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Binding sites of E. coli and B. stearothermophilus ribosomal proteins on B. stearothermophilus 5S RNA

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ABSTRACT

The primary binding sites for Bacillus stearothermophilus proteins B-L5 and B-L22 and the Escherichia coli proteins E-L5, E-L18 and E-L25 on B. stearothermophilus 5S RNA were determined by limited ribonuclease digestion of the corresponding 5S RNA-protein complexes. The results obtained in this study are in agreement with our previous experiments in which the binding sites of E. coli and B. stearothermophilus proteins were determined for E. coli 5S RNA and lead to the conclusion that the proteins interact with the most conserved regions of 5S RNA. A comparison of the results obtained in this study with those of other published experiments suggest that the proposed interaction of nucleotides 16-21 with those of 58-63 is facilitated by protein binding to 5S RNA.

INTRODUCTION

One promising approach to investigate protein-nucleic acid interaction is that by comparative studies, in which similar components of different organisms are permitted to interact. Evolutionally altered regions in the nucleic acid and/or protein components are most likely less important for the interaction than the conserved ones and should therefore find less consideration for the localization of the specific sites of interaction.

Development of methods for the total reconstitution of 30S (1) and 50S (2,3) ribosomal subunits permitted studies on assembly of functionally active ribosomes from components of different bacteria (2,4,5). In addition, it has been possible to construct and characterize hybrid subribosomal particles such as 5S RNA protein complexes from E. coli and B. stearothermophilus 50S ribosomal components (6). These complexes represent an ideal model

system for a comparative study on protein-RNA interaction. The preceding paper on this topic (7) has dealt with the localization of E. coli 5S RNA binding sites for the E. coli proteins E-L5, E-L18 and E-L25 as well as for the B. stearothermophilus proteins B-L5 and B-L22. In this communication we report an extension of such a study in which the E. coli and B. stearothermophilus protein binding sites on B. stearothermophilus 5S RNA are described.

MATERIALS AND METHODS

(a) Materials

If not mentioned otherwise, the biochemical reagents were purchased from Boehringer Mannheim, Germany. RNAse-free DNase and pancreatic ribonuclease as well as some T_1-RNase preparations were obtained from Boehringer Mannheim, while additional T_1-RNase preparations were purchased from Sankyo, Japan or Sigma,USA. Millipore-filters were supplied by Millipore Ltd., England, DEAE-paper (DE 81) and 3 MM paper from Whatman, England, cellulose-acetate from Oxoid Ltd., England, and X-ray film (Curix RPI) from Agfa, Germany.

(b) Buffers and solutions

Tris/Mg/NH_4-I buffer: 10 mM Tris-HCl (pH 7.4 at 23°C), 30 mM NH_4Cl, 10 mM $MgCl_2$, 6 mM ß-mercaptoethanol. Tris/Mg/NH_4-II buffer: same as Tris/Mg/NH_4-I except for $MgCl_2$ which was 0.3 mM. Tris/Mg/K-I buffer: 30 mM Tris-HCl (pH 7.4 at 23°C), 20 mM $MgCl_2$, 1 M KCl, 6 mM ß-mercaptoethanol. Tris-Mg buffer: same as Tris/Mg/K-I except for KCl which was omitted. Tris/Mg/K-II buffer: Tris/Mg-K-I buffer: Tris/Mg buffer = 1:2. Urea/Tris/Mg/K-25 buffer: 6 M urea, 10 mM Tris-HCl (pH 7.4 at 23°C), 1 mM $MgCl_2$, 250 mM KCl, 6 mM ß-mercaptoethanol. Urea/Tris/Mg/K-30 buffer: same as Urea/Tris/Mg/K-25 except for KCl which was 300 mM. Tris/Mg/DNase buffer: 20 mM Tris-HCl (pH 7.4 at 23°C), 20 mM magnesium acetate and 1 µg RNase-free DNase/ml. Tris/boric acid/EDTA buffer: 80 mM Tris-HCl (pH 7.4 at 23°C), 0.1 M boric acid, 2 mM Na_2 EDTA. Tris-Urea buffer: 80 mM Tris-HCl (pH 7.4 at 23°C), 8 M urea. Buffers for high-voltage electrophoresis: First dimension: 7 M urea, 50 mM Na_2EDTA, pH 3.5 (5% acetic acid; v/v). Second dimension: 7% formic acid, pH 1.9; for separation of

nucleotides: 5% acetic acid, pH 3.5, 0.5% pyridine. Elution of nucleotides: 70% triethylamine carbonate, pH 10.

(c) Ribosomes, ribosomal proteins and unlabeled 5S RNA

E. coli (strain A19) and B. stearothermophilus (strain 799) cells were grown and the 50S ribosomal subunits isolated as described previously (7). Before isolation of total 50S protein fractions, the subunits were analyzed for protein synthesis activity (8). B. stearothermophilus 50S ribosomal subunits were also checked for total reconstitutional activities (9). To obtain total 50S protein fractions, the ribosomal subunits (in Tris/Mg/NH_4-I buffer) were mixed with an equal volume of 8 M urea · 4 M LiCl and stored at 0°C for 48 hrs. The precipitated RNA was removed by low speed centrifugation and the resulting supernatants were designated E. coli and B. stearothermophilus UTP50 fraction. Since the B. stearothermophilus UTP50 fraction contains significant amounts of 5S RNA, it was passed through an DEAE-cellulose column to remove the RNA. The unbound protein fraction was designated B. stearothermophilus "AB" fraction (9). Therefore, B. stearothermophilus 5S RNA was complexed with either E. coli UTP50 or B. stearothermophilus "AB" fraction proteins. B. stearothermophilus 5S RNA (not radioactively labeled) was prepared (10) and characterized by disc gel electrophoresis (5).

E. coli UTP50 and B. stearothermophilus "AB" fraction proteins were only used in [^{32}P]-5S RNA protein complex studies after they had previously been analyzed for 5S RNA binding in large scale experiments which enabled two-dimensional electrophoresis of the bound proteins (6).

Purified E. coli proteins E-L5, E-L18 and E-L25 were isolated as previously described (11) and kindly supplied by Prof. H.G. Wittmann.

(d) B. stearothermophilus [^{32}P]-5S RNA

In order to facilitate optimal [^{32}P]-labelling of B. stearothermophilus ribosomal RNA, removal of inorganic phosphate from the yeast extract (Difco) was necessary. Therefore a 10% yeast extract solution was made 10 mM with respect to $MgCl_2$ and the pH adjusted to 9 with NH_4OH. The precipitated magnesium phosphate

was removed by centrifugation (15,000 rpm, 30 min, 4°C), and per 50 ml supernatant 10 g tryptone (Difco), 0.1 mg manganese chloride and 10 g NaCl were added. The pH of this solution (solution A) was adjusted to 7 before sterilization. Separately, 10 g glucose were dissolved in 50 ml water and sterilized (solution B). The final growth medium was prepared by mixing solutions A and B in a 1:1 ratio.

B. stearothermophilus cells were labelled as follows: 1 ml of mid log-phase preculture (rich medium) was transferred to 100 ml phosphate-depleted medium containing 40-50 mCi carrier free [^{32}P]-inorganic phosphate from Amersham Buchler (pH adjusted to 7). The culture was permitted to grow at 61°C until the mid log-phase was reached (generation time of 20 to 30 min). At this time, 60-80% of the [^{32}P]-phosphate was routinely taken up by the cells, as determined by Millipore filtration. The cells were harvested by low speed centrifugation (10,000 rpm, 20 min, 4°C), and frozen at -20°C until further use.

To isolate [^{32}P]-5S RNA, the frozen B. stearothermophilus cell pellet was ground with alumina (from Alcoa, four times the weight of the cell pellet) in the presence of a few drops Tris/Mg/Dnase buffer for 5 min (on ice). The total ribosomal RNA was isolated from the supernatant by phenol extraction. [^{32}P]-5S RNA was further purified by slab gel electrophoresis (7). The specific activity of the radioactive 5S RNA was approximately 0.5 x 10^6 dpm per µg.

(e) Reconstitution of 5S RNA protein complexes

Reconstitution of 5S RNA protein complexes was performed as described (6,7). To B. stearothermophilus [^{32}P]-5S RNA (10^7 cpm; diluted to 3.5 A_{260} units with cold 5S RNA), 100 equivalent units (e.u.) E. coli UTP50 or B. stearothermophilus "AB" fraction proteins were added and incubated for 12 h at 0°C in 3 ml Tris/Mg/K-II buffer. One e.u. protein is defined as that amount of protein obtained from 1 A_{260} unit 50S ribosomal subunits. Complex formation was analyzed by Millipore filtration or slab gel electrophoresis.

(f) Millipore filtration of 5S RNA protein complexes

Retention of B. stearothermophilus [^{32}P]-5S RNA or its fragments

to Millipore filters is due to their interaction with ribosomal proteins. It was measured under identical conditions as described for the E. coli system (7).

(g) Ribonuclease digestion

For standard fingerprint analyses:
Ten µg [^{32}P]-5S RNA were hydrolyzed with 1 µg T_1-RNase or pancreatic RNAse at 37°C for 30 min in 2-10 µl 0.01 M Tris-HCl (pH 7.4) containing 0.001 M EDTA. Fragments obtained after limited digestion of 5S RNA protein complexes were similarly hydrolyzed.

5S RNA protein complexes were hydrolyzed in Tris/Mg/K-II buffer at 37°C for 30 min. Since a T_1-RNase to substrate ratio of 1:10 (w/w) continued to yield significant amounts of non-hydrolyzed 5S RNA, the ratio was decreased to 1:5. The optimal ratio of pancreatic ribonuclease to 5S RNA protein complex was found to be 1:10 (w/w). Standard secondary analysis of T_1-RNase or pancreatic ribonuclease oligonucleotides were carried out as described (12).

(h) Isolation of RNA fragments

The RNA fragments were isolated as described (7). After hydrolysis of the 5S RNA protein complexes with T_1- or pancreatic ribonuclease, the resulting RNA fragments were extracted three times with Tris/Mg/K-II-saturated phenol at 4°C. The RNA was then precipitated from the aqueous phases by addition of two volumes -20°C ethanol (overnight) and pelleted by centrifugation (10,000 rpm, 4°C, 30 min). To remove traces of buffer and ethanol, the pellets were dried under vacuum before dissolving them in Tris/boric acid/ EDTA or Tris/urea buffers. RNA fragments dissolved in Tris/boric acid/EDTA buffer were electrophoresed in 10% polyacrylamide slabs and those dissolved in Tris/urea buffer were electrophoresed in 12% polyacrylamide slabs (7). After electrophoresis, the slab gels were autoradiographed and the RNA fragment bands isolated with 0.6 M NaCl (7).

(i) Sequence determination

Oligonucleotides obtained from T_1- or pancreatic ribonuclease digestion of 5S RNA or RNA fragments were separated by two -dimensional high-voltage paper electrophoresis. The first di-

mension carried out on cellulose acetate-strips was performed at pH 3.5 and the second dimension on DEAE-paper (Whatman DE 81) at pH 1.9 (12). Standard procedures for secondary analysis of T_1- and pancreatic ribonuclease-oligonucleotides were performed as described (12,13).

RESULTS

(a) Preparation and characterization of 5S RNA and 5S RNA protein complexes

Before the B. stearothermophilus and E. coli protein fractions were used for complex formation with [^{32}P]-labelled B. stearothermophilus 5S RNA, they were tested for RNA binding in large reconstitution experiments which enabled isolation and identification of the bound proteins. Therefore, B. stearothermophilus 5S RNA was isolated by Sephadex G-100 chromatography (10), checked by polyacrylamide gel electrophoresis for purity (5) and in 50S reconstitution experiments for biological activity (9). Next, the B. stearothermophilus 5S RNA was incubated with B. stearothermophilus "AB" fraction or E. coli UTP50 proteins and the resulting 5S RNA protein complexes were isolated by sucrose density gradient centrifugation (6). Extraction of the 5S RNA protein complexes with acetic acid followed by two-dimensional gel electrophoresis revealed that B. stearothermophilus proteins B-L5 and B-L22 and E. coli proteins E-L5, E-L18 and E-L25 had interacted with B. stearothermophilus 5S RNA (Table I). These results agreed with our previous findings (6). Therefore, these protein fractions were used for the [^{32}P]-5S RNA protein complex studies described below.

[^{32}P]-labelled B. stearothermophilus 5S RNA was separated from total RNA by slab gel electrophoresis and extracted as described (7). As shown in the autoradiogram (Figure 1), the isolated 5S RNA migrated in slab gel electrophoresis as one homogeneous band. Next, retention of 5S RNA to Millipore filters was measured after reconstitution with B. stearothermophilus "AB" fraction and E. coli UTP50 proteins. The results, summarized in Table II indicate that the radioactive B. stearothermophilus 5S RNA interacted equally well with B. stearothermophilus and E. coli proteins.

Table I

Proteins bound to B. stearothermophilus 5S RNA

Protein source	Proteins bound to B. stearothermophilus 5S RNA		
B. stearothermophilus	B-L5	B-L22	-*
E. coli	E-L5	E-L18	E-L25

B. stearothermophilus 5S RNA was reconstituted with B. stearothermophilus "AB" fraction and E. coli UTP50 proteins, and the resulting 5S RNA~protein complexes were isolated and analyzed for their protein content by two-dimensional gel electrophoresis. * The B. stearothermophilus protein similar to E. coli protein E-L25 is not known, while B-L5 and B-L22 correspond to E-L5 and E-L18, respectively (7).

Hydrolysis of B. stearothermophilus [^{32}P]-5S RNA with T_1-RNase and subsequent two-dimensional high-voltage electrophoresis of the oligonucleotides (12) yielded the fingerprint shown in Figure 2. All T_1-oligonucleotides were isolated and further analyzed by pancreatic ribonuclease and alkali hydrolysis (12). The results of the secondary analysis are summarized in Table III and reflect the observed molar ratio of each nucleotide in the T_1-oligonucleotides. From these data and comparison of our results with previously deduced sequences for B. stearothermophilus 5S RNA (14,15) and published fingerprints (15), we concluded that B. stearothermophilus (strain 799) 5S RNA has the sequence shown in Figure 3 and a T_1-oligonucleotide composition as shown in Table III.

The 5S RNA sequence shown in Figure 3 is in full agreement with the one reported by Stanley & Penswick (14) but differs at positions 8-10, 20 and 59 from that of B. stearothermophilus strain 1430 FV (15).

At the first of these three regions, we found the sequence U-G-G-U-G_{10}-A-U-A-G which, after T_1-RNase hydrolysis, yields fragments T1 (G), T10 (U-G) and T16 (A-U-A-G). The sequence in strain 1430 FV at this region is U-G-A-C-A_{10}-A-U-A-G which, after T_1-ribonuclease digestion, yields the oligonucleotides U-G and A-C-A_{10}-A-U-A-G. The latter heptanucleotide has a characteristic mobility in the fingerprint (15) and migrates

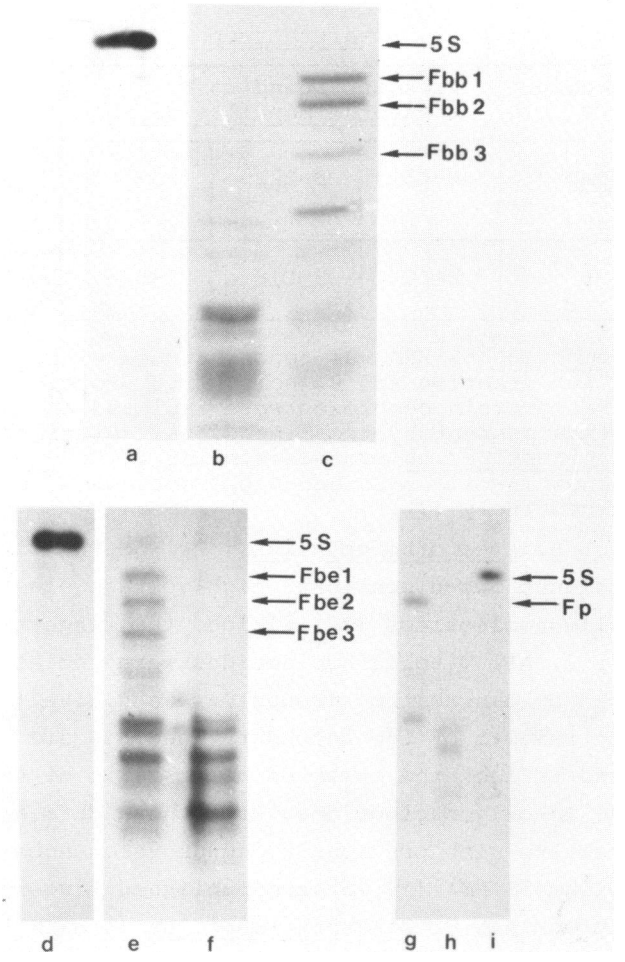

Figure 1. Slab gel electrophoresis of B. stearothermophilus (strain 799) 5S RNA and its fragments obtained after T_1- or pancreatic ribonuclease digestion of 5S RNA protein complexes. Control 5S RNA (a,d and i); 5S RNA hydrolysed with T_1- (b and f) and pancreatic ribonuclease (h); hydrolysis of B. stearothermophilus 5S RNA~B. stearothermophilus protein complexes with T_1- (c) and pancreatic ribonuclease (g); B. stearothermophilus 5S RNA~E. coli protein complex digested with T_1-RNase (e). For experimental details see Materials and Methods.

to a position at which we do not detect any radioactivity. On the other hand, T16 (A-U-A-G) is characteristic for strain 799 since it does not occur in B. stearothermophilus 1430 FV 5S RNA (15).

Table II

Interaction of B. stearothermophilus 5S RNA fragments with E. coli and B. stearothermophilus proteins

Protein source:	B. stearothermophilus 5S RNA fragments retained to Millipore filter (% binding)		
	Fbb1	Fbb2	Fbb3
B.stearo. "AB" fraction	80	38	18
E.coli UTP50	58	38	14
E-L5	12	8	5
E-L18	66	37	13
E-L25	42	10	6

Fragments Fbb1, Fbb2 and Fbb3 were isolated after T_1-RNase hydrolysis of B. stearothermophilus 5S RNA~B. stearothermophilus protein complex, incubated with the proteins indicated and measured by Millipore filter binding. 100% binding corresponds to the retention of the entire fragment to the filter. Background activities (2-4%) obtained by incubation of the fragments with bovine serum albumin were subtracted. For other experimental details see Material and Methods.

The second site at which the sequences differ is at position 20 at which G is present in strain 799 and A present in 1430 FV. The third site of sequence difference is at position 59 which is part of T18 (C-C-C_{60}-U-C-C-A-G). The corresponding T_1-oligonucleotide of B. stearothermophilus 1430 FV has the sequence C-U-C_{60}-U-C-C-A-G_{65} (15) and the presence of the two U's accounts for the significant difference in migration when compared to our T18 oligonucleotide. It is of interest to note that we have other experimental evidence which suggests that strain 799 may have a minor form of 5S RNA which, at position 59, is identical to strain 1430 FV (16). We had previously demonstrated by sequencial hydrolysis of the RNA (10) that B. stearothermophilus strain 799 5S RNA has the 3' terminal nucleotides -G-C_{OH}.

(b) 5S RNA fragments were protected from ribonuclease digestion by B. stearothermophilus and E. coli proteins

B. stearothermophilus 5S RNA was reconstituted with B. stearothermophilus "AB" fraction and E. coli UTP50 proteins. The re-

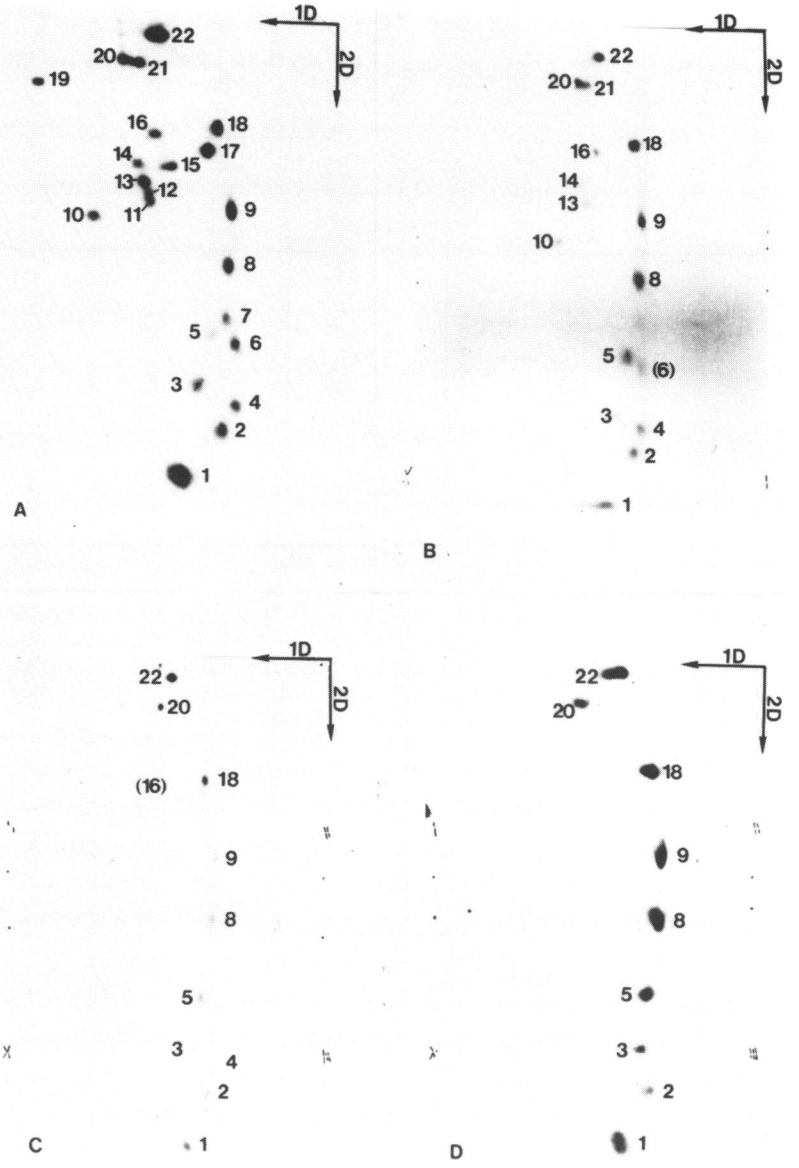

Figure 2. T_1-fingerprint of B. stearothermophilus (strain 799) 5S RNA (A), and fragments obtained after T_1-RNase digestion of B. stearothermophilus 5S RNA~B. stearothermophilus protein complexes. Fingerprint B corresponds to fragment Fbb1, C to Fbb2 and D to Fbb3. Fragments were isolated by slab gel electrophoresis (Figure 1). Molar yield of each T_1-oligonucleotide is summarized in Table IV. Experimental details are given under Materials and Methods.

sulting 5S RNA protein complexes, in Tris/Mg/K-II buffer, were treated with different amounts of T_1-RNase. It was found that at

Table III

Secondary analysis of B. stearothermophilus 5S RNA T_1-oligonucleotides

Spot No.	Pancreatic ribonuclease products	Alkali hydrolysis
T1	G	G
T2	G,C	C,G
T3	A-G	A,G
T4	C_2,G	C_2,G
T5	A-A-G	A_2,G
T6	A-G,C_2	A,C_2,G
T7	A-A-G,C	A_2,C,G
T8	A-A-C,A-C,G	A_3,C_2 G
T9	A-A-A-C,A-C,C_2,G	A_{3-4},C_3,G
T10	G,U	G,U
T11	G,C,U	G,C U
T12	G,C,U	G,C U
T13	A-G,U	A,G,U
T14	A-U,G	A,G,U
T15	A-G,C,U	A,G,C,U
T16	A-G,A-U	A_2,G,U
T17	C_4,G,U	C_4,G,U
T18	A-G,C_{4-5},U	A,C_{4-6},G,U
T19	G,U_2	G,U_2
T20	A-A-G,U_2	A_2,G,U_2
T21	A-G,pC,C,U	A,pC,C,G,U
T22	A-U,G,C_{4-7},U_2	A,C_{5-6},G,U_3

B. stearothermophilus (strain 799) 5S RNA was digested with T_1-ribonuclease and fingerprinted. The separated T_1-oligonucleotides were eluted and further analyzed by pancreatic ribonuclease or alkali hydrolysis. Experimental details are described in Materials and Methods. The results summarized in this table represent the average of two experiments.

0° and 37°C an enzyme to substrate ratio of 1:100, 1:50 or 1:10 (w/w) was not sufficient to partially hydrolyze all of the 5S RNA in the complexes. Best yields of 5S RNA fragments were obtained when the RNase digestion was carried out at 37°C for

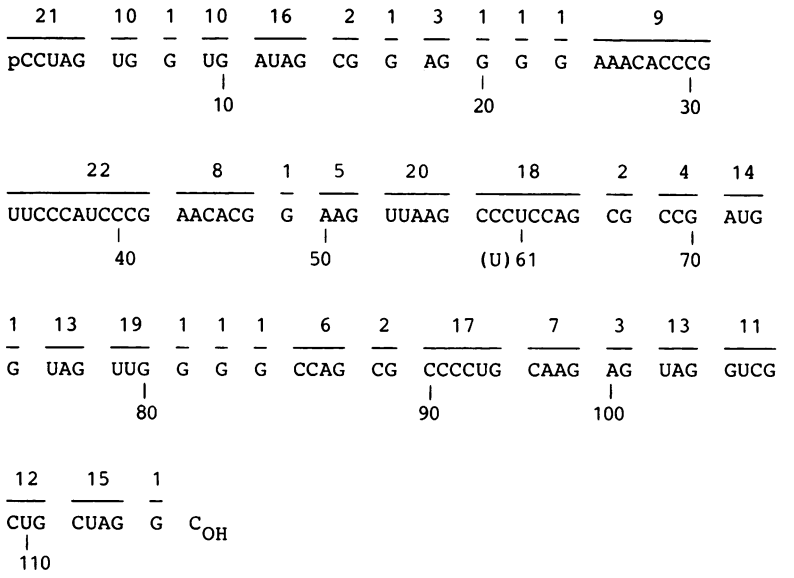

Figure 3. Primary sequence of B. stearothermophilus strain 799 as described in this study and by Stanley and Penswick (14). This sequence differs from that of strain 1430 FV (15), which contains at positions 8-10 A-C-A, at position 20 an A and at position 59 a U. The lines and numbers above the sequence indicate the oligonucleotides obtained after T_1-RNase digestion (see also Figure 2 and Tables III and IV).

30 minutes at an enzyme to substrate ratio of 1:5. As shown in Figure 1, B. stearothermophilus 5S RNA complexed with B. stearothermophilus proteins and hydrolyzed with T_1-ribonuclease yields the three fragments Fbb1, Fbb2 and Fbb3 while hydrolysis of free 5S RNA under identical conditions is complete. On the other hand, treatment of this complex with pancreatic ribonuclease yields only the fragment Fp (Figure 1). T_1-RNase digestion of the 5S RNA E. coli protein complex results also in the production of three distinct fragments Fbe1, Fbe2 and Fbe3 (Figure 1).

The specificity of the different 5S RNA fragments could be demonstrated by protein binding studies in Millipore filter assays (Table II). As expected, the larger fragments protected by B. stearothermophilus and E. coli proteins in the 5S RNA protein complexes were best retained by the filter. Of the proteins E-L5, E-L18 and E-L25 isolated from E. coli, E-L18 was found to bind best to the different B. stearothermophilus 5S RNA fragments (Table II).

Table IV

Molar yields of T_1-oligonucleotides in B. stearothermophilus 799 5S RNA and its various fragments

Spot No.	Sequence	5S RNA (theoretical)	5S RNA (observed)	Fbb1	Fbb2	Fbb3	Fbe1	Fbe2	Fb3
T1	G	12	15	11	8	6	9	8	6
T2	CG	3	3.0	1.9	2.2	1.0	2.2	2.3	0.8
T3	AG	2	2.2	1.4	1.2	0.8	0.9	0.9	0.8
T4	CCG	1	1.2	0.9	0.8	0	1.2	1.5	0
T5	AAG	1	1.1	1.1	1.3	0.9	0.8	0.8	0.6
T6	CCAG	1	0.9	(0.2)*	0	0	1.3	(0.1)*	0
T7	CAAG	1	0.9	0	0	0	(0.4)*	(0.3)*	0
T8	AACACG	1	0.8	0.9	0.8	0.9	0.7	0.7	1.0
T9	AAACACCCG	1	0.8	0.9	0.6	0.9	0.7	0.6	0.6
T10	UG	2	2.2	1.6	0	0	2.1	1.8	0
T11	UCG	1	1.0	0	0	0	0	0	0
T12	CUG	1	0.9	0	0	0	0	0	0
T13	UAG	2	1.8	0.7	0	0	0.8	(0.3)*	0
T14	AUG	1	1.2	0.8	0	0	1.0	0	0
T15	CUAG	1	1.2	0	0	0	0	0	0
T16	AUAG	1	1.0	0.8	(0.3)*	0	0.8	0.7	0
T17	CCCCUG	1	0.9	0	0	0	0	0	0
T18	CCCUCCAG	1	0.8	1.1	0.9	1.1	1.1	1.1	1.0
T19	UUG	1	1.0	0	0	0	0.6	0	0
T20	UUAAG	1	1.0	0.9	0.6	0.6	0.6	0.7	0
T21	(p)CCUAG	1	0.9	0.7	0	0	0.7	0.8	0
T22	UUCCCAUCCCG	1	1.1	1.0	1.3	0.9	1.1	1.0	0.8

B. stearothermophilus (strain 799) ^{32}P-5S RNA or T_1-RNase fragments derived from it due to the presence of B. stearothermophilus (Fbb1, Fbb2, Fbb3) or E. coli (Fbe1, Fbe2, Fbe3) proteins (Figure 1) were fully digested with T_1-ribonuclease and fingerprinted (Figure 2). The T_1-oligonucleotides were isolated and the molar yield of each spot determined. Experimental details are listed under Materials and Methods. *Oligonucleotides present in less than 0.4 moles were assumed not to be part of the fragment. The results represent the average of at least two different experiments.

(c) Sequence determination of 5S RNA fragments obtained after RNase hydrolysis of B. stearothermophilus 5S RNA~B. stearothermophilus protein complex

T_1-ribonuclease digestion of B. stearothermophilus 5S RNA~B. stearothermophilus protein complex yielded the three fragments Fbb1, Fbb2 and Fbb3 as shown in Figure 1. These fragments were isolated, again treated with T_1-RNase and fingerprinted (Figure 2). The radioactive T_1-oligonucleotides were eluted from the fingerprint for sequence and/or molar yield analysis.

Comparison of the Fbb1 fingerprint with that of B. stearothermophilus 5S RNA shows the absence of spots T7 (C-A-A-G), T11 (U-C-G), T12 (C-U-G), T15 (C-U-A-G), T17 (C-C-C-C-U-G) and T19 (U-U-G) (Figure 2). T_1-oligonucleotide T6 (C-C-A-G) was only found in trace amounts (0.2 moles; Table IV) and was therefore assumed to be absent. Since all of these oligonucleotides occur only once at the 3'-end of the 5S RNA (Figure 3), we concluded that B. stearothermophilus proteins did not protect this part of the molecule. From the other T_1-oligonucleotides observed on the fingerprint and their molar amounts recovered (Table IV), we deduced that Fbb1 contains the intact 5'-end of the 5S RNA and includes all nucleotides from position 1 through 77 (Figure 4).

The second largest fragment obtained in the presence of B. stearothermophilus proteins, Fbb2, lacked the characteristic oligonucleotides T10 (U-G) and T21 (pC-C-U-A-G) (Table IV, Figure 3). The absence of T13 (U-A-G) and T14 (A-U-G) suggested that further T_1-RNase hydrolysis had occurred at the 3'-end of fragment Fbb1. Similarly the low recovery of T16 (A-U-A-G) indicated that production of Fbb2 resulted from enzymatic cleavage at the 5'-end of Fbb1. From these results, it is possible to determine the sequence of Fbb2 as one uninterrupted chain which includes nucleotides 11 through 70 (Figure 4).

The smallest fragment isolated, Fbb3, confirms the results obtained for Fbb2, since the T_1-oligonucleotide present in low molar yield at the 5'-end T16 of Fbb2 was found to be absent (Figure 2, Table IV). In addition, T4 (C-C-G) was totally missing from fragment Fbb3. Therefore, we are certain that Fbb3 includes nucleotides 18-65. Since T2 (C-G), which occurred as two copies in Fbb1 and Fbb2, was only found as 1 mole per mole Fbb3

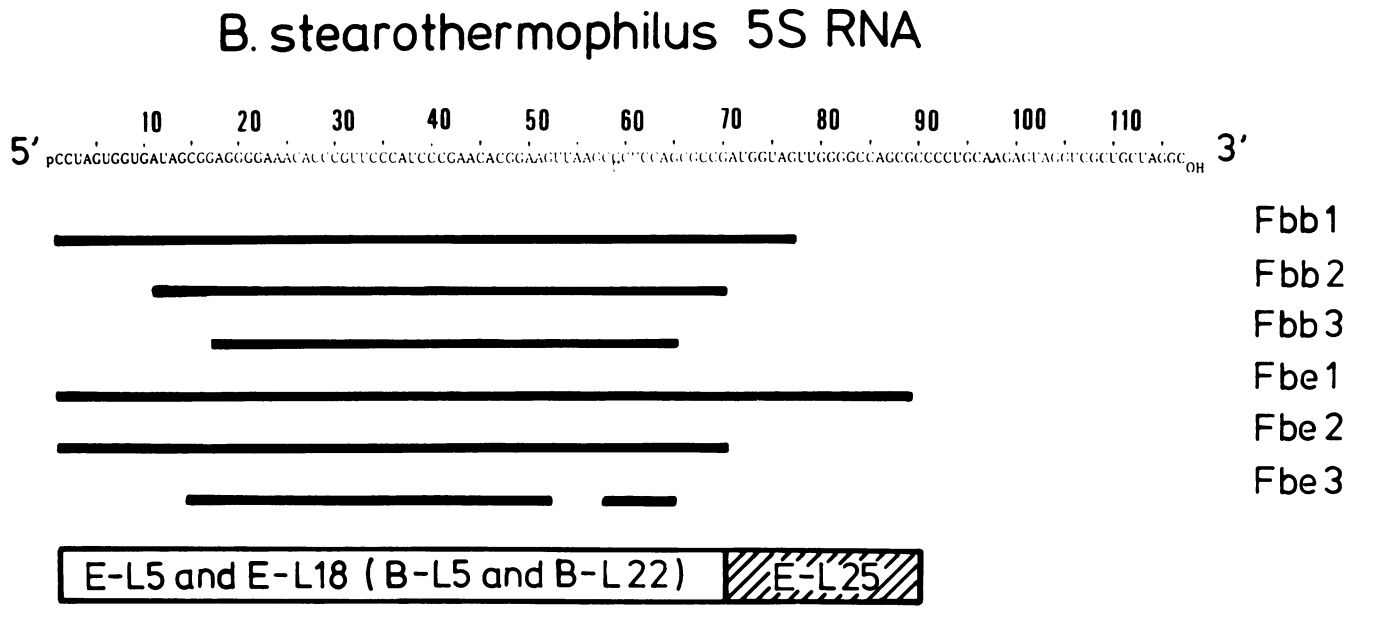

Figure 4. Alignment of 5S RNA fragments obtained by T_1-RNase hydrolysis of B. stearothermophilus 5S RNA~B. stearothermophilus protein complex (Fbb1, Fbb2 and Fbb3) and B. stearothermophilus 5S RNA~E. coli protein complex (Fbe1, Fbe2 and Fbe3) with B. stearothermophilus 5S RNA. Deduced primary binding sites of B. stearothermophilus and E. coli proteins on B. stearothermophilus 5S RNA are shown on the bottom. For other details see Materials and Methods and Discussion.

fragment, it is not clear if this sequence should be placed at the 5'- or 3'-end of the RNA fragment (Figures 3 and 4).

Digestion of the B. stearothermophilus 5S RNA~B. stearothermophilus protein complex with pancreatic ribonuclease yielded only one fragment Fp (Figure 1). Preliminary results have shown that this fragment is similar in size to Fbb1 and that it contains the intact 5'-end and lacks characteristic oligonucleotides of the 3'-end (data not shown).

(d) Sequence determination of 5S RNA fragments obtained after RNase hydrolysis of B. stearothermophilus 5S RNA~E. coli protein complex

B. stearothermophilus 5S RNA~E. coli protein complexes, when hydrolysed with T_1-RNase, permitted the isolation of the three distinct fragments Fbe1, Fbe2 and Fbe3 (Figure 1). All fragments were further digested with T_1-ribonuclease and fingerprinted. The oligonucleotides of the fingerprints were isolated and their molar amounts determined.

The largest fragment, Fbe1, contains all of the typical T_1-oligonucleotides which are part of the 5'-end of 5S RNA and therefore resembles fragments Fbb1 in this region (Table IV, Figure 3). Since it contains T19 (U-U-G) and T6 (C-C-A-G), its 3'-end includes these oligonucleotides. As already observed for the Fbb1 fragment, Fbe1 lacks the other characteristic 3' oligonucleotides T7 (C-A-A-G), T11 (G-U-C-G), T12 (C-U-G), T15 (C-U-A-G) and T17 (C-C-C-C-U-G). Therefore, we conclude that Fbe 1 extends from nucleotide 1 through 87 (Figure 4).

The second largest fragment Fbe2 is unaltered at its 5'-end when compared to Fbe1 (Table IV and Figures 3 and 4). The reduction in length has taken place at its 3'-end, which lacks the additional oligonucleotides T6 (C-C-A-G), T13 (U-A-G), T14 (A-U-G) and T19 (U-U-G). From the T_1-oligonucleotides found in the fingerprint, it is possible to construct one continuous fragment for Fbe2 which encompasses nucleotides 1-70 (Figure 4).

Fragment Fb3, the smallest 5S RNA fragment isolated, showed that cleavages at the 5'-end of Fbe2 were necessary for its production, since the typical 5' T_1-oligonucleotides T21 (pC-C-

U-A-G), T10 (U-G) and T16 (A-U-A-G) are totally absent (Table IV & Figure 3). In addition, the analysis of the fingerprint revealed that T4 (C-C-G) and T20 (U-U-A-A-G) were not part of the fragment and that T2 (C-G) was isolated in molar stoichiometry. It is of interest that T20 was not found on the fingerprint, unlike its two immediate neigbours T5 (5'-side) and T18 (3'-side). It is therefore concluded that Fbe3 contains nicks at positions 51 and 57 and otherwise consists of all nucleotides from 15-65 (Figure 4).

DISCUSSION

The experiments reported in this communication are in agreement with our earlier studies (6) in which we have shown that B. stearothermophilus 5S RNA interacts with B. stearothermophilus proteins B-L5 and B-L22 and with the E. coli 5S RNA binding proteins E-L5, E-L18 and E-L25. Therefore, we analyzed the B. stearothermophilus 5S RNA~B-L5~B-L22 and B. stearothermophilus 5S RNA~E-L5~E-L18 ~ E-L25 complexes by partial ribonuclease digestion. The results were compared with those in our previous study in which E. coli 5S RNA had been complexed with those proteins (7).

T_1-RNase hydrolysis of B. stearothermophilus 5S RNA complexed with B-L5 and B-L22 or with E-L5, E-L18 and E-L25 routinely yields the three distinct fragments Fbb1, Fbb2 and Fbb3 or Fbe1, Fbe2 and Fbe3, respectively (Figure 1). These results are similar to those for the E. coli 5S RNA-protein complexes. The specificity of these fragments was demonstrated by Millipore filter binding assays (Table II). For example, significant amounts of the RNA fragments Fbb1, Fbb2 and Fbb3 were retained by the filters if they had previously been incubated with B. stearothermophilus or E. coli proteins. The percentage of filter binding was dependent upon the length of the fragments, i.e. the longer fragments (Fbb1, Fbb2) were better retained by the filter than the shortest one (Fbb3). This possibly suggests cooperativity of the proteins bound and/or structural differences of these RNA regions when they are part of the whole 5S RNA molecule. Of the isolated E. coli proteins, E-L18 was always found

to bind best, while E-L25 bound only better to the Fbb1 fragment than E-L5. The observation that E-L18 is the best binding protein is expected, since this protein or the corresponding B. stearothermophilus protein B-L22 are always found in the homologous or heterologous 5S RNA protein complexes, while the binding of the other proteins may be variable (6). Since these experiments suggest that the strongest RNA binding occurs with B-L22 (or E-L18), it may be assumed that the smallest fragments mainly contain part of the binding site for this protein.

B. stearothermophilus 5S RNA, when complexed with B. stearothermophilus "AB" fraction proteins, yield a complex in which proteins B-L5 (corresponding to E-L5) and B-L22 (corresponding to E-L18) are bound (Table I). Therefore, partial T_1-ribonuclease digestion of this complex should yield RNA fragments on which the primary binding sites of these two proteins are located. We, therefore, conclude that both B-L5 and B-L22 bind to nucleotides 1-77, which corresponds to Fbb1 (Figure 4). The second largest fragment Fbb2 includes nucleotides 11-70 and suggests that this region of the 5S RNA represents the core of the protein binding sites. This assumption is further supported by fragment Fbb3 (nucleotides 15-65), which resulted by removal of a few nucleotides from the 3'- and 5'-end of Fbb2. We conclude from these experiments that the primary binding sites for B-L5 and B-L22 are located between nucleotides 15-65 and that sequence 1-14 constitutes a secondary binding site. The assigned binding sites of the two proteins to B. stearothermophilus 5S RNA are in good agreement with the one on E. coli 5S RNA (7) and lead to the same conclusion that these parts of 5S RNA, which are strongly conserved during evolution (Figure 5), interact with ribosomal proteins.

Interaction of E. coli proteins E-L5, E-L18 and E-L25 with B. stearothermphilus 5S RNA and subsequent T_1-ribonuclease digestion yields three RNA fragments (Fbe1, Fbe2, Fbe3; see Figure 4), which show similarities to the fragments obtained in the presence of B-L5 and B-L22. The differences are especially interesting since they are due to the presence of E-L25. The largest fragment Fbe1 is considerably longer at its 3'-end when compared to Fbb1, since it includes nucleotides 1-89

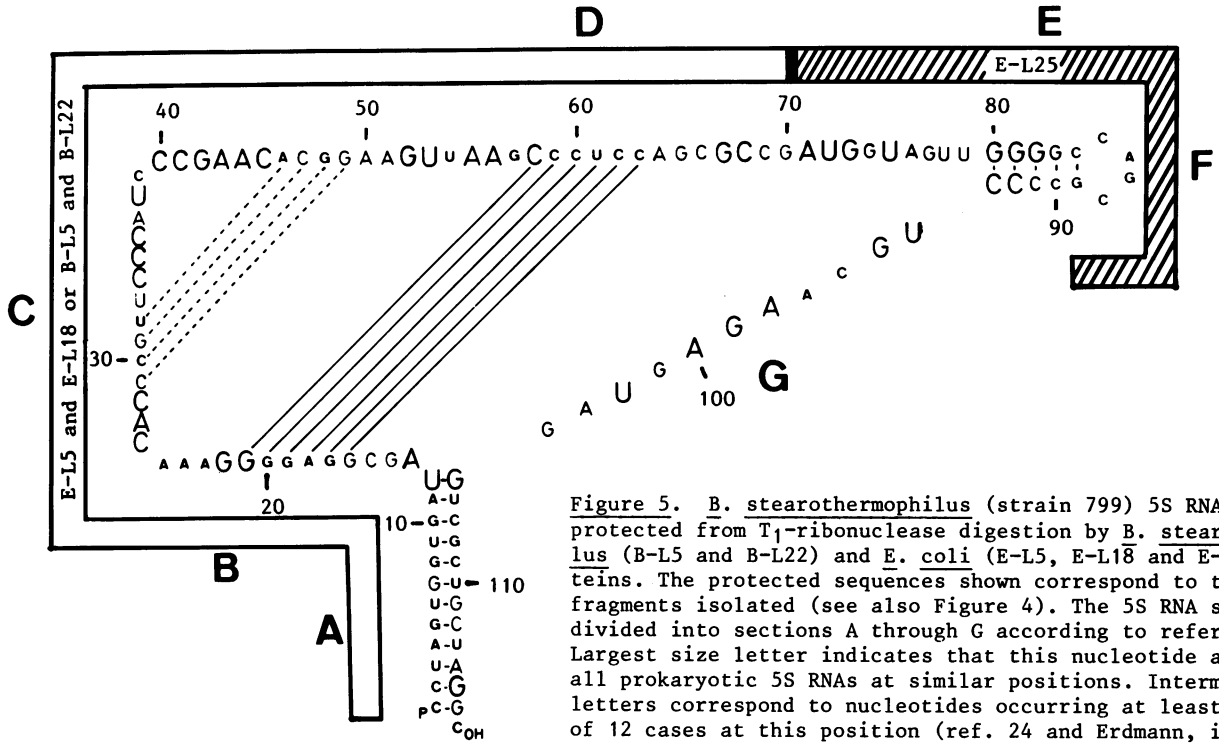

Figure 5. B. stearothermophilus (strain 799) 5S RNA sequences protected from T_1-ribonuclease digestion by B. stearothermophilus (B-L5 and B-L22) and E. coli (E-L5, E-L18 and E-L25) proteins. The protected sequences shown correspond to the largest fragments isolated (see also Figure 4). The 5S RNA sequence is divided into sections A through G according to reference 17. Largest size letter indicates that this nucleotide appears in all prokaryotic 5S RNAs at similar positions. Intermediate size letters correspond to nucleotides occurring at least in 10 out of 12 cases at this position (ref. 24 and Erdmann, in preparation). Small size letters represent variable nucleotides in prokaryotic 5S RNAs. Experimental evidence presented in this communication suggests that the base pairs shown between section B and D occur in 5S RNA-protein complexes. The experiments described here do not permit a similar conclusion to be drawn about the possible base-pairing between section C and D. Both base-paired regions have previously been proposed to occur in prokaryotic 5S RNA (20-22).

(Figure 4). Since the difference between the complexes of B. stearothermophilus 5S RNA ~ B-L5 ~ B-L22 and B. stearothermophilus 5S RNA ~ E-L5 ~ E-L18 ~ E-L25 is the finding that the latter complex contains the additional protein E-L25, we attribute the protection of section 78-89 due to the presence of E-L25. The results are in qualitative agreement with our previous study, in which we concluded that E-L25 binds at the 3' side of E-L18 on E. coli 5S RNA (7).

That E-L25 interacts to a certain degree with the double stranded section A (in this case the 5' strand) of 5S RNA (Figure 5) is also indicated by fragment Fbe2 which, in contrast to Fbb2, still contains nucleotides 1-14. In the case of the E. coli 5S RNA~E. coli protein complexes, we observed that E-L25 protected the 3' strand of section A (7). Since section A is actually a region of 5S RNA which is variable in sequence (17, 24 and Erdmann, in preparation), it seems possible that the protein interacts primarily with the phosphate backbone of this double stranded region and not with its specific bases. This interpretation would be in agreement with a previous study on E. coli 5S RNA protein complexes, in which section A (both the 3' and 5' strands) were protected from ribonuclease digestion (18).

Fragment Fbe3 is especially interesting since this is the only discontinuous fragment isolated. It contains nucleotides 15-52 and 58-65 (Figure 4) and differes from Fbb3 in the absence of T20 (U-U-A_{55}-A-G). Since the fragment was isolated by urea slab gel electrophoresis, it must be assumed that section 58-65 had strongly interacted with the larger part of the fragment. This interaction is theoretically possible, because one can construct 6 base pairs between most of section 58-65 (C_{58}-C-C-U-C-C_{63}) and G_{16}-G-A-G-G-G_{21}, the latter one being the start of the Fbe3 fragment at the 5'-end (Figures 4 and 5). A similar resistance to urea at even elevated temperatures has previously been observed with a hair pin loop in 5.8S RNA, which consisted of 7 G-C, one A-U and one G-U base pairs (19). We therefore feel that it is possible that nucleotides 16-21 are able to base-pair with nucleotides 58-63, which would experimentally support the proposed interactions of these two regions in 5S RNA (20-22).

Since oligonucleotide binding studies do not support this type of interaction in free 5S RNA (16), we suggest that binding of proteins to the RNA facilitates a conformational change which generally enables the base pairing of these two regions in prokaryotic 5S RNA protein complexes. This conclusion is in agreement with other evidence for conformational changes of 5S RNA due to its interaction with proteins (23, 25). It will, therefore, be essential to differentiate between models for 5S RNA which is free in solution or complexed with one or several specific ribosomal proteins.

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