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Structure of 5S rRNA within the *Escherichia coli* ribosome: Iodine-induced cleavage patterns of phosphorothioate derivatives

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ABSTRACT

The protection patterns of 5S rRNA in solution, within the ribosomal 50S subunit, 70S ribosomes, and functional complexes, were assessed with the phosphorothioate method. About 20% of the analyzed positions (G9–G107) showed strong assembly defects: A phosphorothioate at one of these positions significantly impaired the incorporation of 5S rRNA into 50S particles. The reverse has also been observed: A phosphorothioate is preferred over a phosphate residue in the assembly process at a few positions. The results further demonstrate that 5S rRNA undergoes conformational changes during the assembly in the central protuberance of the 50S subunit and upon association with the small ribosomal subunit forming a 70S ribosome. In striking contrast, when the 70S ribosomes are once formed, the contact pattern of the 5S rRNA is the same in various functional states such as initiation-like complexes and pre- and posttranslocational states.

Keywords: phosphorothioate method; protein synthesis; ribosomal assembly; ribosomal elongation cycle; 5S rRNA

INTRODUCTION

5S rRNA is a component of the large ribosomal subunit of all living organisms. Only mitochondrial genomes except those from higher plants do not contain 5S rRNA genes, and the question whether mitochondrial ribosomes can function without 5S rRNA or whether they utilize nuclear-encoded 5S rRNA is far from being settled (for review, see Mason & Sirum-Connolly, 1996). The relatively small molecule (120 nt in *Escherichia coli*) is located in the central protuberance of the large ribosomal subunit (Shatsky et al., 1980; Stöffler-Meilicke et al., 1981) and plays an essential role in the late assembly of the large subunit. Without 5S rRNA, particles of marginal activity are formed (Dohme & Nierhaus, 1976). Mutants harboring altered 5S rRNAs have been described that are severely affected in ribosomal functions (Göringer & Wagner, 1986; Meier et al., 1986). The D-loop of *E. coli* 5S rRNA is adjacent to and possibly interacts with two major functional domains of 23S rRNA—the centers of peptidyl transferase and EF-G binding (Dokudovskaya et al., 1996).

Various experimental strategies have been exploited to unravel the three-dimensional folding of 5S rRNA in solution, of 5S rRNA complexed with binding proteins, and 5S rRNA within the ribosome (for reviews see Ehresmann et al., 1990; Bogdanov et al., 1996; Moore, 1996). 5S rRNA seems to be compactly folded within the ribosome because only a few of its nucleotide bases are accessible for chemical modification (Noller & Herr, 1974; Herr & Noller, 1979; Silberklang et al., 1983). Therefore, the classical footprinting methods based on chemical modification of RNA bases in protein complexes (RNPs) appear to be inappropriate for an investigation of the 5S rRNA state inside the ribosome.

Recently, we have studied the contacts of phosphate residues in 5S rRNA with the binding proteins L5, L18, and L25, specific for 5S rRNA (Shpanchenko et al., 1996), applying the procedure developed by Eckstein and co-workers (Schatz et al., 1991; Rudinger et al., 1992). It is based on the observation that a protein involved in a tight RNA–protein contact can protect phosphorothioated residues from iodine cleavage. Random substitution of phosphorothioates for phosphates does

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not significantly affect the biological activity of mRNAs (Ueda et al., 1991; Alexeeva et al., 1996) and tRNAs (Dabrowski et al., 1995).

Only a limited number of 5S rRNA phosphates are protected in complexes with one or more of the 5S rRNA binding proteins mentioned above (Shpanchenko et al., 1996). New positions of 5S rRNA were found to have protein contacts in addition to those known from chemical modification data (Garrett & Noller, 1979; Peattie et al., 1981). High accessibility of 5S rRNA to iodine and the lack of information about the interactions of phosphate groups with components within assembled 50S particles prompted us to apply this method for an analysis of the contacts of the 5S rRNA sugar–phosphate backbone with ribosomal components within the 50S subunit and 70S ribosomes at different stages of the translational process.

RESULTS

Formation of functional complexes and controls

Modified 5S rRNA with randomly distributed phosphorothioate groups at the 5′ side of the nucleotidyl residues were obtained by T7 transcription from a DNA template, containing *E. coli* 5S rDNA sequence linked to a T7 promoter as described (Dontsova et al., 1994; Shpanchenko et al., 1996). The base pair U1:A119 in the 5S rRNA transcript was changed to the compensatory base pair G1:C119 (e.g., see Fig. 3) to improve the transcription efficiency. Furthermore, U120 was removed, and these changes did not affect the biological activity concerning either assembly or function of the mature particles (Dontsova et al., 1994).

During transcription, one of the four nucleoside triphosphates was present as a mixture of nonmodified compound with the corresponding α-thio-phosphate derivative (about 20%), yielding a random incorporation of up to six modified phosphates into a 5S rRNA molecule (Shpanchenko et al., 1996). The 5S rRNA transcripts were 5′-labeled with [^{32}P] before reconstitution into 50S particles. The transcripts had to be added in a two-molar excess over 23S rRNA in order to yield the maximal amount of active particles during the reconstitution procedure. The particles showed about 80% of the activity observed with native 5S rRNA; the phosphorothioated 5S rRNA yielded the same activity as the unmodified transcripts. The reconstituted 50S particles were associated with a two-fold excess of native 30S subunits forming 70S ribosomes. Both reconstituted 50S subunits and re-associated 70S ribosomes were purified via sucrose gradient centrifugation.

Functional complexes were formed with the help of the heteropolymeric MF-mRNA that contains in the middle the codons for Met and Phe: GGG$(A_4G)_3$AAA-AUG-UUC-$(A_4G)_3$AAAU. Three kinds of complexes were prepared: (1) a 70S complex containing only one tRNA at the P site (P_i complexes, i for initiation), either [^{32}P]tRNA$_f^{Met}$ or Ac[^{14}C]Phe-tRNA^{Phe}; (2) a pretranslocational (PRE) complex carrying a tRNA$_f^{Met}$ at the P site and an Ac-Phe-tRNA^{Phe} at the A site; and (3) a posttranslocational (POST) complex that was obtained from the PRE complex by an EF-G-dependent translocation reaction, thus carrying the tRNA$_f^{Met}$ and the AcPhe-tRNA^{Phe} at the E and P sites, respectively. The homogeneity of the functional complexes in the PRE and the POST state was better than 90% (Table 1).

50S subunits, 70S ribosomes, as well as the P_i, PRE, and POST complexes containing modified 5S rRNA were subjected to iodine treatment under conditions that were found to be optimal in experiments with 5S rRNA–protein complexes (Shpanchenko et al., 1996).

In control experiments, the stability of the modified 5S rRNA during the experimental treatment was tested under identical conditions, but in the absence of iodine. Occasional cleavage was found at position C90 in 50S subunits and 70S ribosomes (Figs. 1, 2, lanes 3), but not in 5S rRNA in solution (Fig. 1, lanes 1) or in 5S rRNA complexed with the 5S rRNA-binding proteins L5, L18, and L25 (Shpanchenko et al., 1996). In all likelihood, this nucleotide marks a position of pronounced stress imposed during the assembly and/or inside the mature 50S subunit. Additional cleavages were also found at A15, A50, A78, U89, and C110 in a few experiments (see, for example, K in Fig. 2).

The phosphates at positions 1–8 and 108–119 (helix I) were not analyzed, because they were not resolved sufficiently in our sequence gels. All experiments were performed at least four times, the autoradiographs were scanned, and the data were processed according Dabrowski et al. (1995). The intensities of the scanned bands were normalized by the intensity of the band corresponding to the noncleaved material. The results of the quantitation are given in Table 2. We considered changes of the intensity of $\geq$30% as significant and arrived at the following classification. A rel-

TABLE 1. N-Ac[^{14}C]Phe-tRNA^{Phe} binding and the specificity of the resulting ribosomal functional complexes.[a]

	AcPhe-tRNA	
State	Binding (tRNA per 70S)	A or P site location (PM reaction)
P_i	0.85	100% P
PRE	0.7	95% A
POST	0.7	91% P

[a] P_i state, AcPhe-tRNA was bound directly to the P site in the presence of MF-mRNA. PRE, AcPhe-tRNA was bound to the A site after pre-filling the P site with tRNA$_f^{Met}$. POST, the PRE state was subjected to an EF-G·GTP-dependent translocation resulting in a P site location of the AcPhe-tRNA. PM, puromycin. For more details, see text.

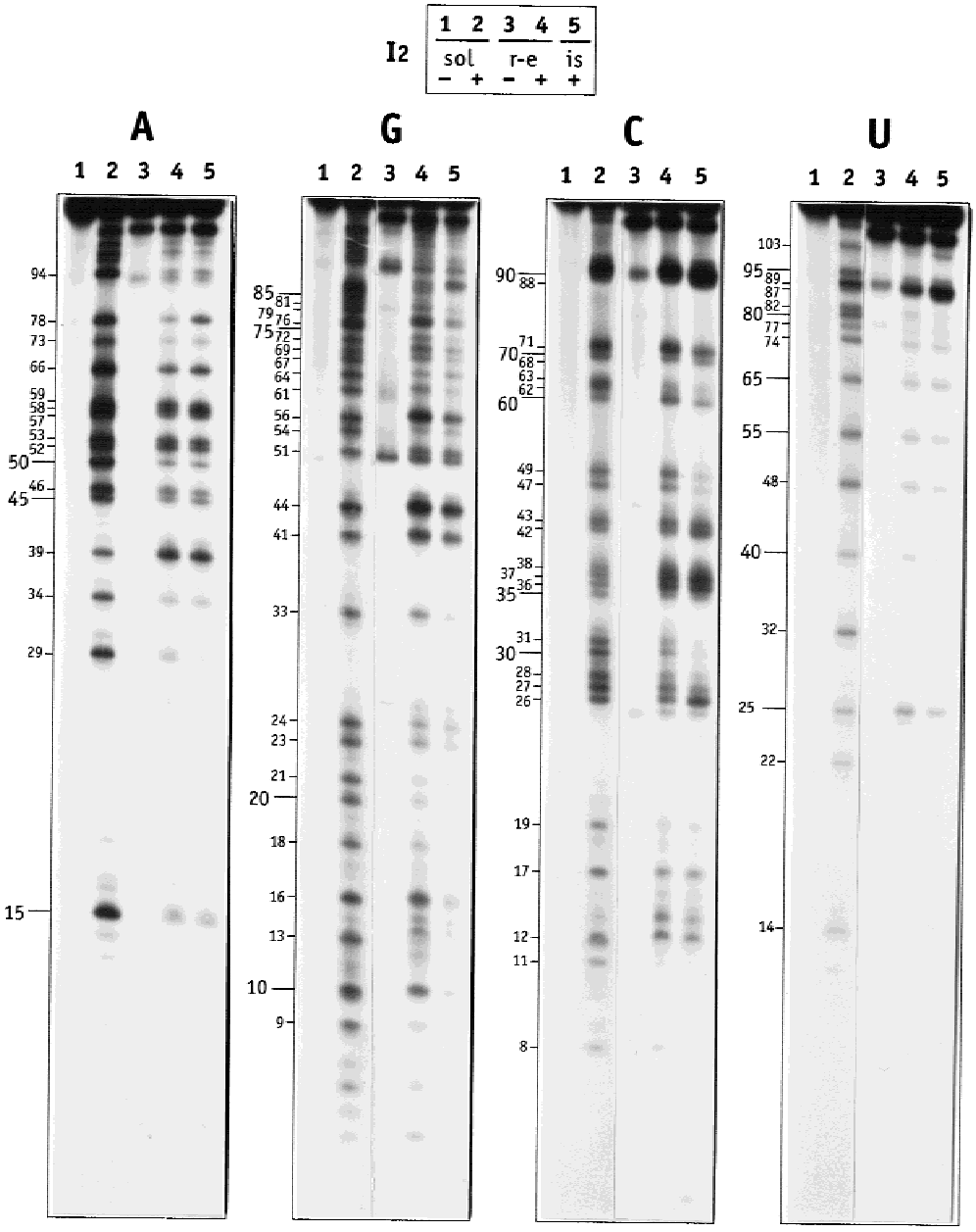

FIGURE 1. Protection pattern of phosphorothioated 5S rRNA within the 50S subunit. Insert at top, and explanation of lanes: Lanes 1 and 2, 5S rRNA in solution (sol) without (−) and with (+) iodine cleavage; lanes 3 and 4, 5S rRNA reconstituted into the 50S particles, extracted via phenolization (r-e), followed by an iodine treatment (+); lane 5, 5S rRNA within the 50S subunit subjected to an iodine treatment (is, in situ). A, G, U, and C, cleavage at these positions, respectively.

ative intensity of <0.3 of the control was called "strong protection;" a relative intensity between 0.3 and 0.7, "slight protection;" an intensity of 1 ± 0.3, "no protection;" an intensity above 1.3, "enhanced accessibility."

Nucleotide positions that require phosphates rather than phosphorothioates during the assembly process

The fact that a twofold molar excess of phosphorothioated 5S rRNA over 23S rRNA was required for complete assembly of the 23S rRNA into active 50S particles might indicate that phosphorothioate residues at some positions of 5S rRNA could interfere with the assembly of the corresponding 5S rRNA molecules. In order to check this possibility and, in a positive case, to identify the critical positions, modified 5S rRNA was isolated from both 50S subunits and 70S ribosomes and subjected to an iodine-induced cleavage. The cleavage pattern to be expected should be identical to that derived from 5S rRNA in solution without a preceding reconstitution step. However, if the modification at a

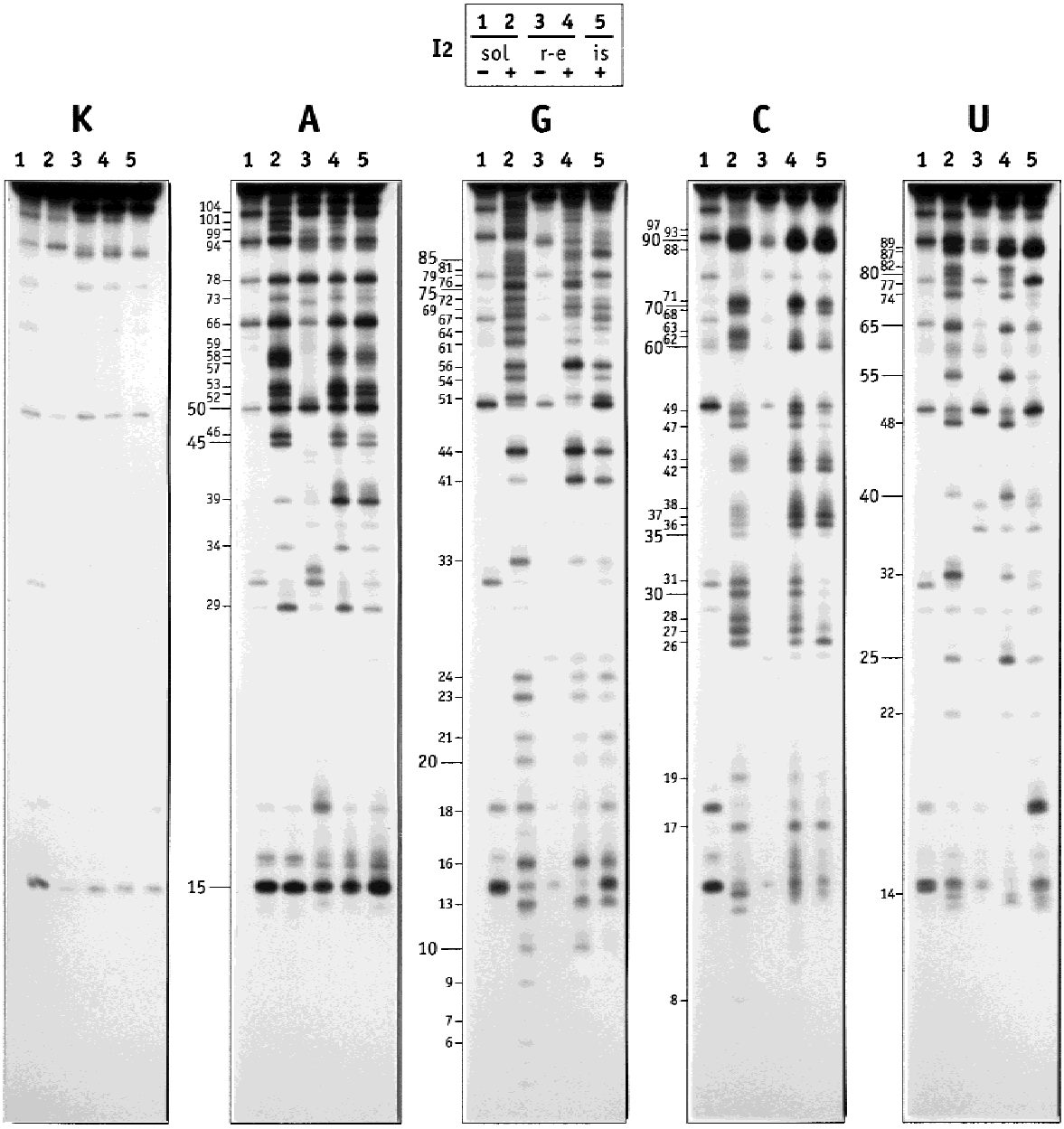

FIGURE 2. Protection pattern of phosphorothioated 5S rRNA within the 70S ribosome. K, controls with transcribed 5S rRNA not containing thioated nucleotides. For further explanations, see legend to Figure 1.

distinct position prevented or impaired the incorporation of the 5S rRNA molecule, no or a reduced cleavage at this position should be observed.

An almost complete exclusion of a 5S rRNA molecule from the assembly process is observed at positions C19 and A99, and significant effects are found at 19 other positions (G9, A15, G20, G21, C28, U32, C35, A50, G54, A57, C62, C63, G64, G72, U80, G81, G96, G98, and U103, see Fig. 1, lanes 4 compared with lanes 2; Table 2, columns Extract/in solution), a total of about 20% of the positions that could be analyzed (G9–G107).

The reverse case has been also observed, i.e., 5S rRNAs with a phosphorothioate at a distinct position are more highly populated in the assembled particles than in the total initial 5S rRNA preparation. This is the case if a cleavage position is stronger in the 5S rRNA derived from 50S subunits than the corresponding position of the total 5S rRNA preparation (5S rRNA in solution; Fig. 1, compare lanes 4 with lanes 2; Table 2, columns Extract/in solution). A conspicuous example is A39, and less strong effects were observed at positions C36, C37, G44, and G56. The same strengthening of the cleavage pattern was also observed at the

TABLE 2. Relative accessibility of the phosphate of the 5S rRNA in the 50S and 70S ribosomes.[a]

		50S		70S	
No.	Nucleotide	Extract/in solution	In situ/extract	Extract/in solution	In situ/extract
1	G	n.d.	n.d.	n.d.	n.d.
2	G	n.d.	n.d.	n.d.	n.d.
3	C	n.d.	n.d.	n.d.	n.d.
4	C	n.d.	n.d.	n.d.	n.d.
5	U	n.d.	n.d.	n.d.	n.d.
6	G	n.d.	n.d.	n.d.	n.d.
7	G	n.d.	n.d.	n.d.	n.d.
8	C	n.d.	n.d.	n.d.	n.d.
9	G	0.38 ± 0.04	0.58 ± 0.03	0.45 ± 0.05	0.61 ± 0.06
10	G	0.74 ± 0.03	0.18 ± 0.09	1.12 ± 0.06	0.22 ± 0.03
11	C	0.98 ± 0.05	1.03 ± 0.06	0.89 ± 0.04	1.11 ± 0.02
12	C	0.95 ± 0.07	0.92 ± 0.02	0.97 ± 0.05	0.99 ± 0.08
13	G	0.95 ± 0.09	0.49 ± 0.04	0.91 ± 0.10	1.02 ± 0.05
14	U	0.81 ± 0.02	1.08 ± 0.03	0.74 ± 0.01	1.08 ± 0.09
15	A	0.34 ± 0.01	0.80 ± 0.05	0.43 ± 0.04	0.76 ± 0.01
16	G	1.12 ± 0.04	0.55 ± 0.02	1.07 ± 0.07	0.84 ± 0.09
17	C	0.81 ± 0.03	0.76 ± 0.01	1.16 ± 0.07	0.73 ± 0.02
18	G	0.74 ± 0.02	0.24 ± 0.05	0.87 ± 0.07	0.26 ± 0.03
19	C	0.11 ± 0.01	0.52 ± 0.03	0.14 ± 0.03	0.53 ± 0.01
20	G	0.52 ± 0.05	0.52 ± 0.02	0.65 ± 0.01	0.97 ± 0.08
21	G	0.47 ± 0.09	0.57 ± 0.06	0.58 ± 0.05	1.13 ± 0.10
22	U	0.75 ± 0.01	1.18 ± 0.03	0.79 ± 0.05	1.06 ± 0.07
23	G	0.78 ± 0.05	0.60 ± 0.02	0.75 ± 0.01	0.63 ± 0.04
24	G	0.82 ± 0.03	0.86 ± 0.05	0.76 ± 0.02	1.05 ± 0.08
25	U	1.19 ± 0.07	0.99 ± 0.03	1.18 ± 0.02	0.75 ± 0.01
26	C	0.82 ± 0.04	1.49 ± 0.08	0.80 ± 0.04	1.40 ± 0.05
27	C	0.75 ± 0.03	0.54 ± 0.05	0.74 ± 0.02	0.51 ± 0.03
28	C	0.42 ± 0.04	0.24 ± 0.01	0.58 ± 0.02	0.27 ± 0.02
29	A	0.86 ± 0.08	0.24 ± 0.03	0.97 ± 0.05	0.17 ± 0.01
30	C	0.81 ± 0.04	0.11 ± 0.03	0.77 ± 0.04	0.14 ± 0.02
31	C	0.76 ± 0.02	0.09 ± 0.02	0.73 ± 0.01	0.16 ± 0.03
32	U	0.42 ± 0.05	0.25 ± 0.02	0.62 ± 0.01	0.22 ± 0.03
33	G	0.86 ± 0.09	0.27 ± 0.01	0.76 ± 0.03	0.25 ± 0.03
34	A	0.82 ± 0.03	0.62 ± 0.03	1.19 ± 0.07	0.21 ± 0.08
35	C	0.49 ± 0.04	1.01 ± 0.03	0.56 ± 0.03	0.87 ± 0.09
36	C	1.42 ± 0.06	1.11 ± 0.02	1.80 ± 0.09	0.83 ± 0.07
37	C	1.44 ± 0.08	1.11 ± 0.01	1.60 ± 0.12	0.91 ± 0.06
38	C	1.23 ± 0.02	0.93 ± 0.05	1.20 ± 0.04	0.56 ± 0.03
39	A	2.93 ± 0.25	1.01 ± 0.04	3.61 ± 0.15	0.98 ± 0.05
40	U	0.97 ± 0.07	0.64 ± 0.01	1.15 ± 0.05	0.27 ± 0.01
41	G	1.23 ± 0.05	0.81 ± 0.05	1.15 ± 0.09	0.83 ± 0.05
42	C	1.28 ± 0.01	1.17 ± 0.04	1.20 ± 0.05	0.86 ± 0.01
43	C	1.25 ± 0.02	1.19 ± 0.06	1.22 ± 0.01	0.90 ± 0.02
44	G	1.48 ± 0.05	0.80 ± 0.06	1.50 ± 0.08	0.76 ± 0.02
45	A	0.92 ± 0.04	0.82 ± 0.06	0.80 ± 0.01	0.76 ± 0.02
46	A	0.94 ± 0.06	0.77 ± 0.03	0.89 ± 0.04	0.55 ± 0.05
47	C	0.71 ± 0.01	0.15 ± 0.04	1.07 ± 0.05	0.16 ± 0.04
48	U	0.89 ± 0.08	0.88 ± 0.04	1.07 ± 0.06	0.08 ± 0.05
49	C	0.83 ± 0.05	0.17 ± 0.01	1.25 ± 0.02	0.26 ± 0.01
50	A	0.39 ± 0.02	0.88 ± 0.05	0.44 ± 0.06	0.95 ± 0.07
51	G	0.99 ± 0.09	0.70 ± 0.08	0.86 ± 0.03	1.16 ± 0.05
52	A	1.09 ± 0.03	0.87 ± 0.05	1.05 ± 0.06	1.07 ± 0.02
53	A	0.85 ± 0.04	1.03 ± 0.07	1.19 ± 0.01	0.81 ± 0.02
54	G	0.47 ± 0.06	0.76 ± 0.02	0.35 ± 0.03	1.54 ± 0.07
55	U	0.91 ± 0.04	1.05 ± 0.08	1.18 ± 0.01	0.11 ± 0.02
56	G	1.69 ± 0.15	0.35 ± 0.02	1.64 ± 0.07	0.52 ± 0.04
57	A	0.57 ± 0.04	0.61 ± 0.01	0.42 ± 0.02	0.48 ± 0.06
58	A	0.82 ± 0.07	1.07 ± 0.04	0.76 ± 0.01	0.81 ± 0.03
59	A	0.93 ± 0.01	0.56 ± 0.08	0.95 ± 0.07	0.48 ± 0.01
60	C	1.14 ± 0.04	0.72 ± 0.01	1.11 ± 0.07	0.81 ± 0.06
61	G	0.88 ± 0.08	0.13 ± 0.03	0.79 ± 0.02	0.19 ± 0.06

(*continued*)

TABLE 2. *Continued.*

		50S		70S	
No.	Nucleotide	Extract/in solution	In situ/extract	Extract/in solution	In situ/extract
62	C	0.48 ± 0.05	0.18 ± 0.02	0.55 ± 0.06	0.26 ± 0.02
63	C	0.45 ± 0.01	0.22 ± 0.04	0.48 ± 0.03	0.23 ± 0.03
64	G	0.43 ± 0.07	0.78 ± 0.03	0.39 ± 0.03	1.17 ± 0.08
65	U	1.02 ± 0.04	1.15 ± 0.07	0.99 ± 0.03	0.75 ± 0.02
66	A	0.82 ± 0.04	1.14 ± 0.02	0.94 ± 0.02	0.98 ± 0.12
67	G	0.76 ± 0.05	0.19 ± 0.02	0.91 ± 0.07	0.21 ± 0.01
68	C	0.83 ± 0.01	0.81 ± 0.04	1.21 ± 0.07	0.81 ± 0.04
69	G	1.02 ± 0.10	0.75 ± 0.02	0.83 ± 0.02	0.83 ± 0.05
70	C	0.98 ± 0.07	1.02 ± 0.04	0.94 ± 0.06	0.96 ± 0.04
71	C	1.03 ± 0.06	0.52 ± 0.01	1.18 ± 0.09	0.56 ± 0.04
72	G	0.57 ± 0.04	0.08 ± 0.01	0.55 ± 0.03	0.16 ± 0.02
73	A	0.78 ± 0.05	0.46 ± 0.05	0.81 ± 0.04	0.47 ± 0.01
74	U	0.79 ± 0.08	1.14 ± 0.03	0.79 ± 0.01	0.32 ± 0.02
75	G	1.02 ± 0.03	0.23 ± 0.01	1.23 ± 0.02	0.21 ± 0.04
76	G	0.98 ± 0.07	0.19 ± 0.02	1.17 ± 0.07	0.23 ± 0.02
77	U	0.89 ± 0.04	0.83 ± 0.07	0.76 ± 0.01	0.74 ± 0.02
78	A	0.74 ± 0.01	1.53 ± 0.15	0.81 ± 0.04	0.96 ± 0.07
79	G	0.74 ± 0.01	0.81 ± 0.06	0.98 ± 0.02	0.95 ± 0.03
80	U	0.58 ± 0.01	0.67 ± 0.03	0.55 ± 0.05	0.27 ± 0.02
81	G	0.62 ± 0.03	0.08 ± 0.01	0.58 ± 0.01	0.13 ± 0.01
82	U	0.82 ± 0.04	0.68 ± 0.01	0.91 ± 0.07	0.17 ± 0.02
83	G	0.83 ± 0.02	0.17 ± 0.02	1.01 ± 0.05	0.22 ± 0.04
84	G	0.98 ± 0.04	0.23 ± 0.05	0.89 ± 0.07	0.15 ± 0.01
85	G	0.86 ± 0.06	0.78 ± 0.01	1.06 ± 0.10	1.45 ± 0.10
86	G	1.05 ± 0.07	0.95 ± 0.07	1.02 ± 0.02	1.56 ± 0.08
87	U	1.01 ± 0.08	0.97 ± 0.04	1.15 ± 0.09	0.93 ± 0.03
88	C	0.89 ± 0.04	0.97 ± 0.02	1.08 ± 0.05	0.99 ± 0.05
89	U	0.99 ± 0.08	1.02 ± 0.04	1.15 ± 0.06	0.88 ± 0.09
90	C	1.17 ± 0.05	1.03 ± 0.04	1.21 ± 0.04	0.09 ± 0.03
91	C	0.97 ± 0.05	0.95 ± 0.01	1.12 ± 0.09	0.91 ± 0.04
92	C	1.01 ± 0.08	0.89 ± 0.05	1.04 ± 0.01	0.93 ± 0.03
93	C	1.11 ± 0.01	0.97 ± 0.04	1.03 ± 0.07	0.94 ± 0.04
94	A	0.76 ± 0.02	1.01 ± 0.05	0.87 ± 0.08	1.09 ± 0.11
95	U	0.86 ± 0.04	1.05 ± 0.04	0.92 ± 0.05	0.99 ± 0.04
96	G	0.56 ± 0.04	1.08 ± 0.09	0.47 ± 0.03	0.87 ± 0.01
97	C	1.04 ± 0.07	0.92 ± 0.09	0.99 ± 0.05	1.04 ± 0.02
98	G	0.54 ± 0.06	0.47 ± 0.03	0.50 ± 0.01	0.49 ± 0.05
99	A	0.17 ± 0.01	0.63 ± 0.04	0.14 ± 0.02	0.67 ± 0.01
100	G	0.93 ± 0.05	0.38 ± 0.03	1.04 ± 0.05	0.42 ± 0.07
101	A	0.84 ± 0.05	0.57 ± 0.02	0.90 ± 0.08	0.21 ± 0.03
102	G	1.05 ± 0.07	0.54 ± 0.02	0.95 ± 0.09	0.15 ± 0.01
103	U	0.67 ± 0.01	1.20 ± 0.01	0.61 ± 0.04	1.09 ± 0.13
104	A	0.86 ± 0.07	1.08 ± 0.01	0.91 ± 0.02	0.99 ± 0.11
105	G	0.95 ± 0.10	0.86 ± 0.05	1.02 ± 0.08	0.89 ± 0.03
106	G	0.89 ± 0.10	1.02 ± 0.05	0.97 ± 0.03	0.96 ± 0.09
107	G	0.87 ± 0.01	1.05 ± 0.05	0.92 ± 0.02	1.09 ± 0.10
108	A	1.04 ± 0.07	0.95 ± 0.10	1.10 ± 0.09	0.89 ± 0.15
109	A	0.94 ± 0.04	0.87 ± 0.08	1.03 ± 0.01	0.89 ± 0.03
110	C	n.d.	n.d.	n.d.	n.d.
111	U	n.d.	n.d.	n.d.	n.d.
112	G	n.d.	n.d.	n.d.	n.d.
113	C	n.d.	n.d.	n.d.	n.d.
114	C	n.d.	n.d.	n.d.	n.d.
115	A	n.d.	n.d.	n.d.	n.d.
116	G	n.d.	n.d.	n.d.	n.d.
117	G	n.d.	n.d.	n.d.	n.d.
118	C	n.d.	n.d.	n.d.	n.d.
119	C	n.d.	n.d.	n.d.	n.d.

[a]The deviations from averaged values are given.

same positions of 5S rRNA isolated from 70S and then subjected to iodine cleavage (Fig. 2, lanes 4 and 2; Table 2, compare columns 50S Extract/in solution and 70S Extract/in solution); no position, in addition, was observed. It is therefore the assembly process that prefers 5S rRNA molecules modified at these positions; there is no additional position that is selected in its modified state for the association of the subunits to 70S ribosomes. The positions of thioated phosphates that affect the assembly in a positive or negative way are summarized in Figure 3A.

Contact patterns within the 50S subunits and the 70S ribosome

50S subunits containing modified 5S rRNA were subjected to iodine-induced cleavage. Bands that were significantly protected in comparison with 5S rRNA in solution indicate that the access of iodine of the corresponding phosphate was limited due to tertiary interactions of 5S rRNA within the ribosome or due to the tight neighborhood of ribosomal proteins or 23S rRNA. Because we compared the intensity of a distinct band after iodine-induced cleavage before and after extraction from the reconstituted particle (lanes 5 and 4 in Fig. 1, respectively, and Table 2, columns In situ/extract), a possible impairment of the assembly as described in the preceding section was taken into consideration. Strong protections were found at the 5′-phosphates of 21 positions (G10, G18, C28, A29, C30, C31, U32, G33, C47, C49, G61, C62, C63, G67, G72, G75, G76, G79, G81, G83, and G84); some weak protections were also found at 22 other positions (G9, G13, G16, C19, G20, G21, G23, C27, A34, U40, G56, A57, A59, C71, A73, U80, U82, G98, A99, G100, A101, and G102; see Fig. 1, lanes 5; Table 2, columns In situ/extract).

Some positions are more exposed inside the 50S particle and thus are more easily cleaved than the corresponding positions on the 5S rRNA isolated from 50S particles before the cleavage reaction was performed (Fig. 1, compare lanes 5 and 4; Table 2, columns In situ/extract). Examples are C26 and A78.

Some phosphates showed a conspicuous change in their protection patterns when the 50S subunit was incorporated into the 70S ribosome. For example, U55 shows the same accessibility in 5S rRNA extracted from reconstituted particles and in 5S rRNA within the 50S subunit (Fig. 1, lanes 4 and 5, respectively; Table 2, columns In situ/extract). In contrast, the corresponding band in 70S ribosomes is strongly protected (Fig. 2, compare lanes 5 and 4; Table 2, columns In situ/extract). Altogether, the following phosphate residues were newly protected in the 70S ribosome: C38, A46, U48, U55, and U74. The strongest protection was observed for U55 followed by U48. Some protections become stronger in the 70S ribosome in comparison with that in the 50S particle: A34, U40, U80, U82, A101, and G102.

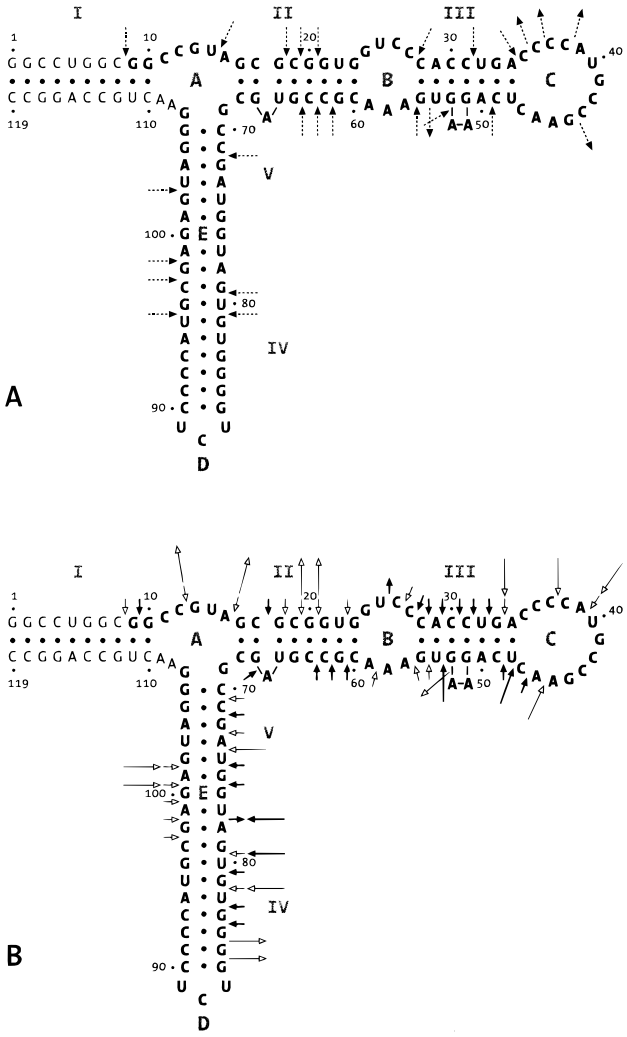

FIGURE 3. Summary of the protection patterns projected onto the model of the secondary structure of 5S rRNA (Moore, 1996). A, assembly effects of 5S rRNA. Arrows directed to the RNA chain indicate a position where the presence of a phosphorothiate residue impairs the incorporation of the corresponding 5S rRNA during the assembly process. Arrows pointing away from the RNA chain indicate a favored incorporation, when a phosphorothiate is at this position. B, protection pattern of 5S rRNA within the 50S subunit (short arrows) and changes seen after the association with a native 30S subunits forming 70S ribosome (long arrows). Arrows directed to the RNA chain, protection of the phosphorothioate from iodine cleavage. Arrows directed away from the RNA chain, enhanced accessibility of the phosphorothioate residue to the iodine atack. Open and closed arrows, slight and strong effects, respectively. For example, head-to-head arrangement of arrows at the position 78: enhanced accessibility for iodine only in the 50S subunit but not in the 70S ribosome (compare Figs. 1 and 2).

On the other hand, some positions that were protected in the 50S subunit became easily accessible in the 70S subunit: G13, G16, G20, and G21 (compare Fig. 1, lanes 5 with the corresponding lanes in Fig. 2; Table 2, columns In situ/extract). Similar but less pronounced cases are G54, G85, and G86, which were not protected in 50S subunits, but are more accessible in 70S ribosomes. A reverse change was also ob-

served. The enhanced accessibility seen with A78 changed to a normal accessibility in the 70S ribosome.

The protection pattern of 5S rRNA within the 50S subunit and the changes seen when the 50S subunits were associated with the small subunits to form 70S ribosomes are summarized in Figure 3B.

The fact that association of the 30S and 50S subunits can increase the accessibility of some positions and decrease the accessibility of others is a strong indication for a conformational change of the central protuberance upon association. In addition, the small subunit might directly protect some exposed regions of the 5S rRNA in the central protuberance.

Patterns of 5S rRNA within functional complexes

The protection patterns of 5S rRNA within functional complexes of the ribosome were almost identical to the pattern inside the associated but otherwise empty 70S ribosome except for a few minor changes. In contrast to empty 70S ribosomes, position C26 was slightly protected in all three complexes, viz. the P_i, PRE, and POST states; slight protections were observed in positions A46 and A99 in PRE and POST states. The accessibility was slightly enhanced at C63 in PRE and POST complexes compared with empty 70S ribosomes (data not shown).

It follows that the sugar–phosphate backbone of 5S rRNA undergoes conformational changes during the assembly of the 50S subunit and within this subunit upon association with the small subunit forming 70S ribosomes, but otherwise seems to adopt the same conformation, at least in the three defined functional states, P_i, PRE, and POST.

DISCUSSION

5S rRNA does not contain modified bases and thus is appropriate to be transcribed in vitro and to be used for the assembly of 50S ribosomal subunits. This feature opens an interesting array of applications: A homogenous population of reconstituted 50S particles can be obtained, all carrying an altered 5S rRNA according to mutations in the 5S rRNA gene (e.g., Zvereva et al., 1998) or modified 5S rRNA, as in this study.

The advantages and limitations of the phosphorothioate method were discussed previously by Dabrowski et al. (1996). Although the substitution of a nonbridging oxygen by sulfur is usually considered as a minimal alteration in an RNA structure, one has to keep in mind that there are several remarkable differences between a phosphate and a phosphorothioate. The negative charge in the latter tends to localize largely on the sulfur atom. The polarizability of sulfur is higher than that of oxygen. There is also a remarkable difference in the ability of the phosphate and the phosphorothioate to coordinate metal ions: Mg^{2+} as well as other hard ions coordinate to oxygen, whereas soft metal ions coordinate either predominantly to sulfur (e.g., Cd^{2+}) or to both sulfur and oxygen (e.g., Mn^{2+}; for review, see Heidenreich et al., 1993).

Control experiments, where about 90% of the magnesium ions were replaced by manganese ions, revealed a set of thioated nucleotides impairing the assembly process that was practically identical to that observed in the presence of Mg^{2+} (not shown). It follows that at least most of these positions are not involved in the coordination of Mg^{2+} ions, but rather fulfill delicate requirements of the tertiary and quaternary structure involving the 5S rRNA; these requirements cannot be fulfilled by a sulfur atom.

In addition to the coordination of a metal ion involving a phosphate group hydrogen bonds, a change of the tertiary structure of the RNA or a contact with another ribosomal component, either rRNA or ribosomal protein, can prevent an iodine-induced cleavage. In general, cleavage in the complex depends on the accessibility of the sulfur to the reagent as well as the spatial arrangement of the 2′-OH group such that it can participate in the expulsion of the 5′ oxygen. For example, if a tight contact of a distinct phosphate group with a ribosomal protein prevents the access of iodine, no cleavage is observed at this position ("protection"). The position might be protected either directly by a tight contact to the binding protein or by a conformational change of the RNA induced by the incorporation of the 5S rRNA during the assembly process. Protected phosphate residues can be identified by comparing the intensities of the bands corresponding to the RNA fragments released after iodine cleavage and separated by gel electrophoresis. An enhancement of the band intensity of a bound RNA compared to the one in solution suggests a conformational change of the RNA resulting from binding to the protein, so that this position is more easily accessible to iodine in the bound state.

Phosphorothioates at 21 positions affected the assembly and reduced the efficiency of 5S rRNA incorporation into the 50S subunit by at least 30%. These positions are clustered in helix II (C19 shows a particularly strong effect) and at the transition of E loop and helix IV with an outstanding effect at A99 (arrows pointing to the 5′ side of the respective nucleotide in Fig. 3A).

The structural requirements for integrating 5S rRNA into the ribosome are much more stringent than those for binding a tRNA to a ribosomal site, for which less than 10% of critical positions were found (Dabrowski et al., 1995; M. Schäfer, S. Patzke, & K.H. Nierhaus, manuscript in prep.; for the anticodon stem-loop structure, see von Ahsen et al., 1997).

Interestingly, the reverse case was also observed. Phosphorothioates at a few positions improved the in-

corporation of the corresponding 5S rRNA molecule. There is only one cluster of this kind of positions at the C-loop (arrows pointing away from the 5′ side of the respective nucleotide in Fig. 3A). We have no simple explanation for this observation. A similar case was described by Milligan and Uhlenbeck (1989) concerning the interaction of an RNA fragment of the R17 phage and its coat protein.

5S rRNA, together with its binding proteins L5, L18, and L25, are important components of the central protuberance (Stöffler & Stöffler-Meilicke, 1986). Only 10 phosphate positions are protected in the isolated tetrameric complex (Shpanchenko et al., 1996); eight of these comprise a subgroup of the more than 40 protected phosphates of the 5S rRNA observed after assembly into the 50S subunit (Fig. 3B). For example, in the complex with the three 5S rRNA binding proteins, only a few phosphate residues (A73, U80, U82, A99, and A101, Shpanchenko et al., 1996) were protected in helix V–loop E–helix IV structural elements known as a binding site of the protein L25 (Huber & Wool, 1984), whereas, inside the ribosome, many more protections have been observed in this area (Fig. 3B). Similarly, only a limited number of phosphates protected in the complexes with three proteins (U22 and A34, Shpanchenko et al., 1996) were found in the helix II–loop B–helix III region known as a binding site of the protein L18 (Huber & Wool, 1984). This region is strongly protected both in 50S subunit and 70S ribosomes. The reverse case has been also observed. Phosphates at the positions U22 and A39, protected in the 5S rRNA–protein complex, become fully accessible to iodine cleavage in 50S subunits and 70S ribosomes.

These data clearly indicate that the 5S rRNA–protein complex undergoes further conformational changes when it becomes a domain of 50S subunit. The tight packing of the tetrameric complex within the central protuberance contrasts with the loose structure of the 5S rRNA–(L5, L18, L25) complex in solution. This observation agrees well with the strong protection of L5, L18, and L25 in the ribosome against tritium bombardment (Agafonov et al., 1997).

About 15% of the positions changed their protection pattern on the way from the 50S subunit to the 70S ribosome via association with the small subunit. Changes occur in both directions. Some phosphates are more protected in the 70S ribosome (long arrows pointing to the 5′-side of the respective nucleotide in Fig. 3B), others are less (long arrows pointing away). The most essential changes occurred at the phosphate residues of U48 and U55. The latter was accessible to iodine cleavage in 5S rRNA within the 50S subunit. This residue belongs to a 5S rRNA region devoid of strongly protected phosphate residues within the 50S subunit. A likely explanation is that the protection of this single phosphate is caused by a direct contact of 5S rRNA with the 30S subunit within 70S ribosome. The phosphate of residue U48, which became strongly protected in the 70S ribosome, is conspicuously surrounded by phosphates that were protected already in the 50S subunit (A45–C49). This observation suggests that this region of the 5S rRNA becomes more tightly packed upon formation of 70S ribosomes. Other changes in the protection pattern of 5S rRNA in 70S subunit are not as significant and are located at areas of interactions with proteins L18 and L25. For instance, the increase of the protections in loop E–helix IV (Fig. 3B) region can be explained by a further stabilization of interactions with protein L25 inside the 70S ribosome, whereas the increase of the protections in helix II region (Fig. 3B) may indicate more tight interactions with protein L18.

The phosphate residues at G85 and G86 with enhanced reactivity are located next to the very strongly protected phosphates of U80–G84 (most probably caused by protein L25). Note that, according to cross-linking data (Dokudovskaya et al., 1996), the nearby nt U89 may be involved in stacking interactions with nucleotides of 23S rRNA. It is conceivable that such quaternary interactions increase the accessibility of G85 and G86 for iodine attack.

G54 in loop B also shows an enhanced accessibility in 70S ribosomes. The adjacent nt U55 is, as mentioned above, highly protected and probably involved in interactions with the 30S subunit. C26, with an enhanced accessibility in both 50S subunits and 70S ribosomes, is also located in loop B, but in the opposite strand of RNA. The enhanced reactivity of two opposite phosphate groups in loop B may reflect the bending of the helix II–loop B–helix III–loop C.

This striking set of changes observed during association of the subunits contrasts with the stable protection pattern observed with various functional complexes. These programmed ribosomal complexes contained a single tRNA at the P site (P_i state) with an occupancy of 0.85 (i.e., 85% of the ribosomes were in the P_i state), or two tRNAs at the A and P sites (PRE state), or P and E sites (POST state, see Table 1). The corresponding protection patterns of 5S rRNA did not show any significant differences between that of empty ribosomes and those of the various functional states. This clearly demonstrates that, after the 70S ribosome has been formed, the central protuberance is in the same conformation at the main stages of protein synthesis. This finding stands against some speculations concerning conformational changes of 5S rRNA during functional transitions (Weidner et al., 1977; Garrett et al., 1981; Christensen et al., 1985).

A rigid structure of the central protuberance might be important for a stable and defined organization of functional centers of the ribosome, because the 5S rRNA contacts two important functional domains of 23S rRNA, viz. the peptidyl transferase and EF-G-binding domain

(U89 of 5S rRNA; Dontsova et al., 1994; Dokudovskaya et al., 1996). Furthermore, it probably also contacts the head of the 30S subunit (U55, this work; Frank et al., 1995; Stark et al., 1995).

This detailed view of the central protuberance agrees with the general view of the gross structure of the 70S ribosome derived from electron-microscopy studies (Stark et al., 1997; R. A. Agrawal, P. Penczek, R.A. Grassucci, S. Srivastava, A. Malhotra, N. Burkhardt, R. Jünemann, K.H. Nierhaus, & J. Frank, submitted) and analysis with neutron small-angle scattering (Svergun et al., 1997; Wadzack et al., 1997), viz. the shape of the 70S ribosome does not change significantly in the course of elongation within the limits of resolution (down to 12 Å). However, transient alterations in the 5S rRNA structure upon binding of elongation factors to the ribosome cannot be excluded.

MATERIALS AND METHODS

Preparation of modified 5S rRNA

The DNA template containing the *E. coli* 5S rDNA sequence linked to a T7 promoter was prepared as described by Dontsova et al. (1994) and transcribed by T7 polymerase using the procedure of Shpanchenko et al. (1996). 5S rRNA was purified by electrophoresis in 4% PAAG with 7 M urea (Alexeeva et al., 1996). Radioactive labeling at the 5′ end was performed using [γ-^{32}P]ATP and T4 polynucleotide kinase (Alexeeva et al., 1996). After the second purification in 4% PAAG with 7 M urea, the 5S rRNA was used for reconstitution of 50S ribosomal subunits.

Reconstitution of 50S ribosomal subunits and 70S ribosomes

23S rRNA was isolated from crude 70S ribosomes and total proteins of 50S ribosomal subunit (TP50) were isolated as described by Nierhaus (1990). Twofold molar excess of the ^{32}P-labeled modified 5S rRNA was mixed with 23S rRNA and TP50 and reconstituted following the standard procedure (Nierhaus, 1990) except that the reconstitution was scaled up to 200 pmol of 23S rRNA. The reconstitution mixture was applied to a 10–30% sucrose gradient in 20 mM Hepes-KOH, pH 7.6, 20 mM magnesium acetate, 400 mM NH_4Cl, 0.2 mM EDTA, 4 mM 2-mercaptoethanol, and centrifuged for 20 h, 4 °C at 23,000 r.p.m. in an SW 40 Ti rotor (Beckman). After fractionating (fraction size was about 500 μL), the radioactivity and optical density were determined and the fractions with the 50S ribosomal subunit were collected. The 50S ribosomal subunits were precipitated by centrifugation for 20 h, 4 °C at 35,000 r.p.m. in an SW 60 Ti rotor (Beckman). The activity of the reconstituted 50S subunits was measured by the poly(U) dependent poly(Phe) synthesis assay according to Bommer et al. (1996). In order to obtain 70S ribosomes, the reaction mixture after reconstitution of the 50S subunits was diluted into a buffer resulting in final concentrations of 20 mM Hepes-KOH, pH 7.6, 20 mM magnesium acetate, 100 mM NH_4Cl, 0.2 mM EDTA, 4 mM 2-mercaptoethanol. Twofold molar excess of 30S subunits was added in the same buffer, and the mixture was incubated 30 min at 40 °C. The re-association mixture was applied to a 10–30% sucrose gradient in the same buffer and centrifuged for 23 h, 4 °C at 18,000 r.p.m. in an SW 40 Ti rotor (Beckman). After fractionating (fraction volume was about 500 μL), the radioactivity and optical density were determined and the fractions with the 70S ribosomes were collected. The 70S ribosomes were precipitated via centrifugation for 27 h, 4 °C at 20,000 r.p.m. in an SW 60 Ti rotor (Beckman) and dissolved in a proper buffer for the functional tests.

Functional complexes of the ribosome

Formation of the functional ribosomal complexes, the estimation of the amount of bound N-AcPhe-tRNA^{Phe}, and tRNA$_f^{Met}$ and determination of the peptidyltransferase and translocation activity followed the procedures described by Bommer et al. (1996).

Iodine cleavage

Iodine cleavage and analysis of RNA fragments were performed as described by Shpanchenko et al. (1996), except that a buffer was used that contained 20 mM Hepes-KOH, pH 7.6, 6 mM magnesium acetate, 150 mM NH_4Cl, 4 mM 2-mercaptoethanol, 2 mM spermidine, and 0.05 mM spermine.

The autoradiographs were scanned using an Epson GT-8000 scanner. The data were processed as described by Dabrowski et al. (1995).

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