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PREPARATION AND SEQUENCING OF THE CLOACIN FRAGMENT OF STREPTOMYCES AUREOFACIENS 16S RNA

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A fast method for isolation of a 3′-terminal fragment of Streptomyces aureofaciens 16S RNA was developed. The procedure involves reaction of 70S ribosomes with cloacin DF13 and subsequent fractionation of the reaction mixture by polyacrylamide gel electrophoresis. The cloacin fragment was eluted from the gel and used directly for 3′-end labeling with cytidine-3′,5′-[5′-^{32}P]bisphosphate. The labeled RNA fragment was sequenced by the enzymatic method. It consists of 50 nucleotides and has the sequence 5′-GUCGUAACAAGGUAACCGUACCGGA-AGGUGCGGUUGGAUCACCUCCUUUC$_{OH}$. The differences from the E.coli and Bacillus sequences and their possible influence on the rate and specificity of polypeptide synthesis are discussed.

The rate of protein synthesis in various Streptomyces species is considerably lower than in Escherichia coli (1,2). Ribosomes of S.aureofaciens, a Gram-positive bacterium which differs from ordinary eubacteria by high G+C content (3), are inefficient in in vitro translation of Qβ or MS2 RNA.

There are several differences between the translational systems of streptomycetes and E.coli. Only partial homology was found between ribosomal proteins of E.coli and S.granaticolor (4) or S.aureofaciens (5). In Streptomyces is apparently no functional equivalent of E.coli ribosomal protein S1 that plays an important role in polypeptide chain initiation (6) and elongation (7). The 30S ribosomal protein of S.aureofaciens which resembles protein S1 by size, low isoelectric point and loose binding to the ribosome (5) exhibited only poly(U) binding comparable with that of S1 but

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did not stimulate the polyphenylalanine-synthesizing activity (8). Initiation factors from S.aureofaciens showed different properties than did the factors from E.coli and were only partially interchangeable (9). An in vitro translation system derived from S.aureofaciens required more than 10 times higher amounts of poly(U) for maximum polyphenylalanine synthesis than did an analogous system of E.coli (1).

Another component of the translational apparatus that might be responsible for the observed peculiarities of streptomycete protein synthesis is the 3′ terminus of 16S RNA. From its part in mRNA selection (6), tRNA binding (10) and ribosomal subunit interaction (11), it is possible to infer that this part of the 16S RNA molecule may strongly influence the rate and efficiency of protein synthesis.

In this communication, we describe a simple yet powerful procedure for the isolation of a 3′-terminal fragment of streptomycete 16S RNA which enables rapid sequencing. Thus, the "colicin fragment" from S.aureofaciens was prepared and its nucleotide sequence was determined.

MATERIALS AND METHODS

Cultivation of Streptomyces aureofaciens 84/25 and isolation of ribosomes was described elsewhere (1). 70S ribosomes washed with the standard buffer containing 1 M NH_4Cl were used in this study.

Conditions for the cloacin cleavage reaction were optimized in 20-µl mixtures containing 10 mM Tris-HCl, pH 7.4, 60 mM NH_4Cl, 6 mM 2-mercaptoethanol, 15 mM magnesium acetate, 1.5 A_{260} units of ribosomes, and cloacin DF13 (a gift from Prof. P. H. Van Knippenberg) in concentrations of 0 - 0.3 µg per 1 A_{260} unit of ribosomes. Cloacin DF13 was heat-activated prior to the reaction (12). The reaction mixtures were incubated at 42°C for 45 min, then 15 µl of the solution containing 10 M urea, 0.4 % sodium dodecyl sulfate, 0.025 % each xylene cyanol and bromophenol blue was added to each tube and the incubation continued for 30 min. The reaction was stopped by chilling the tubes in dry ice and the suspensions were clarified by centrifugation (10,000 x g, 10 min). The supernatants were loaded on a 10 % polyacrylamide gel slab and fractionated by electrophoresis (see Fig.1). For preparation, a larger reaction mixture containing the buffer, 150 A_{260} units of ribosomes and 30 µg cloacin DF13 in a total volume of 0.4 ml was used and, after the reaction, loaded across the whole gel. After the run, a narrow strip was cut off alongside the gel and stained for 10 min in the dark with 0.005 % "Stains-all" (13). The non-stained bulk of the

cloacin fragment was thus located, excised, and RNA was eluted with 0.5 M ammonium acetate, 0.1 % sodium dodecyl sulfate, 0.1 mM EDTA. The cloacin fragment was recovered by precipitation with ethanol.

The 3´-end labeling was carried out essentially according to D´Alessio (14) with the exception that bovine serum albumin was omitted. Cytidine-3´,5´-[5´-^{32}P]bisphosphate (111 TBq/mmol) was purchased from Amersham, T4 RNA ligase was from Pharmacia. The labeled fragment was purified by electrophoresis in 5 % polyacrylamide gel (14).

The Expanded RNA Sequencing Kit from Pharmacia was used for the enzymatic sequencing and the reaction conditions described in ref.14 were observed. The oligonucleotides were separated in 20 % polyacrylamide gel (see Fig.2).

All electrophoreses were performed in an LKB Macrophor.

RESULTS AND DISCUSSION

Isolated streptomycete ribosomal RNA´s tend to be unstable in aqueous solutions which may be due to the presence of ribonucleases that are associated even with washed ribosomes (15). When we attempted at 3´-terminal labeling of isolated 16S RNA from S.aureofaciens we always observed considerable degradation of the RNA molecule after electrophoretic fractionation of the ligase reaction mixture. We assumed that the risk of RNA damage would be minimized using a shorter fragment and a fast purification procedure.

A 3´-terminal fragment of 16S RNA can be prepared e.g. by cleavage of isolated 16S RNA with 3 M NaCl in anhydrous hydrazine (16) or by the action of bacteriocins on intact 70S ribosomes (17, 18). Baan et al. (18) described a reliable technique for the isolation of the "colicin fragment" from various bacteria that involves centrifugations in a large-volume zonal rotor. This method, however, is rather laborious and time-consuming. It was proved (16) that the fragment purified by polyacrylamide gel electrophoresis may be used directly for sequencing. Thus, most of the purification steps may be omitted if rapid determination of the nucleotide sequence is only intended.

Older data (17) suggested that ribosomes from some Gram-positive bacteria could be resistant to cloacin DF13. This bacteriocin produced no RNA fragments from ribosomes of Bacillus licheniformis and Saccharomyces carlsbergensis while cloacin fragments were read-

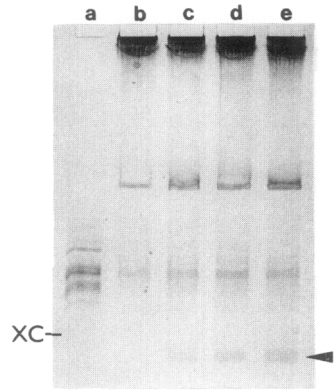
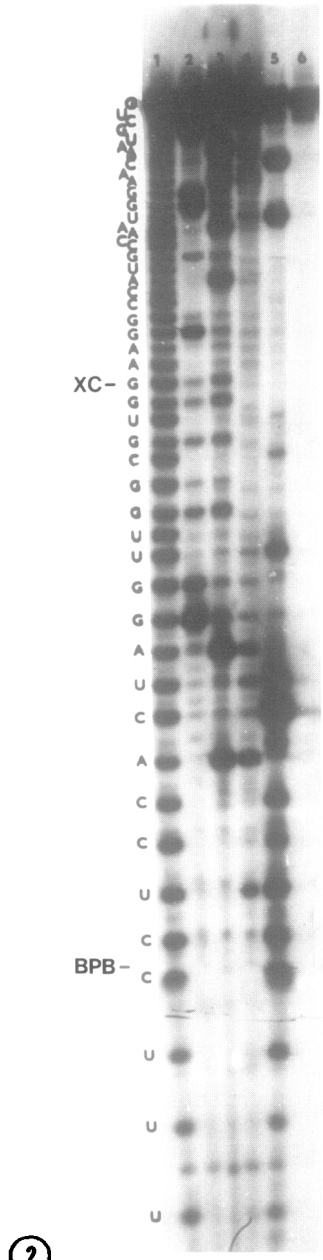


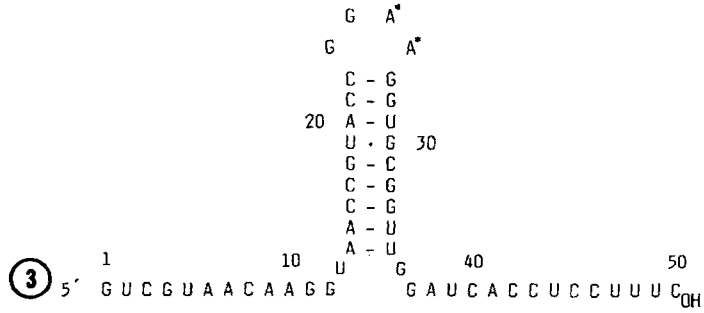
Fig.1. Action of cloacin DF13 on 70S ribosomes from S.aureofaciens. Aliquots of the cloacin reaction mixtures were loaded on 10 % polyacrylamide gel containing 7 M urea and electrophoresed in TBE buffer (14) at constant voltage of 600 V for 2 h. After the run, the gel was stained with "Stains-all". a - E.coli tRNA marker (SERVA; 5 µg); b - cloacin DF13 omitted; c, d, e - 0.1, 0.2, 0.3 µg cloacin DF13 per 1 A_{260} unit of ribosomes, respectively. XC, xylene cyanol FF. The arrow indicates the cloacin fragment band.

ily formed when ribosomes of other microorganisms were used (19). We showed that cloacin DF13 splits off an RNA fragment from S.aureofaciens 70S ribosomes (Fig.1). Optimum cleavage was achieved at cloacin DF13 concentration of 0.2 µg per 1 A_{260} unit of ribosomes. The well-separated cloacin fragment band could be easily eluted (without phenol extraction) and used for 3´-end labeling. By the method described here, we obtained about 1 A_{260} unit of pure cloacin fragment from 150 A_{260} units of S.aureofaciens ribosomes.

Sequencing of the fragment was carried out by the enzymatic method (14) since it yields also some information on the secondary structure of the RNA sequenced. A typical sequencing gel pattern is shown in Fig.2. It can be seen that the bands corresponding to nucleotides 14 - 22 and 27 - 35, which are supposed to be engaged in the double-stranded stem of a hairpin (20), are weaker than those of nucleotides in the single-stranded regions. Complete nucleotide sequence of the S.aureofaciens cloacin fragment in the presumptive loop structure is presented in Fig.3.



(2)



(3)

Fig.2. Sequencing of the S.aureofaciens cloacin fragment. The 3′-terminally labeled fragment was sequenced by the enzymatic method and the oligonucleotides were separated in 20 % gel containing 7 M urea (14). The gel was run at 60°C at constant voltage of 3 kV for 2 h and then autoradiographed. Lane 1 - partial alkaline digest; 2 - ribonuclease T_1; 3 - ribonuclease U_2; 4 - ribonuclease Phy M; 5 - B.cereus ribonuclease; 6 - no enzyme control. BPB, bromophenol blue, XC, xylene cyanol FF.

Fig.3. Structure of the S.aureofaciens cloacin fragment. Some nucleotides (e.g. the two adenines marked with asterisks) may be methylated.

The fragment consists of 50 nucleotides, or it is larger than the colicin fragment of E.coli (21) and shorter than that of B.stearothermophilus (22). The nucleotide sequence of the three fragments is very similar: when compared with E.coli, the S.aureofaciens fragment differs only by the two inverted base-pairs $\begin{matrix}C\text{-}G\\C\text{-}G\end{matrix}$ in positions $\begin{matrix}22\text{-}27\\21\text{-}28\end{matrix}$, which are, in turn, in the same configuration as in Bacillus (20), and by the 3´-terminal trinucleotide UUC_{OH} instead of A_{OH}. This latter difference might be meaningful in mRNA recognition. The Shine-Dalgarno sequence CCUCC is fully conserved. The free energy of the secondary structure of the S.aureofaciens cloacin fragment (Fig.3) as calculated according to Tinoco´s rules (23) is despite the inversion of the two base-pairs identical to that of E.coli. However, the actual conformational stability may be affected by methylation (24) which has not yet been examined in S.aureofaciens and by the different interactions of the C_{22}-G_{27} base-pair with the adjacent G_{23} and A_{26} in the loop.

We conclude from our data that the 3´ terminus of S.aureofaciens 16S RNA is not the sole determinant of the streptomycete ribosome that is responsible for selective mRNA translation.

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