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Chemical crosslinking of elongation factor G to the 23S RNA in 70S ribosomes from *Escherichia coli*

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ABSTRACT

Elongation factor G was crosslinked to the 23S RNA of 70S Escherichia coli ribosomes with the bifunctional, cleavable reagent diepoxybutane (DEB). The EF-G-23S RNA complex was isolated and digested with ribonuclease A. After digestion, an RNA fragment, protected by EF-G was cleaved from the complex and isolated. The nucleotide sequence of this RNA fragment was determined by partial ribonuclease digestion. It proved to be 27 nucleotides long and it could be identified with residues 1055 to 1081 of the nucleotide sequence of E. coli 23S RNA. In the presence of thiostrepton, which prevents binding of EF-G to the ribosome, there was a dramatic decrease in the yield of this complex.

INTRODUCTION

Many investigations have been carried out to localize the site of the ribosome to which elongation factor G (EF-G) binds during protein synthesis (1-8). The common feature of most of these studies has been to focus on the ribosomal proteins localized at or near the site for the EF-G interaction. Less studied but probably equally important are the functions and structure of the rRNA. Thus, it is now thought that rRNA not only contributes to structural organisation during assembly but that it may also participate directly to the function of the ribosome (9-12).

In the present work, we have used the chemical crosslinker diepoxybutane to examine the interaction between EF-G and the ribosomal RNA (13-14). Binary complexes of the factor crosslinked to ribosomal RNA were isolated, digested with ribonuclease A, and the protected RNA fragment bound to EF-G was identified after cleavage of the crosslink. In this way an RNA fragment corresponding to the 23S RNA sequence at 1055 to 1081 was identified as part of the ribosomal neighbourhood to which EF-G binds (15).

MATERIALS AND METHODS

Crosslinking of EF-G to 70S ribosomes

The 70S ribosomes and the elongation factor G(EF-G) were prepared and labelled as described previously (7).

The reaction mixtures for the crosslinking experiments contained 3000 pmol 70S ribosomes, 800 µg poly(U) (poly-uridylic acid), 1.0 µmol guanosine 5'-(β,γ-methylene) triphosphate ($p(CH_2)ppG$), 200pmol (^{125}I)-labelled EF-G and 3000 pmol unlabelled EF-G in a total volume of 800 µl of buffer I (20mM triethanolamine-HCl pH 7.8, 15 mM $Mg(OAc)_2$, 50 mM KCl and 1 mM dithioerythritol). This mixture was preincubated for 5 min at 37°C and then it was allowed to cool down to room temperature. The crosslinking reaction was initiated by the addition of the cleavable reagent diexpoxybutane (DEB) (13,14) to a final concentration of 0.5% v/v. The crosslinking reaction was terminated after 1h at 37°C by the addition of at least a five fold molar excess of methylamine-HCl (pH 7.5). Control experiments were performed as above except that thiostrepton (Squibb/Sweden) was added to the mixture at a final concentration of 25 µM prior to the addition of EF-G (15).

Isolation and purification of crosslinked ribosomal RNA-EF-G complexes.

After the termination of the crosslinking reaction, the mixtures were layered onto 5-30% sucrose gradients in 20 mM NaOAc pH 5.6, 5 mM $Mg(OAc)_2$, 400 mM NaCl, 10 mM NH_4Cl and 2 mM dithioerythritol and then centrifuged in a SW 27 Beckman rotor at 21 000 rpm at 1°C for 18h. Ammonium chloride was included in the gradients in order to inactivate any residual unreacted crosslinking reagent. Fractions containing 30S respectively 50S subunits were collected on an Isco fraction collector model UA-4. The 30S and 50S subunits were pooled separately and precipitated with two volumes of ethanol after adjusting the $Mg(OAc)_2$ concentration to 100 mM. After centrifugation the subunits were dissolved in 10 mM NaOAc pH 5.6 and 2% sodium dodecylsulphate, heated 10 min at 56°C and subsequently centrifuged in 5 to 30% sucrose gradients containing 20 mM NaOAc pH 5.6 and 0.2% dodecylsulphate in a SW 27 Beckman rotor at 25 000 rpm at 18°C for 18h.The 16S and 23S RNA were then collected as described above. Finally, the RNA and RNA-EF-G complexes were ethanol-precipitated several times to remove the dodecylsulphate before the pellets were redissolved in a small volume of buffer II containing 20 mM triethanolamine-HCl pH 7.5; 0.5 mM $Mg(OAc)_2$ and 50 mM KCl.

RNase treatment of the 23S RNA-EF-G complex

The RNA in the 23S RNA-EF-G complex was incubated in buffer II for 5 min

and slowly cooled to 37°C; then it was placed on ice (17). The 23S RNA was digested with ribonuclease A in the same buffer at 0°C for 20 min at an RNase/RNA ratio of 1:100-1500 µg of RNase A per µg of RNA. The RNase digestion was stopped by adding sodium dodecylsulphate to a final concentration of 2%.

Isolation of 23S RNA fragments crosslinked to EF-G

Immediately after digestion, the RNA-EF-G mixture was made 7 M in urea, heated at 90°C for 1 min, and loaded on a dodecylsulphate 8% polyacrylamide gel (18). After electrophoresis and autoradiography the gel pieces containing radioactivity were cut from the gel and shaken in an elution buffer according to (19). The elution was done in order to remove comigrating RNA fragments not crosslinked to EF-G. The gel pieces containing the EF-G-RNA complex were then soaked in sample buffer (18) containing urea and run over a second dodecylsulphate 10% polyacrylamide gel. After electrophoresis the gel pieces containing the EF-G-RNA fragment complexes were again excised from the gel and then cleaved with 15 mM $NaIO_4$ at pH 6.6 for 20 min at room temperature. After cleavage each gel piece was washed in water to remove excess $NaIO_4$ and subsequently shaken in a small volume of elution buffer (19) for several hours at 37°C in order to elute the RNA fragments. After elution, the RNA fragments were precipitated twice with two volumes of 96% ethanol at -20°C overnight and finally washed with 70% ethanol before 5'end labelling.

Labelling 5'ends

The RNA fragments were 5'end labelled according to (19) except that the final volume of the mix was 30 µl with 100 µCi (γ-^{32}P) ATP (2900 Ci/mmol, New England Nuclear).

After incubation at 37°C for 30 min, 30 µl 4 M NH_4Cl was added to the mixture just before the addition of 5 µg carrier tRNA and three volumes of ethanol. After centrifugation the pellet was redissolved in 100 µl 0.3 M NaOAc pH 5.6 and the RNA was reprecipitated with 3 volumes of ethanol. The subsequent pellet was rinsed with 70% ethanol and dried under vacuum. Finally the pellet was redissolved in 10 µl sample buffer(20).

Purification of 5'end labelled RNA fragments

The labelled RNA fragments were run on a 20% polyacrylamide gel according to (21). After electrophoresis the gel was subjected to autoradiography, the radioactive bands were located by superposition of the developed film over the gel and were cut out. The radioactive RNA fragments were then eluted from their respective gel piece and prepared for a second 20% poly-

acrylamide gel as above. This second electrophoresis was done in order to further purify the fragments. Each fragment was then eluted and precipitated again as above. The pellets were redissovled in 30 µl 0.1% dodecylsulphate solution and desalted on a 300 µl sephadex G 25 column (Pharmacia) in water. Finally fragments were precipitated and subsequently redissolved in 5 µl water and stored at -80°C.

Identification of the 23S RNA fragments

For sequence analysis on polyacrylamide gel the following enzymes were used for controlled digestions: RNase T1, and U_2 as described in (21) at concentrations of 2.5×10^{-3}u and 0.1-0.2u respectively per µg of RNA; RNase PhyM and that from B.cerus as described in (22,23) at concentrations of 1u and 0.5u respectively per µg of RNA. Carrier tRNA was added to all samples. The concentration of 5'end labeled RNA was considered negligible. The reactions were done at 50°C for 15 min in 10 µl aliquots containing 4 µg RNA. From each sample a reference ladder (23) was prepared by boiling one fraction in water for 4 min at an RNA concentration of 0.8 µg/µl water. Before loading onto the 20% polyacrylamide gel, urea was added to the B. cerus sample as well as one volume of two fold concentrated sample buffer to the sample containing the ladder RNA.

RESULTS

Elongation factor G was crosslinked to 70S ribosomes as described in Materials and Methods. As shown in fig.1 EF-G is recovered bound to both 16S and 23S RNA after a dodecylsulphate gradient wash. Parallel experiments performed in the absence of the crosslinking reagent show a residual binding of EF-G of about 60% to 16S and 15% to 23S RNA. As the nonpermissive conditions we chose crosslinking in the presence of the antibiotic thiostrepton, which is known to be a very strong inhibitor of EF-G binding (16). As also shown in fig. 1 thiostrepton decreased the binding of EF-G to both ribosomal RNA's by at least 95%. Since thiostrepton dramatically decreases the extent of crosslinking we conclude that the crosslinked complexes obtained in its absence are site-specific. Because of the small difference in recovery between crosslinked and non-crosslinked EF-G-16RNA complexes those complexes are of questionable significance. For that reason and because of their low yield they were not further analysed.

After RNase treatment the EF-G-23S RNA fragment complexes migrate in a dodecylsulphate 8% gel as a broad band at a position above EF-G (Fig. 2). To ensure that EF-G was not crosslinked to the 23S RNA via another ribosomal

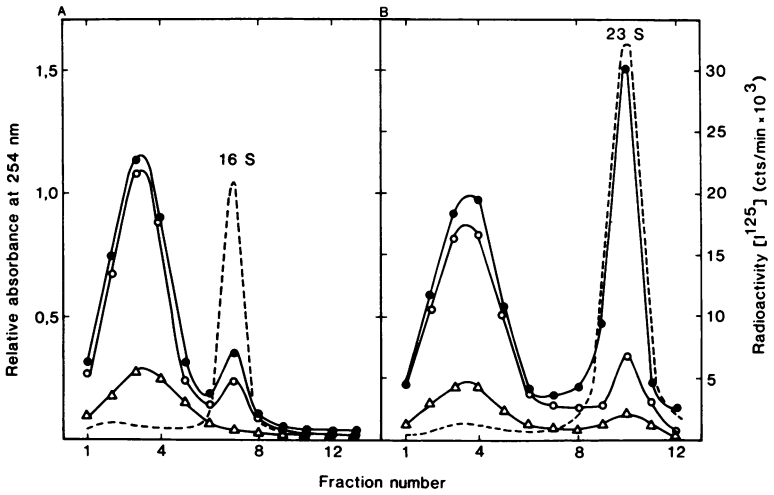

Fig. 1A,B. After in situ crosslinking of EF-G to 70S ribosomes from E. coli the 30S and 50 S subunits were isolated from a sucrose gradient and subsequently treated with 2% sodium dodecylsulphate in a NaOAc buffer pH 5.6. The subunits were then analysed by scanning for absorbance and radioactivity on a 5-30% sucrose gradient containing sodium dodecylsulphate. A:30S, B:50S. Dashed lines: relative absorbance at 254 nm; (●) (^{125}I) radioactivity in the presence of 3 mM DEB and (o): in the absence of crosslinker. (Δ): control experiment in the presence of 3 mM DEB after preincubation of the 70S ribosomes with 25 μM thiostrepton. The position of 16S and 23S RNA are indicated. Migration in the gradients was from left to right.

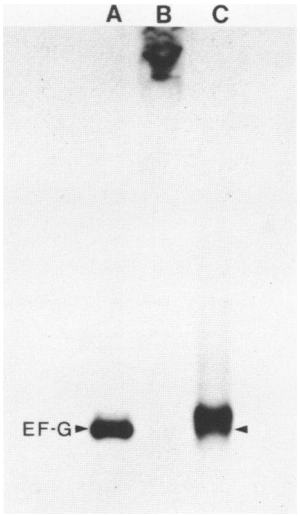

Fig.2. Autoradiograph of the EF-G-23S RNA complex in a sodium dodecylsulphate 8% polyacrylamide gel. A: (^{125}I) EF-G, B: (^{125}I) EF-G-23S RNA complex in the the absence of RNase and C:(^{125}I) EF-G-23S RNA complex after RNase A digestion. The samples are prepared and electrophoresed as described in Materials and Methods.

protein (i.e. trimeric complexes) we ran a parallel experiment where the ribosomes were labelled with ^{35}S. We could not find any trimeric complexes, at the position where the EF-G-23S RNA complexes were isolated (data not shown).

The RNA fragments liberated from EF-G after cleavage were then carefully purified and isolated before 5'end labelling and application onto a 20% polyacrylamide gel for electrophoresis (see Materials and Methods). As can be seen in fig. 3 two main bands, A and B, appear on the gel. A couple of larger and a few smaller fragments were also observed but these bands contained too little material to allow reasonable recovery from the gel. The fragments A and B were then, after further purification, isolated and sequenced by partial digestion as described in Materials and Methods. The RNA fragment A is 27 nucleotides long and the first 17 nucleotides determined at its 5'end have a sequence corresponding to G1055 to G1071 in the previously determined 23S RNA sequence (15) (fig. 4). Noteworthy is the finding that adenosine 1067 is missing in this sequence. Fragment B proved to be the same fragment as A except that it was 5 or 6 nucleotides shorter at the 3'end. (Data not shown.) We also tried to study the sequences of the weaker bands (fragments) released by RNase T1 digestion. The results indicate that they are shorter or longer fragments that overlap with fragment A. (Data not shown.)

Fig. 3. Autoradiography on the 5'end labelled fragments isolated from the EF-G-23S-RNA complex after RNase digestion and cleavage of the crosslinker. Fragment A and B were calculated to be approximataly 27 and 22 nucleotides long respectively. For sample preparation and the electrophoresis see Materials and Methods.

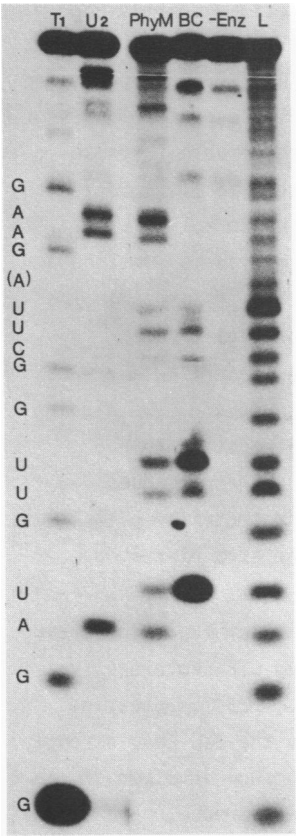

Fig. 4. Autoradiograph of the 5'end labelled 23S-RNA framgent A (see fig. 3) partially digested with ribonuclease T_1, U_2, Phy M and B cerus (BC).-Enz lane: incubation - of fragment A in the absence of RNase and L: ladder prepared from fragment A. The samples are prepared and electrophoresed as described in Materials and Methods.

DISCUSSION

Most of the studies on the binding of ribosomal proteins to ribosomal RNA have been done by using the technique of nuclease protection (25). The results from these studies vary, but the fragments protected are generally large, which makes it difficult to determine the contact point between proteins and RNA. Crosslinking by UV irradiation or treatment with specific bifunctional reagents have also been used in a few cases to obtain a more precise position for proteins on the rRNA (25). In the present investigation we have tried by chemical crosslinking to localize more precisely the region of ribosomal-RNA where EF-G interacts with the 70S ribosome. Results obtained with the crosslinking reagent DEB can be summarized as follows: EF-G seems to be in close contact with rRNA in both subunits but most clearly with the 23S RNA of the large subunit. In addition, we have isolated and sequenced two fragments of 23S RNA crosslinked to and protected by EF-G

against digestion with ribonuclease A.

These two fragments, which are approximately 22 and 27 nucleotides long, have sequences that correspond to the part of the 23S RNA sequence published by Brosius et al., at positions 1055-1076 and 1055-1081, respectively. Even though the nucleotides close to the 3'end were not identifiable in these fragments the remaining nucleotides identified correspond to a unique region on the 23S RNA. Furthermore, this sequence can according to Noller et al., form a stem plus loop in region II (26). Such a conformation may well be a specific binding site for EF-G and thereby nicely explain its crosslinking ability as well as its protection by EF-G against RNase digestion.

A very attractive pattern seems to emerge when the present data are correlated with those of Schmidt et al., (27). Their studies of the binding site for protein L11 on the 23S RNA have suggested that this protein protects an RNA fragment from ribonuclease T1 which includes residues 1052-1112. Even if this fragment is longer than that protected by EF-G against RNase A, they both contain the sequence for the stem plus loop discussed above.

These data, together with the data from Maasen & Möller (2,3) indicating that L11 can be labelled by both EF-G dependent GDP and GTP photoreactive analogues, as well as the results from Thompson et al., (28), suggesting that L11 is involved in the binding of thiostrepton to the 23S RNA, strongly support the original suggestion that protein L11 is somehow involved in the EF-G-dependent GTPase activity on the ribosme (29). Furthermore, more recent studies of Thompson et al., have identified a methylase enzyme that modifies adenosine-1067 of E. coli 23S RNA and is responsible for antibiotic protection in the thiostrepton-producing Streptomyces azureus (30). This modification of adenosine-1067 which is associated with thiostrepton resistence is not only situated in the 23S RNA loop discussed above, but it corresponds to the adenosine that is missing from the sequence in the present EF-G associated RNA fragment. This might be a coincidence, but it could also reflect a modification of the adenosine caused by the DEB crosslinking reaction between this adenosine and EF-G.

Another protein that possibly is a component of this functional site is protein L7/L12. From crosslinking experiments it is already known that L7/L12 is a close neighbour of both EF-G and protein L11 (1,7,31). Furthermore, Dijk et al., demonstrated that the binding of the L7/L12-L10 protein complex to the 23S RNA is stimulated by protein L11 and vice-versa (32). This is compatible with earlier results on the binding of elongation factor

G to protein-depleted ribosomal cores. Thus, the binding of the factor required the prior assembly of L7/L12 and this in turn was stimulated by proteins L10 and L11 (32,33). All of these observations together with the present results suggest that the ribosomal domain associated with EF-G embraces at least residues 1055 to 1081 of the 23S RNA as well as the proteins L10, L11 and L7/L12 in the large ribosomal subunit.

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