80S ribosome was associated with substantial changes in the chemical footprinting patterns of 18S, 5.8S and 28S rRNA. The only molecule not influenced by subunit joining is 5S rRNA (4, 11, 25) even though it is suggested to interact with 18S rRNA over the ribosomal interface (26). The alterations in chemical accessibility of the rRNAs, reported here, are consistent with the view that the rRNAs are involved in subunit association.

The central domain of 18S rRNA

The central domain of the prokaryotic 16S rRNA is essential for 70S ribosome formation (7, 8, 27, 28). The chemical footprinting pattern of the central domain in 18S rRNA was extensively altered after subunit association and both protections and enhanced exposures were observed (Fig. 2). Specific protections were found in the internal loops of helices 23 and 25 and at the 3'-side of helix 21. This agrees with data from the prokaryotic ribosome where base changes in helix 25 reduces the ability of the 30S particle to interact with the large ribosomal subunit (3, 6-8, 28-30). Furthermore, the apical loop of helix 25 is suggested to interact with complementary bases in the apical loop of helix 102 in 23S rRNA (27). However, as both homologous loops in 18S and 28S rRNA were accessible for modification within the 80S ribosomes, our data does not support a tertiary interaction between the two loops across the interface of the eukaryotic ribosome. The only protection seen in helix 25 was localized to the internal bulge. Instead, we found substantial protections in helix 21. In this region, all bases that were accessible for modification in the 40S subunit became less exposed after 80S ribosome formation.

Subunit association induced exposure of the rRNA was seen in the region composed of the unstructured expansion segment and hairpin 22 (Fig. 2), indicating that this part of the central domain undergoes structural changes upon subunit association. The influence of the 60S subunit on the 40S particle could also be observed as an increased number of natural stops in helix 22 (Fig. 4). The occurrence of new stop sites could result from a limited nucleolytic attack or reflect an altered dynamic stability of the rRNA as suggested by Baudin et al. (6). In the case of helix 22 nuclease degradation is unlikely since 18S rRNA extracted from the 80S ribosomes used for preparation of isolated subunits showed identical natural stops to those present after joining of the dissociated ribosomal subunits (Fig. 4). Therefore, the natural stops seen in the 18S rRNA preparations from both isolated 80S monosomes and 80S ribosomes formed in vitro are likely to reflect a stabilization of helix 22 in the 80S particles and hence a decreased ability of reverse transcriptase to copy this part of the rRNA sequence.

The decoding region of the ribosome

The decoding region of the prokaryotic ribosome is considered localized in the 3'-domain of 16S rRNA and contains part of the rRNA homologous to hairpin 47 in 18S rRNA and the preceding interhelical sequence (1). In prokaryotes, helix 47 has been found
to undergo allosteric transitions upon binding of the 50S subunit (31) and both the nucleotides and the phosphate backbone of this region are protected from modification by the 50S subunit (3, 6, 29). Furthermore, removal of the helix or perturbation of its secondary structure by point-mutations abolishes or reduces 70S ribosome formation (32–34).

Association of the 40S and 60S subunits resulted in specific protection of helix 47 (Fig. 2). In the prokaryotic ribosome the base of helix 47 is juxtaposed to helix 48 (14, 35) and both protection of helix 47 (Fig. 2). In the prokaryotic ribosome the association induced changes in chemical reactivity. Thus, this 75 and the adjacent side of hairpin 74 (41). In mouse ascites E.coli puromycin (40). Interestingly, in the apical loop of helix 48 has been cross-linked to a region in 23S rRNA homologous to the terminal loop of helix 73 in domain IV (9), a region also affected by subunit association in eukaryotes.

The accessibility of the region containing helices 73 and 74 were reduced upon formation of the 80S ribosome (Fig. 3), supporting the notion that this part of the 28S rRNA is positioned at the ribosomal interface. Some observed protections in the eukaryotic ribosome coincided with or were located adjacent to sites protected by peptidyl-tRNA in the homologous region of the 23S rRNA (20). This is analogous to the situation in E.coli where bases affected by subunit-association are often positioned in close vicinity to bases influenced by the binding of tRNA (30, 37).

The data reported here show that subunit association protects sequences in 18S and 28S rRNA homologous to sequences in the prokaryotic RNAs that have been directly cross-linked across the ribosomal interface and that are functionally linked to the decoding region of the ribosome.

The peptidyl transferase region

The peptidyl transferase function of the ribosome is associated with the central ring of domain V (1). Domain V in 28S rRNA was the only domain that contained a cluster of nucleotides that were more exposed in the 80S ribosome than in the 60S subunit. The peptidyl-transferase ring contained two bases, A1402 and U1403, whose reactivity were induced by the presence of the 40S subunit (Fig. 3). The homologous nucleotides in 23S rRNA have been cross-linked to peptidyl-tRNA (38) and should thus be expected to be exposed in the ribosome. This assumption is supported by the observation that this part of domain V is open for hybridization to oligo nucleotides (39). Furthermore, U1452 in the apical loop of helix 96 was exposed in the 80S ribosome. The homologous nucleotide in prokaryotes is cross-linked to puromycin (40). Interestingly, in E.coli the apical loop of helix 96 has been cross-linked to a part of domain IV containing hairpin 75 and the adjacent side of hairpin 74 (41). In mouse ascites ribosomes, the homologous part of domain IV showed no subunit association induced changes in chemical reactivity. Thus, this region was still open for chemical modification in the 80S ribosomes while the proximate sequence forming hairpin 73 and the adjacent side of hairpin 74 became protected in the complete ribosome. Based on these observations it is tempting to speculate that the interaction between the large and the small subunit causes a conformational change in the region around the peptidyl-transferase ring that opens up this part of domain V for interaction with translational components.

Domain V also contained a cluster of bases in the region containing helices 87 to 93 that became protected from modification after subunit association (Fig. 3). Subunit association induced protections of domain V have also been observed in Xenopus laevis 28S rRNA (13). The extensive influence of the small subunit on the modification pattern of domain V in 28S rRNA and the observation that point-mutations in the conserved peptidyl-transferase ring of E.coli 23S rRNA abolish formation of the 70S ribosome (5) suggests that domain V is involved in interaction between the two subunits.

Domain 1 of 28S rRNA

Subunit joining mainly altered the chemical modification pattern of the 5.8S rRNA part of domain I. This part of the domain was more exposed in the reassOCIated 80S particles than in the derived 60S subunits (Fig. 3). This is in contrast to the situation reported for rat ribosomes in which the 5.8S rRNA is less accessible for modification in the complete 80S ribosome than in the isolated subunits (12). However, as the rat liver 80S ribosomes were isolated as monosomes and not formed by reassociation of derived subunits, the reduced accessibility for modification may reflect protection exerted by other non-ribosomal translational components rather than by the complementary 40S subunit.

Eukaryote specific sequences in 28S rRNA

Although the eukaryote specific sequences present in mouse 28S rRNA constitute more than one third of the nucleotides in the molecule, subunit association had no influence on the chemical reactivity of these sequences with one major exception. The eukaryote specific helices 28.2 and 28.3 showed substantial alterations in their chemical modification patterns after subunit association (Fig. 3). However, as the mechanism behind subunit association is most likely similar in both prokaryotes and eukaryotes the involvement of eukaryote specific sequences in the primary interaction between the two subunits is unlikely. Nevertheless, as the eukaryotic rRNAs are considerably larger than the prokaryotic homologs it cannot be excluded that a part of these additional sequences is positioned directly at the ribosomal interface.

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