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Specific *in situ* cleavage of 16S ribosomal RNA of *Escherichia coli* interferes with the function of initiation factor IF-1

(3′ end of 16S rRNA/initiation of protein synthesis/function of IF-1)

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ABSTRACT **Specific *in situ* cleavage of 16S rRNA of *E. coli* has been accomplished by *in vitro* treatment of 70S ribosomes ("tight couples") with the bacteriocin cloacin DF13. The defective ribosomes, which have fully lost their ability to sustain polypeptide synthesis, are still able to form initiation complexes with MS2 RNA, but the kinetics are altered. This is apparently due to an improper functioning of initiation factor IF-1, for the defective ribosomal couples respond normally to dissociation by IF-3 but the dissociation is not stimulated by IF-1. The initiation complexes formed with defective ribosomes are fully reactive with puromycin. Their ability to bind alanyl-tRNA is reduced by about 50% at all concentrations of elongation factor Tu studied. Cleavage of the 16S rRNA, not the release of the terminal fragment from the ribosome, causes the block of protein synthesis and the aberrations observed during initiation and elongation.**

The evidence is growing that the 3′-OH end of 16S rRNA of *Escherichia coli* is part of an important functional site on the 30S ribosome subunit. Specific cleavage of the 16S rRNA by the bacteriocin colicin E3, 49 nucleotides from the 3′-OH end, results in a total loss of the activity of the ribosome in translation (1). The presence of two adjacent methylated adenine residues near the 3′ terminus of 16S rRNA determines sensitivity towards the antibiotic kasugamycin, a potent inhibitor of chain initiation (2). Crosslinking experiments indicate that a number of ribosomal proteins and initiation factors that have been implicated in initiation events, namely, S1 (3, 4; G. van Dieijen, P. H. van Knippenberg, and J. van Duin, to be published), S12 (5), IF-2 (6, 7), and IF-3 (6, 8), cluster around this end of 16S rRNA in the 30S particle (9–11, 30). Sequence analysis has led Shine and Dalgarno (12) to suggest that part of the 3′-OH terminus of the 16S rRNA is implicated in the recognition of conserved sequences found in the ribosome binding sites of various mRNAs.

In this context it seemed of great importance to know which step in polypeptide synthesis is actually blocked after bacteriocin treatment of the ribosome. Up to the present, no clear answer to this question can be found in the literature. Early experiments by Konisky (thesis 1968) referred to by Nomura in a recent review (13) showed that ribosomes isolated from colicin E3-treated cells are inactive in poly(AUG)-dependent fMet-tRNA binding. Tai and Davis (14), working *in vitro* with isolated polysomal ribosomes and free ribosomes, were able to distinguish between a block in polypeptide chain elongation that is relieved at elevated Mg^{++} concentrations and an inhibition of ribosomes initiating on natural messenger that is not relieved at high Mg^{++}. According to these authors, this may imply that some step in initiation in addition to a step in elongation is affected, or that transition of initiation complexes into elongation ones is inhibited. On the other hand, Turnowsky and Högenauer (15) reported that colicin E3-treated ribosomes bind fMet-tRNA in response to the trinucleotide ApUpG, whereas their ability to bind phenylalanyl-tRNA in a poly(U)-dependent and EF-T catalyzed reaction is reduced.

In this paper we have studied the consequences of ribosome damage inflicted by the bacteriocin cloacin DF13, known to cleave the 16S rRNA at the same site as colicin E3 (16). In agreement with others (17) it is shown that this cleavage results in an absolute loss of overall amino-acid incorporation in a phage MS2 RNA dependent system. Furthermore, as has been suggested by others (14), cloacin treatment also affects initiation, although to a far less extent. It is shown here that the kinetics of phage RNA dependent binding of fMet-tRNA to ribosomes are affected by cloacin treatment and that this effect can be ascribed to an improper functioning of the initiation factor IF-1. The observed functional defects of cloacin treated ribosomes cannot be attributed to the loss of the 3′-OH terminal fragment of 16S rRNA from the ribosome during any stage of protein synthesis studied so far.

MATERIALS AND METHODS

E. coli MRE 600 was grown in 40-liter batches in a New Brunswick fermentor. The medium contained per liter: bactopeptone 5.0 g, yeast extract 1.0 g, glucose 10 g, $MgSO_4$ 0.13 g, NH_4Cl 3.6 g, KH_2PO_4 3.0 g, and $Na_2HPO_4 \cdot 10H_2O$ 15.1 g. During growth the pH of the medium was maintained at 7.0–7.2 with 5 M NH_4OH. At the beginning of the logarithmic phase of growth 5 ml of a saturated solution of NH_4Cl was added per liter of medium. Cells were harvested at midlogarithmic phase of growth by centrifugation in a Sorvall RC2-B centrifuge using an SS-34 rotor equipped with a continuous flow system. The cell paste was washed immediately with a buffer containing 10 mM Tris·HCl, pH 7.4, 60 mM NH_4Cl, 10 mM Mg acetate, 6 mM 2-mercaptoethanol (standard buffer), centrifuged once more, and frozen at −80°.

Preparation of Ribosomes. An S30 extract was obtained by grinding the cell paste with alumina in the presence of DNase (0.2 mg/50 g of cells) at 4°, and subsequent centrifugation. Ribosomes were isolated from the S30 by centrifugation for 6 hr at 30,000 rpm and washed once with 1 M NH_4Cl in standard buffer. Tight couples were prepared by zonal centrifugation in 6 mM Mg acetate, in a B XIV zonal rotor of an MSE 65 centrifuge. Ribosomes were stored at −80° in standard buffer.

Initiation factors were purified from the ribosomal wash according to the start procedure described by Benne *et al.* (18) except that Ultrogel AcA34 replaced Sephadex G-200 and G-100 in the case of IF-2 purification. Factors were ho-

Abbreviations: IF, initiation factor; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis[2(5-phenyloxazolyl)]benzene; A_{260} unit, that amount of material that gives an absorbance of 1 when dissolved in 1 ml of solvent when the light path is 1 cm; EF, elongation factor.

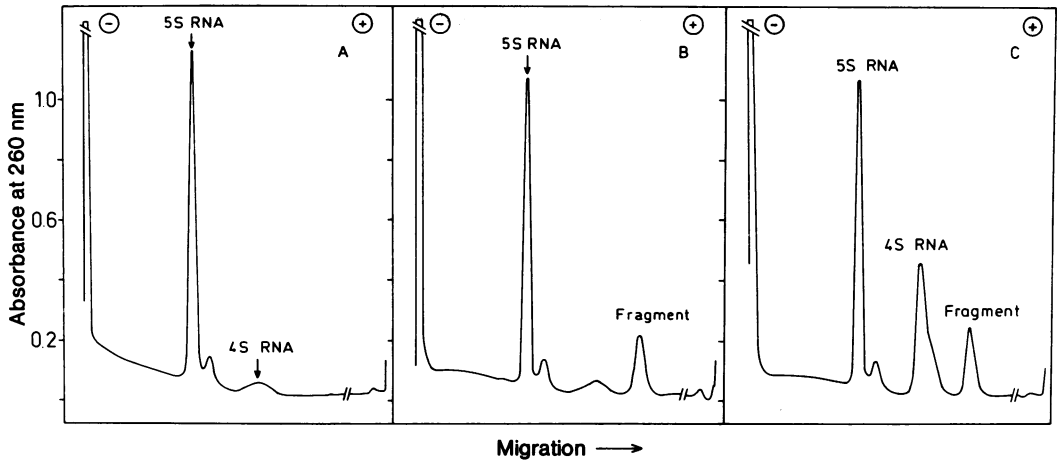

FIG. 1. Analysis of 16S rRNA cleavage. (A and B) Samples containing 3 A_{260} units of 70S ribosomes incubated in the presence or absence of cloacin DF13 (1 $\mu g/A_{260}$ unit) for 30 min at 37° were analyzed on 15% polyacrylamide gels containing 0.1% sodium dodecyl sulfate, as described in *Materials and Methods*. Gels were scanned at 260 nm immediately after the run. Panel A: incubation without cloacin. Panel B: incubation with cloacin. (C) Samples containing 3 A_{260} units of cloacin-treated ribosomes incubated with all components for polypeptide synthesis (see *Materials and Methods*) were concentrated by centrifugation at 40,000 rpm for 15 hr. Pellets were resuspended and aliquots were analyzed as described above.

mogeneous, as shown by electrophoresis on sodium dodecyl sulfate-polyacrylamide gels. Protein concentration was determined according to Lowry *et al.* (19), with lysozyme as standard.

MS2 RNA and MS2 [^{3}H]RNA (specific activity 6500 dpm/pmol) were prepared as described by Vermeer *et al.* (20). Labeled aminoacyl-tRNAs, namely, f[^{3}H]Met-tRNA (specific activity 11.6 × 10^3 dpm/pmol) and [^{14}C]Ala-tRNA (specific activity 400 dpm/pmol) were isolated as described by Voorma *et al.* (21). Purified EF-Tu, prepared according to Miller and Weissbach (22), was a gift of Miss J. Talens. Purified cloacin DF13 prepared from *Enterobacter cloacae* DF13 was a gift of Dr. F. K. de Graaf, Microbiology Department, Free University, Amsterdam. The bacteriocin was dissolved in standard buffer.

Assays. Binding of formyl[^{3}H]methionyl-tRNA was tested in a total volume of 0.1 ml containing 50 mM Tris·HCl, pH 7.6, 40 mM NH_4Cl, 5 mM Mg acetate, 6 mM 2-mercaptoethanol, and about 3% glycerol. The reaction components were 0.4–0.5 A_{260} unit of 70S ribosomes, 15 pmol of f[^{3}H]Met-tRNA, 15 μg of MS2 RNA, 0.02 μmol of GTP, 0.9 μg of IF-1, 2.3 μg of IF-2, and 0.6 μg of IF-3. The mixtures were incubated for 20 min at 37°, unless indicated otherwise. The reaction was stopped by the addition of 3 ml of cold incubation medium, and the mixtures were subsequently filtered over Selectron BA85 filters. Radioactivity was determined in 10 ml of toluene containing 4 g of 2,5-diphenyloxazole (PPO) and 50 mg of 1,4-bis[2(5-phenyloxazolyl)]benzene (POPOP) per liter. Formation of f[^{3}H]methionylpuromycin was tested in reaction mixtures with the same composition as those described above, except that 50 μg of puromycin was present during the incubation. f[^{3}H]Metpuromycin was extracted with ethylacetate as described by Leder and Bursztyn (23). Radioactivity was determined in 10 ml of a mixture containing Triton X-100/toluene/H_2O (10:20:3) supplemented with 8.5 g of PPO and 0.5 g of POPOP per liter of toluene.

Binding of [^{14}C]alanyl-tRNA was tested in mixtures containing the same components as those described for f[^{3}H]Met-tRNA binding, except that unlabeled fMet-tRNA was used and 22 pmol of [^{14}C]Ala-tRNA, 25 μg of phospho*enol*pyruvate, and 0.015 μg of pyruvate kinase were added. The Mg concentration was 7 mM. Radioactivity of [^{14}C]Ala-tRNA bound was determined on Selectron filters.

Incorporation of [^{14}C]alanine into polypeptide was measured in a total volume of 0.05 ml containing 40 mM Tris-acetate, pH 7.6, 10 mM Mg acetate, 60 mM NH_4Cl, 6 mM 2-mercaptoethanol, 1 mM ATP, 0.12 mM GTP, 5 mM phospho*enol*pyruvate, 0.1 μg of pyruvate kinase, 3 μl of leucovorin (Ca-salt; Lederle), 1 × 10^{-3} μmol each of all amino acids minus alanine, and 0.3 × 10^{-3} μmol of [^{14}C]alanine (171 mCi/mmol), 20 μg of unlabeled fMet-tRNA, 30 μg of uncharged tRNAs, 30 μg of MS2 RNA, 1 μg each of the three initiation factors, 4 μg of S100 supernatant protein, and 1 A_{260} unit of ribosomes. Mixtures were incubated for 30 min at 37°. The reaction was stopped with 1 ml of 5% trichloroacetic acid. After heating for 20 min at 90° the samples were filtered on Whatman GF/A filters. Radioactivity was determined in a toluene-PPO-POPOP scintillation liquid.

Analytical Methods. Low-molecular-weight RNA was analyzed on 15% gels containing 0.1% sodium dodecyl sulfate according to a procedure described by van Diggelen (24) with some modifications. This method permits the separation of 5S rRNA, tRNA, and the 49-nucleotide fragment resulting after cloacin treatment. Electrophoresis was carried out at 2 mA/gel (75–80 V) for 15–16 hr. Gels were scanned at 260 nm with the aid of a Gilford 2400 spectrophotometer equipped with a gel scanner. Sucrose gradient analysis was performed on 19-ml, 10–30% linear gradients in a buffer containing 50 mM Tris·HCl, pH 7.6, 50 mM NH_4Cl, and 5 mM Mg acetate. Centrifugation was at 20,000 rpm for 15 hr at 4° using an SW-27 rotor in a Beckman L2-65 centrifuge. The gradients were monitored at 260 nm with the aid of a Gilford 2400 spectrophotometer equipped with a flow cell. When radioactive material was present, fractions were collected directly in counting vials. Radioactivity was determined after addition of 7 ml of Triton-toluene scintillation liquid.

RESULTS

Cleavage Affects Protein Synthesis and Initiation to Different Extents. The studies described in this paper were performed with ribosomes prepared according to Noll and Noll (25). These so-called "tight couples" can be converted

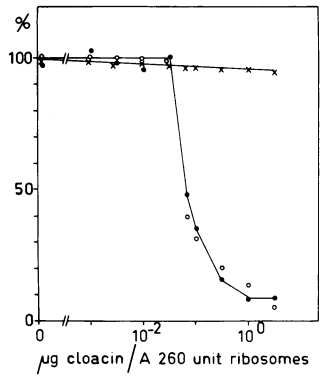

FIG. 2. Effect of 16S rRNA cleavage on initiation and translation. Samples containing 0.5, 1, or 3 A_{260} units of ribosomes were incubated for 30 min at 37° with increasing amounts of cloacin and assayed during a second 30-min incubation for f[^{3}H]Met-tRNA binding (×) and [^{14}C]alanine incorporation (●), both with MS2 RNA as messenger, and for 16S rRNA cleavage (○), as described in the *text*. Results are expressed as percentage of the value at zero concentration of cloacin.

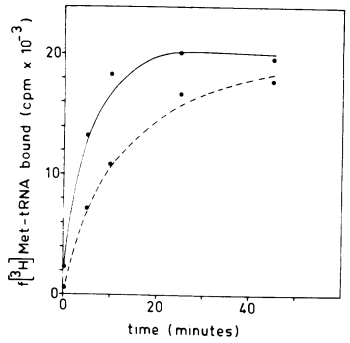

FIG. 3. Kinetics of initiation complex formation. Ribosomes treated with cloacin (as described in the legend to Fig. 1) and control ribosomes were tested in MS2 RNA-directed binding of f[^{3}H]Met-tRNA after different times of incubation. The amount of initiation complex formed was determined by retention on Selectron filters (see *Materials and Methods*). (——) Control ribosomes; (- - -) cloacin-treated ribosomes.

with high efficiency (60–85%) into initiation complexes containing phage RNA and fMet-tRNA.

In vitro treatment of these ribosomes with cloacin DF13 resulted in specific cleavage of 16S rRNA (Fig. 1A and B).

The extent of cleavage was computed using the 5S rRNA peak as an internal standard. Corrections were made for a small amount (about 10%) of free 50S subunits present in the 70S preparation. At a ratio of 0.8 molecule of cloacin per ribosome (1 $\mu g/A_{260}$ unit) virtually all 16S rRNA was cleaved after a 30-min incubation.

Consequences of cleavage for MS2 RNA-directed incorporation of [^{14}C]alanine and binding of f[^{3}H]Met-tRNA to the ribosome were different (Fig. 2). In these experiments ribosomes were incubated for 30 min with varying amounts of cloacin. Components were then added for initiation or polypeptide synthesis. To control treated samples no addition was made. After incubation for another 30 min, f[^{3}H]Met-tRNA binding and [^{14}C]alanine incorporation were determined. The extent of cleavage was analyzed in the control sample. Under conditions where virtually all ribosomes contained a nick in their 16S rRNA and protein synthesis was completely inhibited, initiation complex formation was only slightly diminished (Fig. 2, crosses). Since Jakes and Zinder (26) have shown that mRNA promotes the action of colicin E3, cleavage in the control samples may have been somewhat slower at limiting concentrations of cloacin. Nonetheless the decrease in protein synthesis (Fig. 2, closed circles) was found to be roughly parallel with the disappearance of intact 16S rRNA (Fig. 2, open circles).

After the incorporation experiment, the 3′-terminal fragment was still present on the ribosome (Fig. 1C). Hence, the abolition of polypeptide synthesis was caused by cleavage of 16S rRNA and not by the loss of the 3′-terminal fragment. All experiments described below were performed with 70S ribosomes pretreated with cloacin for 30 min at 37° to give complete cleavage (conditions of Fig. 1). These treated ribosomes also retained the 3′-OH terminal fragment during sucrose gradient centrifugation. When the experiments were repeated with ribosomes purified in this way, the same results were obtained.

Cleavage and Improper Functioning of IF-1. Although initiation complex formation seemed to be hardly affected by cloacin (Fig. 2), the kinetics were found to be altered (Fig. 3). Treated ribosomes bound fMet-tRNA at a lower rate, but at 40 min, binding was only slightly lower than to control ribosomes (compare also Fig. 2). The possibility was considered that one of the initiation factors did not function properly with the defective ribosomes. As is shown in Fig. 4A–D, dissociation into subunits on the addition of IF-3 occurred to the same extent with control and cloacin-treated ribosomes. However, the stimulating effect of IF-1 on IF-3-mediated dissociation of 70S couples (27, 28) is lost (Fig. 4E and F).

The joining of the 50S ribosomes to 30S initiation com-

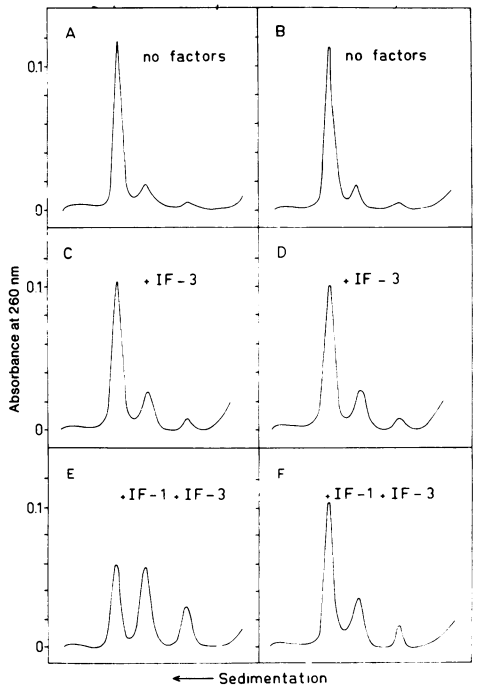

FIG. 4. Effect of IF-3 and IF-1 on dissociation of 70S ribosomes. Ribosomes (1 A_{260}) preincubated with or without cloacin (as indicated in the legend to Fig. 1) were incubated for 20 min at 37° at 5 mM Mg with IF-1 (1.5 μg) and IF-3 (1.2 μg) where indicated. Sucrose gradient analysis was carried out as described in *Materials and Methods*. No dissociation occurred in the presence of IF-1 alone. Dissociation in the absence of IF-1 showed a linear dependence on IF-3. Panels A, C, and E: control ribosomes. Panels B, D, and F: cloacin-treated ribosomes.

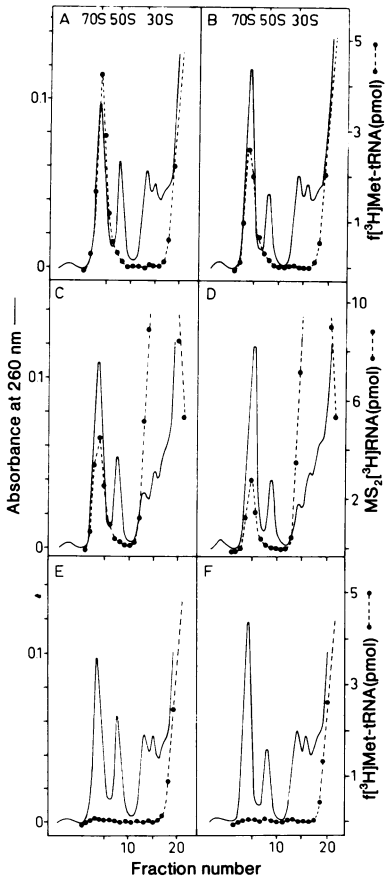

FIG. 5. Formation of 70S initiation complexes; reactivity with puromycin. Cloacin-treated and control ribosomes (1 A_{260} unit) were incubated for 20 min at 37° with components for initiation complex formation as described in *Materials and Methods*, with either f[^{3}H]Met-tRNA and unlabeled MS2 RNA (panels A, B, E, and F) or unlabeled fMet-tRNA and MS2 [^{3}H]RNA (panels C and D). After fixation with 0.25% glutaraldehyde, samples were analyzed by sucrose gradient centrifugation (see *Materials and Methods*). Panels A, C, and E: control ribosomes. Panels B, D, and F: cloacin-treated ribosomes. Panels E and F: puromycin (50 μg) was present during the incubation.

plexes is unaffected after cloacin treatment. This is shown in Fig. 5A–D, in which 70S initiation complex function was followed by sucrose gradient analysis. The reduction in f[^{3}H]Met-tRNA binding (Fig. 5A and B), which is still substantial after 20 min of incubation (see Fig. 3), equaled that in MS2 [^{3}H]RNA binding (Fig. 5C and D). The initiation complexes are fully reactive with puromycin (Fig. 5E and F), showing that peptidyltransferase is not affected by 16S rRNA cleavage.

If the main effect of cloacin treatment is improper functioning of IF-1, the difference in fMet-tRNA binding observed with normal and defective ribosomes might be less in the absence of IF-1 than in its presence. This assumption was borne out by the experiments shown in Fig. 6. Under these conditions the binding of fMet-tRNA to control and defective ribosomes was much slower (see *Discussion*), but the difference in kinetics vanished completely on omission of IF-1.

Binding of Alanyl-tRNA to the Ribosomes. IF-1 is known to exert at least one other effect during initiation. Benne *et al.* (29) showed that IF-1 is involved in the release of IF-2 from the 70S initiation complex. An indirect method to determine IF-1 activity in this respect is to measure the

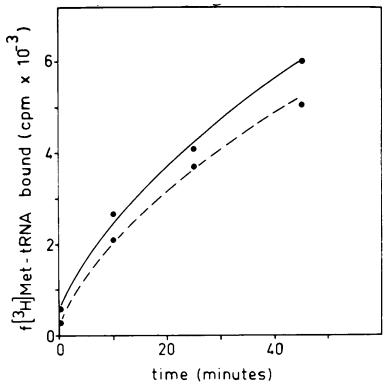

FIG. 6. Kinetics of initiation complex formation in the absence of IF-1. Cloacin-treated and control ribosomes were tested in MS2 RNA-directed binding of f[^{3}H]Met-tRNA after different times of incubation. Mixtures contained components as described in *Materials and Methods*, except that IF-1 was omitted. The amount of initiation complex formed was determined by retention on Selectron filters. (——) Control ribosomes; (- - -) cloacin-treated ribosomes.

elongation factor EF-Tu dependent binding of the second aminoacyl-tRNA, namely, alanyl-tRNA to the initiation complex, since it is known that this binding is reduced on 70S initiation complexes still carrying IF-2. Fig. 7 shows that, indeed, binding of alanyl-tRNA to initiation complex is reduced to 50% with cloacin-treated ribosomes as compared with control ribosomes. This reduction is not due to a kinetic effect (not illustrated).

DISCUSSION

In this investigation we studied the consequence of a specific cleavage of 16S rRNA for polypeptide chain initiation and elongation. The primary effect with regard to initiation seems to be the abolition of the stimulatory action of IF-1 on IF-3-mediated dissociation of 70S ribosomes. This lack of response to IF-1 can explain the altered kinetics of initiation complex formation. It may also explain the discrepancy in the literature between the results of Konisky and those of Turnowsky and Högenauer (15) referred to in the introduction. Fig. 3 shows that the inhibition of fMet-tRNA binding to the ribosome can amount to approximately 40% after short incubation periods, whereas after 1 hr hardly any effect can be detected.

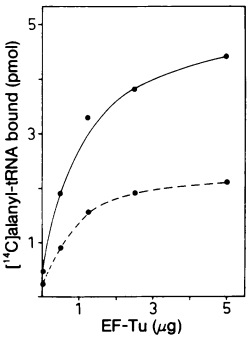

FIG. 7. Binding of [^{14}C]alanyl-tRNA to initiation complexes. Cloacin-treated and control ribosomes were tested in MS2 RNA-directed binding of [^{14}C]alanyl-tRNA in the presence of increasing amounts of EF-Tu, as indicated on the abscissa. Reaction conditions and determination of [^{14}C]alanyl-tRNA binding were as indicated in *Materials and Methods*. (——) Control ribosomes; (- - - -) cloacin-treated ribosomes.

The observation that ribosomes defective in their 16S rRNA can form 70S initiation complexes does not necessarily imply that they are also able to complete a full round of initiation and thus are able to enter the elongation cycle. The first reaction in this process, namely, binding of [^{14}C]alanyl-tRNA, was found to be decreased to about 50% at all concentrations of EF-Tu studied. Turnowsky and Högenauer (15) reported that E3-treated ribosomes no longer synthesize the R17 RNA-coded dipeptide fMet-Ala, although initiation complexes are formed. These authors inferred that colicin E3 acts by damaging the tRNA-specific binding region of the ribosomal A site. Our experiments with cloacin-treated ribosomes (to be published) show, quite to the contrary, that dipeptide formation does occur in defective ribosomes. The 50% inhibition of [^{14}C]alanyl-tRNA binding may be interpreted in the light of the findings by Benne *et al.* (29). They showed that 70S initiation complexes, which in the absence of IF-1 did not succeed completely in releasing IF-2, also displayed a reduced binding of alanyl-tRNA. Under these conditions the puromycin reaction is not interfered with. Release of IF-2 is necessary before the ternary complex alanyl-tRNA·EF-Tu·GTP can be accommodated in the acceptor site of the ribosome. Such a release requires hydrolysis of GTP and the presence of IF-1. We found the uncoupled IF-2-dependent GTPase activity of cloacin-treated 70S particles unimpaired (not shown). Turnowsky and Högenauer (15) obtained comparable results for EF-T- and EF-G-dependent GTPase. Furthermore, cloacin-treated ribosomes are also able to convert fMet-tRNA into fMet-puromycin.

At the start of this investigation, the fate of the 3′-OH terminal fragment formed upon cleavage of 16S rRNA was obscure. From our results it is clear that this fragment is retained by the ribosome after cloacin treatment and that it does not leave the particle under conditions that normally permit initiation and elongation. Apparently it is the cleavage of the 16S rRNA rather than the loss of the fragment which causes the block of protein synthesis and the aberrations observed during initiation and elongation. The protein S1 remains attached to cloacin-treated ribosomes after the initiation reaction and can be recovered from these particles as a complex with the 49-nucleotide fragment (see ref. 10 and our unpublished results). Furthermore, two-dimensional electrophoresis did not show any difference in protein composition of cloacin-treated and control ribosomes.

It is interesting that the 3′-OH end of the 16S rRNA, which is connected to a number of proteins that are important for initiation, is also linked to IF-1 function. Up to now no crosslinking data were available to show that IF-1 belongs to the protein cluster (S1, S12, IF-2, and IF-3) that has tentatively been located in the 30S ribosomes near the 3′-OH terminus of 16S rRNA.

After submission of this paper we learned of recent studies by Dr. P.-C. Tai (31) with colicin E3-treated ribosomes. In short incubations, R17 RNA-directed amino acid incorporation, binding of fMet-tRNA, and formation of fMet-puromycin were strongly reduced. Moreover, formation of the 30S initiation complex was inhibited. We have seen a similar effect at the 30S level. In addition, formation of 30S initiation complexes is stimulated by IF-1 (Van der Hofstad and Voorma, to be published), and cloacin-treated particles are much less responsive (our unpublished results).

We are much indebted to Dr. F. K. De Graaf, Microbiology Department, Free University of Amsterdam, for his generous gift of purified cloacin DF13. We thank Miss J. Talens for the preparation of purified EF-Tu and Mr. J. M. Gubbens for that of MS2 RNA, MS2 [^{3}H]RNA, and labeled aminoacyl-tRNAs.

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