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Interaction of unfolded tRNA with the 3'-terminal region of *E. coli* 16S ribosomal RNA

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ABSTRACT

Fragments of tRNA possessing a free TΨC-loop or a free D-loop form stable complexes with the colicin fragment (1494-1542) of 16S ribosomal RNA from E. coli. The colicin fragment does not bind to tRNA in which the T-loop and the D-loop are involved in tertiary interactions. Colicin cleavage of the 16S rRNA from E. coli is inhibited by aminoacyl-tRNA or tRNA fragments, indicating that a similar interaction may take place on the intact 70S ribosomes. The oligonucleotide d(G-T-T-C-G-A)homologous to the conserved sequence G-T-Ψ-C-Pu-(m^1)A in the TΨC-region of many elongator tRNAs binds to the conserved sequence U-C-G-mU-A-A-C (1495-1501) of the 16S rRNA. It is suggested that the 3'-end of the 16S rRNA may provide the part of the binding site for the elongator tRNAs on bacterial ribosomes.

INTRODUCTION

Almost all tRNAs contain a nearly ubiquitous sequence in the T-loop (1). The role of this G-T-Ψ-C sequence in the formation of the tRNA tertiary structure (2,3), for the expression of tRNA genes in eucaryots (4) and tRNA processing (5) was convincingly demonstrated. The involvement of this sequence in the interaction of tRNA with ribosomes during polypeptide elongation was also suggested (6). This hypothesis was supported by the observation that the oligonucleotide T-Ψ-C-G, excised from tRNA, inhibits the binding of elongator tRNAs to the ribosomal A-site (7) and is able to participate as an effector in the ribosomal synthesis of ppGpp (8). As a binding site for this oligonucleotide, the 5S RNA from the large ribosomal subunit was implicated (6). This suggestion could not be confirmed by recent investigations in which in vitro modified 5S RNA lacking the potential binding site was studied (9,10). Furthermore it was shown that the C-56 residue of a

suppressor tRNA is not absolutely essential for its function in vivo (11), however as tested in in vitro experiments the mutation at position 56 affects the normal ribosomal function of an elongator tRNA.

In the course of our systematic study of the structure, properties and function of the invariant regions in tRNA sequences, such as C-C-A (12) and G-T-Ψ-C (7), we attempted to detect possible sequences in ribosomal RNAs where a binding of the T-region of tRNA can occur. Here we report the results of experiments indicating that the C-G-mU-A-A-C sequence (1496-1501) of the 16S RNA from the small subunit of E. coli ribosomes has the potential to bind the G-T-Ψ-C-G sequence of an elongator tRNA.

MATERIALS AND METHODS

$tRNA^{Phe}$ from bakers yeast was isolated from $tRNA^{bulk}$ obtained from Boehringer Mannheim, Germany (13). Cleavage of the $tRNA^{Phe}$ at the m^7G-46 residue and separation of the fragments (1-45) and (47-76) by gel filtration on a Sephadex G-100 column was described previously (7). 70S ribosomes were isolated from E. coli MRE 600 grown to late logarithmical phase according to Jelenc (14). T4 RNA-ligase (EC 6.5.1.3) was isolated from T4-infected E. coli cells essentially according to Mc Coy et. al (15). It was free of ribonuclease and had a specific activity of 108 units/mg protein. The following purification steps were used: streptomycin precipitation, chromatography on DEAE-cellulose, chromatography on DEAE-Sepharose CL-6B, gel filtration on Ultrogel AcA 34 and chromatography on hydroxylapatite.

Ribonuclease H (EC 3.1.4.34) from E. coli was obtained from PL-Biochemicals (Milwaukee, U.S.A.). Colicin E 3 was from Dr. G. Sander (Berlin), the oligonucleotide d(G-T-T-C-G-A) was a synthetic product obtained from Dr. H. Köster, Hamburg. Carrier-free [^{32}P]orthophosphoric acid was from NEN (Boston, U.S.A.). It was used for the preparation of γ-[^{32}P]ATP (16) and 5'-[^{32}P]pCp (17). All other enzymes were from Boehringer (Mannheim, Germany). The chemicals were of analytical grade and were obtained from Merck (Darmstadt, Germany). X-ray film Curix RP1

from Agfa-Geveaert (Leverkusen, Germany) was used for autoradiography. The amplyfier sheet Titan HS was from Siemens (Erlangen, Germany).

30S ribosomal subunits were labelled in situ on the 3'-end of 16S rRNA according to Backendorf et. al (18) with 5'-[^{32}p]pCp of a specific activity of 300 Ci/mmol. [^{14}C]Phe-tRNA^{Phe}, specific activity 5.04 Ci/mol, was prepared as described (19). The treatment of 70S ribosomes with colicin E3 was performed in a reaction mixture containing 30 mM Tris-HCl pH 7.6, 100 mM KCl, 20 mM $MgCl_2$, 1 mM dithiothreitol, 2.15 µM ^{32}P-labelled 30S subunits, 2.46 µM 50S subunits, 0.183 mg/ml poly(U), 4.3 mg/ml colicin E3 and [^{14}C]Phe-tRNA^{Phe} or tRNA fragments as described in the legends to the figures. After incubation, the RNA was isolated and analysed by polyacrylamide gel electrophoresis and autoradiography. For quantitative evaluation the radioactivity was determined by Cerenkov counting.

The treatment of 70S ribosomes, 30S ribosomal subunits or 16S rRNA with RNase H in the presence of the deoxyhexanucleotide d(G-T-T-C-G-A) was performed in a reaction mixture containing 30 mM Tris-HCl pH 7.6, 100 mM KCl, 20 mM $MgCl_2$, 1 mM dithiothreitol, 60 µM d(G-T-T-C-G-A) and 215 units/ml RNase H. 30S subunits, 70S particles and 16S rRNA were present in concentrations of 2.1 µM, 2.4 µM and 3.3 µM, respectively. In all experiments, the 16S rRNA had been labelled on its 3'-end (17) with 5'-[^{32}P]pCp and T4 RNA ligase. About $2x10^6$ cpm were used in one experiment. The reaction mixtures (12 µl) were incubated at 4 ^{o}C for 18 h (20). The reaction was stopped by addition of 30 µl of ethanol. After centrifugation the radioactively labelled oligonucleotides were deproteinized by phenol extraction and analysed by gel electrophoresis and autoradiography. The new fragment formed by the cleavage of 16S rRNA was identified by limited digestion with T1 ribonuclease (21).

The ^{32}P-labelled, 49-nucleotide long, colicin fragment was isolated by gel electrophoresis as described above. For the investigation of its interaction with tRNA^{Phe} and tRNA^{Phe} fragments, a reaction mixture containing 2.5 µM colicin fragment,

125 μM $tRNA^{Phe}$ or $tRNA^{Phe}$ fragments, 30 mM Tris-HCl pH 7.6, 100 mM KCl, 10 mM $MgCl_2$ and 1 mM dithiothreitol was incubated for 30 min at 37 °C. The samples were applied to 15 % polyacrylamide gels which were prepared according to Backendorf et al. (18) in a buffer containing 5 mM Tris-acetate pH 8.0 and 5 mM Mg-acetate. The electrophoresis buffer was also composed of 5 mM Tris-acetate pH 8.0 and 5 mM Mg-acetate. The electrophoresis was performed for 19 h at 40 V/cm and 4 °C in an apparatus allowing a buffer circulation with a flow rate of 2 ml/min.

RESULTS

Treatment of intact *E. coli* 70S ribosomes with colicin E3 results in cleavage of the phosphodiester bond between residue 1493 and 1494 of the 16S rRNA and a formation of a 49-nucleotide long 3'-terminal fragment (22). After the cleavage, the function of the initiation factor-1 on 70S ribosomes is impaired (23) and the elongation cyclus is severely inhibited (24). The inactivation of ribosomes with colicin E3 is *in vitro* effectively inhibited by binding of the aminoacyl-tRNA to the ribosomal A-site (25). If namely the colicin treatment is performed in the presence of A-site bound aminoacyl-tRNA, the ribosomes are not deactivated in respect to their ability to promote poly(U)-dependent poly(Phe)-synthesis. We analysed the protective effect of aminoacyl-tRNA against the colicin cleavage of ribosomes by polyacrylamide gel electrophoresis. For this purpose the 3'-end of 16S rRNA was labelled with 5'-[^{32}P]pCp and T4 RNA ligase. The labelled 30S subunits were associated with 50S particles to provide active 70S ribosomes, which were then treated with colicin E3. The colicin fragment was identified by electrophoresis and sequencing. The results in Fig. 1 show that the presence of increasing amounts of Phe-$tRNA^{Phe}$ in the reaction mixture inhibit the formation of the colicin fragment, since the amount of formed fragments is gradually decreasing with the increasing concentration of Phe-$tRNA^{Phe}$ in the assay. About 50 % of the cleavage can be avoided in the presence of an excess of Phe-$tRNA^{Phe}$. A complete protection, however, was not achieved, probably due to a

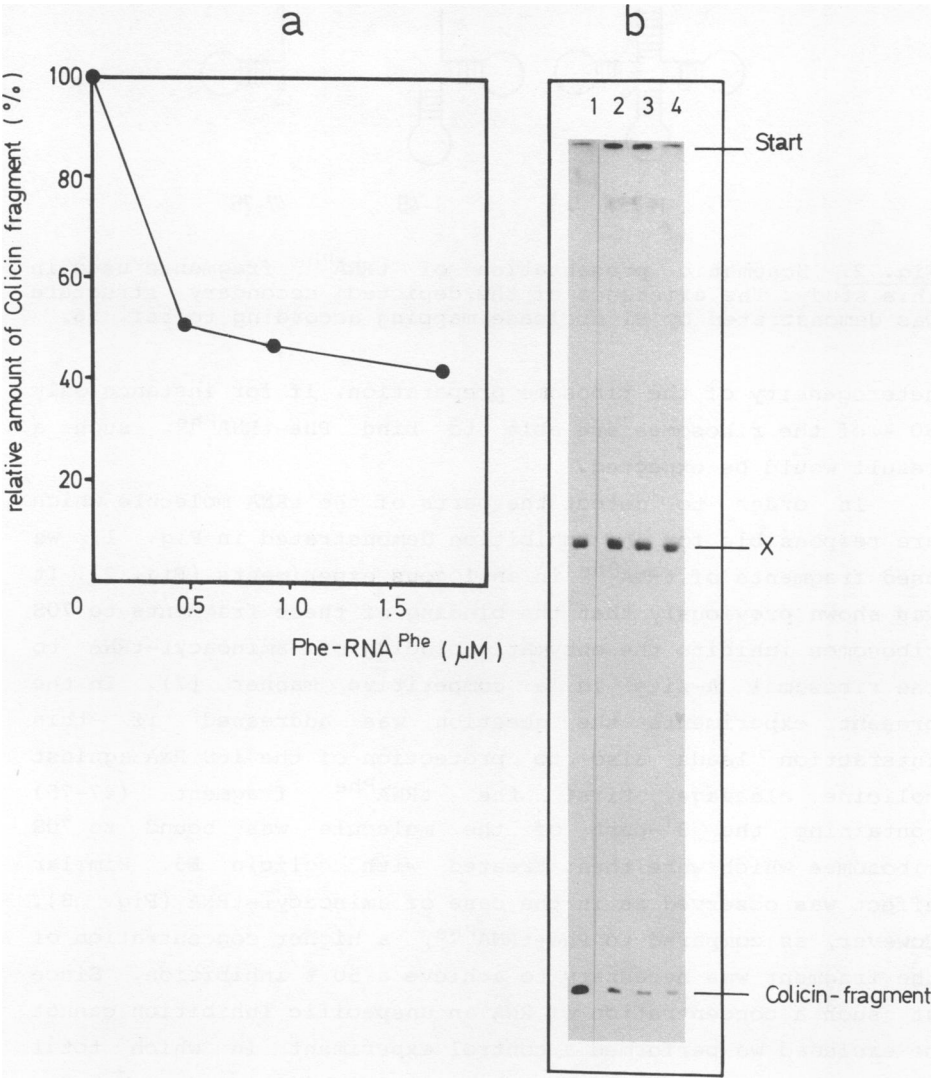

Fig. 1. Inhibition of the formation of colicin fragment by Phe-tRNA^{Phe}. 70S ribosomes were incubated with colicin E3 as described in "Methods". Lane 1: without Phe-tRNA^{Phe}; lanes 2, 3 and 4: with 0.46 µM, 0.92 µM and 1.75 µM Phe-tRNA^{Phe}, respectively. a) relative amount of the colicin fragment as determined by Cerenkov counting; b) autoradiography of the gel. x corresponds to a fragment formed by spontaneous hydrolytic cleavage of the 16S rRNA.

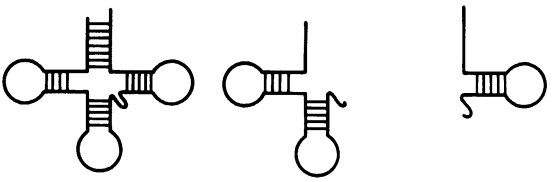

Fig. 2. Schematic presentation of $tRNA^{Phe}$ fragments used in this study. The existence of the depicted secondary structure was demonstrated by S1 nuclease mapping according to ref. 26.

heterogeneity of the ribosome preparation. If for instance only 50 % of the ribosomes are able to bind Phe-$tRNA^{Phe}$, such a result would be expected.

In order to detect the parts of the tRNA molecule which are responsible for the inhibition demonstrated in Fig. 1, we used fragments of $tRNA^{Phe}$ in analogous experiments (Fig. 2). It was shown previously that the binding of these fragments to 70S ribosomes inhibits the enzymatic binding of aminoacyl-tRNA to the ribosomal A-site in a competitive manner (7). In the present experiments the question was addressed if this interaction leads also to protection of the 16S RNA against colicine cleavage. First the $tRNA^{Phe}$ fragment (47-75) containing the 3'-part of the molecule was bound to 70S ribosomes which were then treated with colicin E3. Similar effect was observed as in the case of aminoacyl-tRNA (Fig. 3). However, as compared to Phe-$tRNA^{Phe}$, a higher concentration of the fragment was necessary to achieve a 50 % inhibition. Since at such a concentration of RNA an unspecific inhibition cannot be excluded we performed a control experiment in which total yeast RNA was added to the assay. No inhibition was observed with this RNA (Fig. 3). $tRNA^{Phe}$ (1-45) fragment inhibited the colicin reaction in a similar manner as it was the case with the $tRNA^{Phe}$ (47-75) fragment (Fig. 4). The inhibition was slightly more effective as that with the 3'-fragment since lower concentration was necessary to achieve 50 % inhibition. The reason for this may lie in the presence of the anticodon loop in the $tRNA^{Phe}$ (1-45) fragment which probably facilitates its

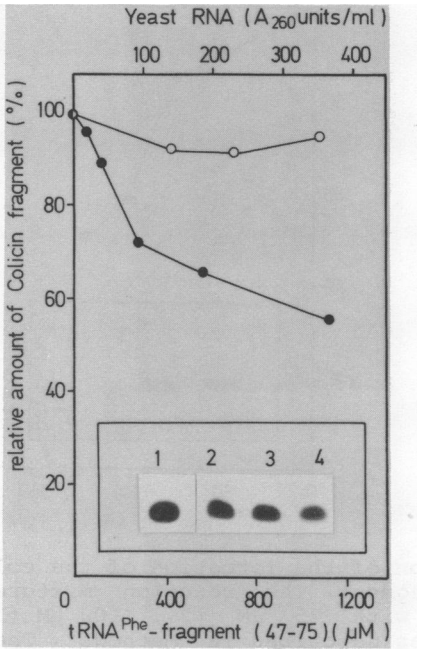

Fig. 3. Inhibition of the formation of colicin fragment by $tRNA^{Phe}$ (47-75) fragment. The reaction mixture was incubated without (1) or with 283 μM (2), 567 μM (3) and 1133 μM (4) $tRNA^{Phe}$ fragment as described in "Methods". The insert shows the autoradiography of the part of the gel containing the colicin fragment. The values (-●-) represent the Cerenkov counts of the appropriate colicin fragment bands, (-o-) are the values obtained in the presence of total yeast RNA.

binding to the ribosomes. Nevertheless, this fragment is still a less efficient inhibitor of the colicin-reaction as the native aminoacyl-tRNA.

One possible explanation for the inhibitory effect of the tRNA fragments in the colicin-induced cleavage of the 16S rRNA could be the direct interaction of partial sequences of tRNA with the 3'-terminal part of 16S rRNA. To test this possibility, we isolated the radioactively labelled 49-nucleotide long colicin fragment of the 16S rRNA. This oligonucleotide was incubated with an unlabelled $tRNA^{Phe}$ from yeast or its fragments and the mixture was analysed by polyacrylamide gel electrophoresis under non-denaturing conditions. The results of these experiments are shown in Fig.

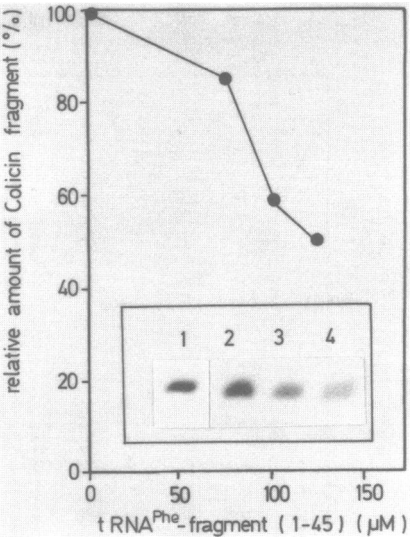

Fig. 4. Inhibition of the formation of the colicin fragment by $tRNA^{Phe}$ (1-45) fragment. The reaction mixture was incubated without (1) or with 75 μM (2), 100 μM (3) and 125 μM (4) $tRNA^{Phe}$ fragment as described in "Methods". The values -o- were obtained by Cerenkov counting of the polyacrylamide gels. In the insert the autoradiography of the appropriate part of the gel is shown.

5. The colicin fragment does not form a detectable complex with the intact $tRNA^{Phe}$. It has in the absence as well as in the presence of this tRNA the same electrophoretic mobility (Fig. 5, lanes 1 and 2). Addition of the $tRNA^{Phe}$ fragments to the labelled colicin fragment leads, however, to a complex formation (Fig. 5, lanes 3 and 4) resulting in an apparent increase of the molecular weight of the colicin fragment and to a decrease of its electrophoretic mobility. The complex of the colicin fragment and the three quarter fragment of $tRNA^{Phe}$ moves slightly slower (Fig. 5, lane 3) as compared to the complex which contains the shorter $tRNA^{Phe}$ (47-75) fragment. Typical for the formation of a non covalent complex is the tailing of the spots indicating a partial dissociation of the complexes during the electrophoresis. The experiment in Fig. 5 demonstrates the necessity for an unfolding of the tertiary structure of tRNA in order to form complexes with the colicin fragment. This suggests that the invariant nucleotide residues G_{18}, G_{19} of the D-loop and the G-T-Ψ-C-Pu residues of the T-region are involved

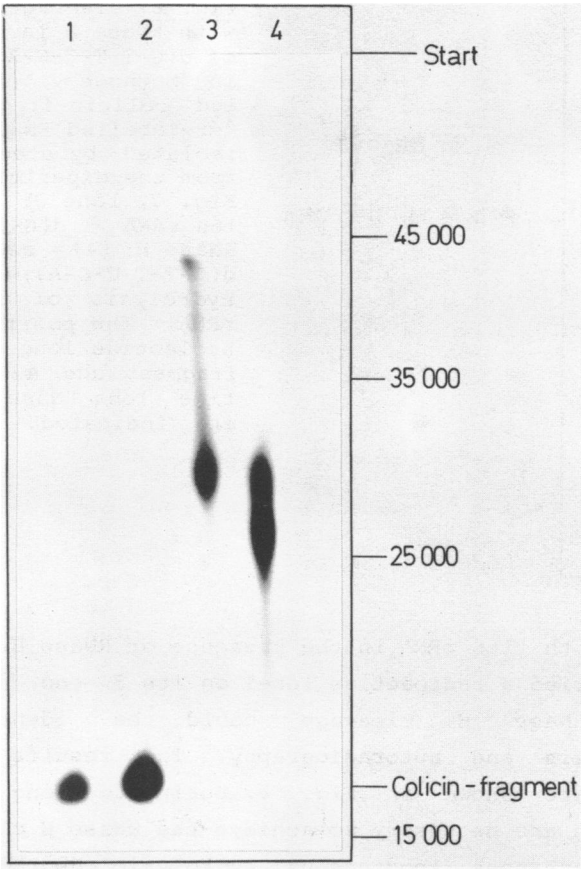

Fig. 5. Complex formation between ^{32}P-labelled colicin fragment of 16S rRNA and unlabelled $tRNA^{Phe}$ or $tRNA^{Phe}$-fragments as analysed by polyacrylamide gel electrophoresis under non-denaturing conditions (1) = ^{32}P-labelled colicin-fragment, (2) = ^{32}P-labelled colicin-fragment + $tRNA^{Phe}$, (3) = ^{32}P-labelled colicin-fragment + $tRNA^{Phe}$ (1-45) fragment, (4) = ^{32}P-labelled colicin-fragment + $tRNA^{Phe}$ (47-75) fragment.

in these interactions. The secondary structure of the T- and D-loop which is still preserved in the fragments (26), is probably important for the complex formation as well.

In order to identify the sequence in the colicin fragment where the T-region of tRNA may bind, we used the hexanucleotide d(G-T-T-C-G-A) which is homologous to the conserved sequence in the T-loop of elongator tRNAs. This oligonucleotide was

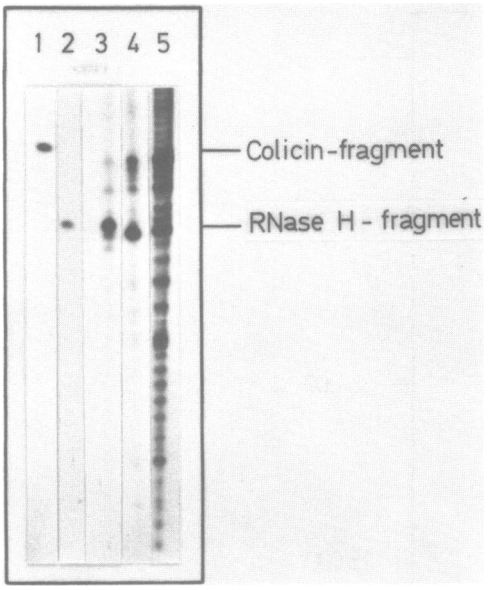

Fig. 6. Cleavage of 16S rRNA with RNase H in the presence of d(G-T-T-C-G-A) as described in "Methods". (1) = ^{32}P-labelled colicin-fragment; (2) = ^{32}P-labelled RNaseH fragment isolated by electrophoresis from the experiment shown in Fig. 7, lane 3; (3) = 3'- ^{32}p 16S rRNA + d(G-T-T-C-G-A) + RNase H; (4) = control without d(G-T-T-C-G-A); (5) = alkaline hydrolysis of 3'- ^{32}P 16S rRNA. The positions of 49-nucleotide long colicin fragment and 42 (41) nucleotide long RNase H fragments are indicated.

incubated with 16S rRNA in the presence of RNase H. Since the 16S rRNA carried a radioactive label on its 3'-end, a fragment formed by RNase H cleavage could be identified by electrophoresis and autoradiography. The results of these experiments are shown in Fig. 6. During the long incubation times, which are necessary to achieve the RNase H cleavage the 16S rRNA undergous spontaneous hydrolytic degradation in aqueous solutions (27). Fragments formed by this unspecific reaction are visible in a control experiment which was performed in the absence of deoxyhexanucleotide (Fig. 6, lane 4). In the presence of RNase H and deoxyhexanucleotide a new main cleavage product is formed (Fig. 6, lane 3) which is not present in considerable amount in the control (lane 4). In order to see the fragments formed by unspecific hydrolysis the lane 4 is overexposed. The additional cleavage product (lane 3) was characterized by sequencing which revealed that the RNase H cleavage site is at position 1501 (or 1500) of the 16S rRNA. Similar results as with 16S rRNA were obtained when 30S ribosomal subunits of 70S ribos particles were incubated with RNase H in the presence of d(G-T-T-C-G-A) (Fig. 7). Beside the fragments formed by spontaneous hydrolysis of 16S rRNA in the

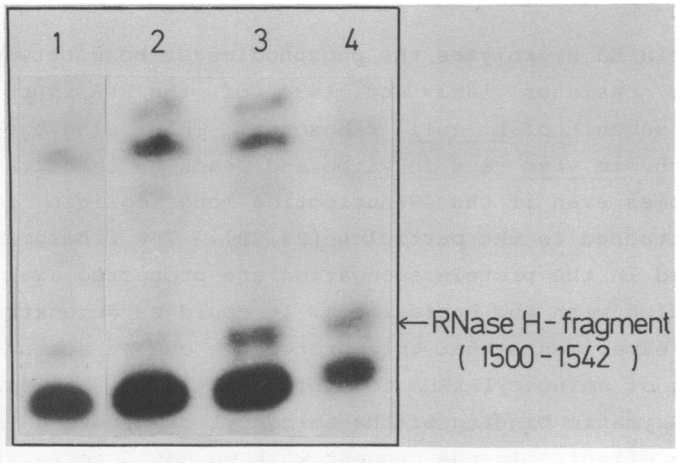

Fig. 7. Cleavage of ^{32}P 16S rRNA with RNase H in the presence of d(G-T-T-C-G-A) as described in "Methods". RNase H in the presence of $tRNA^{Phe}$ (47-75) fragment. (3) = 30S ribosomal subunits incubated with d(G-T-T-C-G-A) in the presence of RNase H. (2) = as (3), but without d(G-T-T-C-G-A). (4) = 70S ribosomes incubated with d(G-T-T-C-G-A) and RNase H. The reaction mixtures were deproteinized by phenol extraction and analysed by polyacrylamide gel electrophoresis.

presence of RNase H and oligohexanucleotide, a new fragment which is indicated in Fig. 7, is formed. The cleavage of 16S rRNA in the 30S subunits which occurs in the presence of d(G-T-T-C-G-A) and RNase H can be inhibited by $tRNA^{Phe}$ (47-75) fragment (Fig. 7, lane 1). This experiment may indicate that the T-loop of the fragment and the synthetic hexanucleotide compete for the same binding site at the 16S rRNA. It is interesting to note that in the case of the 30S subunits the yield of the RNase H fragment was reproducibly higher as compared to the yield in the case of 70S ribosomes. Apparently the single stranded region of 16S rRNA where the cleavage occurs, is better accessible in the 30S particles.

Control experiments were also performed in which the 3'-ends of 5S rRNA and/or 23S rRNA were radioactively labelled. Such 50S subunits or 70S ribosomes were incubated with d(G-T-T-C-G-A) and RNase H. No cleavage could be detected in the 5S rRNA or in the 3'-terminal region (up to 150 nucleotides) of the 23S rRNA (not shown).

DISCUSSION

Colicin E3 hydrolyses the phosphodiester bond between the nucleotide residues 1493 and 1494 of the RNA in the small ribosomal subunit of E. coli ribosomes. This cleavage takes place both in vivo and in vitro and leads to inactivation of the ribosomes even if the 49-nucleotide long colicin fragment remains attached to the particles (23,28). The ribosomes which are engaged in the protein elongation are protected against the desactivation with the bacteriocin. It could be demonstrated by in vitro experiments that this protection can be achieved also by binding of aminoacyl-tRNA to ribosomes (25). Both enzymatic and non enzymatic binding of the aminoacyl tRNA have a similar protective effect. In the present work we could correlate this protective activity of the aminoacyl-tRNA bound to the ribosomal A-site with the amount of colicin fragment formed during the incubation of 70S ribosomes with the bacteriocin. More important, we found that fragments of tRNA, when bound to 70S ribosomes, possess similar inhibitory activities as the intact tRNA. This observation is in agreement with the previous reports in which the ability of tRNA-fragments to compete with aminoacyl-tRNA in the A-site-directed binding reaction was demonstrated (7). The protection of the 3'-terminal region of 16S rRNA against cleavage with colicin may be a result of a direct interaction of the aminoacyl-tRNA or tRNA fragments with this part of 16S rRNA. Such an interaction could prevent the colicin to access the cleavage site. It is, however, also possible that the binding of aminoacyl-tRNA or tRNA fragments only indirectly influences the structure of the 16S rRNA in the vicinity of the colicin cleavage site. With the present techniques we are not able to distinguish between these two possibilities. However, the experiments, in which we demonstrate the complex formation between the colicin fragment of 16S rRNA and the tRNA-fragments, are in support of a view according to which a direct interaction between parts of tRNA sequences and the 3'-end of 16S rRNA takes place.

It is remarkable that the complex formation between the colicin fragment and intact tRNA does not occur whereas both fragments of $tRNA^{Phe}$ bind very efficiently. The secondary

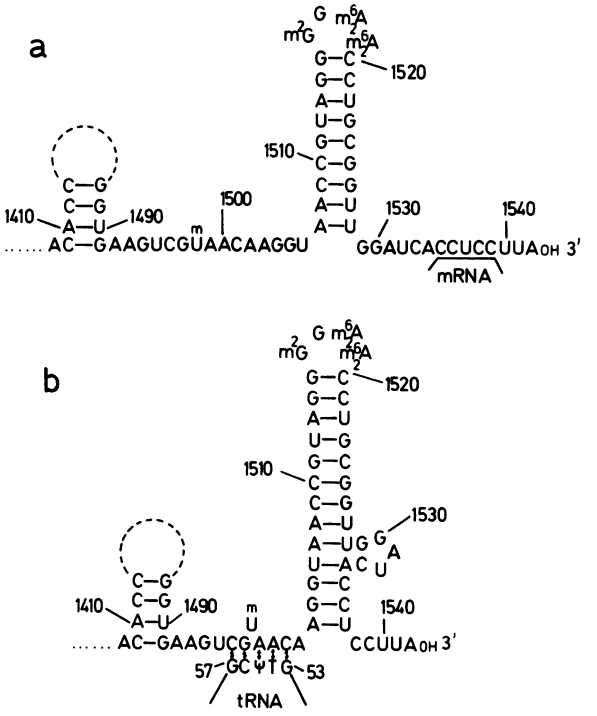

Fig. 8. a) Structure of the 3'-end of E. coli 16S rRNA during the initiation according to ref. 31, and b) the proposed structure of the same part of the 16S rRNA during the binding of aminoacyl-tRNA to the ribosomal A-site.

structure of the $tRNA^{Phe}$ fragments is preserved a depicted in Fig. 2. The former D-stem and anticodon stem of the tRNA exist in the 1-45 fragment and the secondary structure of the T-stem is preserved in the 47-75 fragment. Thus these fragments contain free D- and T-loops (26). Actually the cleavage of the tRNA into two halves and separation of the fragments is the only unambiguous method to avoid intermolecular interaction between the T- and D-loops of tRNA. The free loops can then be tested for their ability to participate in intermolecular interactions with other RNA molecules.

The complex formation between the tRNA (47-75) fragment and the colicin-fragment of 16S rRNA, as well as the RNase H cleavage of 16S rRNA at position 1500 (1501) in the presence of

d(G-T-T-C-G-A) point to the sequence U-C-G-mU-A-A-C of the 16S rRNA as a potential interaction site. This sequence has several interesting features (Fig. 8):

a. It is highly conserved in procaryotic and eucaryotic 16S rRNAs (29).
b. This single stranded region forms a link between two double-stranded parts of 16S rRNA. Such a configuration is favourable for a binding of a complementary sequence. If namely an intermolecular interaction takes place in this region, the newly formed double strand can be stabilized by stacking to the adjacent stems.
c. In E. coli 16S rRNA this sequence contains a modified uridine residue at position 1498. Its structure corresponds probably to N^1-methyluridine (30) which cannot form a Watson-Crick type base pair. This feature would fit to the base pairing scheme between the T-loop of tRNA and nucleotides 1495-1501 of the 16S rRNA as depicted in Fig. 8.
d. The initiation factor 3 (IF-3) from E. coli interacts with the colicin fragment in this region (31).

It is possible that the elongator tRNAs react via the G-T-Ψ-C-(G) sequence with the nucleotides C-G-mU-A-A-C (1496-1501) of the 16S rRNA as depicted in Fig. 8. Such interaction would explain the observations reported in this work concerning the inhibition of the colicin cleavage of 16S rRNA with aa-tRNA or the $tRNA^{Phe}$ (47-75) fragment. The complex formation between the $tRNA^{Phe}$ (47-75) fragment and the colicin fragment of 16S rRNA as well as the binding of d(G-T-T-C-G-A) to this region of the 16S rRNA also suggest that an interaction as depicted in Fig. 8 is feasible.

There are strong indications that the tertiary structure of tRNA in the vicinity of the D-loop: T-loop contacts is changed upon binding of the anticodon to the codon sequence (32,33). This suggests that these conserved parts of tRNAs can become involved in other than intramolecular interactions during their residence time on ribosomes. We can show that the 3'-fragment of the 16S rRNA can participate as a partner for such an intermolecular interaction. The binding of the T-loop of tRNA to the 16S rRNA according to the scheme depicted in

Fig. 8 could also explain how this ribosomal RNA is transfered from its "initiation" structure in which contacts with m-RNA and initiation factor 3 are taking place (31) to the "elongation" structure which facilitates the binding of the elongator tRNAs the ribosomal A-site.

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