Communication

Release Factor Binding to Ribosome Requires an Intact 16 S rRNA 3' Terminus*

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SUMMARY

Cloacin DF13 cleavage of Escherichia coli f13HMet-tRNA A U G·ribosome complexes affects this substrate for in vitro peptide chain termination. Codon-directed release factors (RF) 1 and 2 release of f13HMetmethylionine is inhibited by cloacin. Since cloacin inhibits RF1 and -2 binding to ribosomes but not RF-directed f13HMetmethylionine release from f13HMet-tRNA A U G·ribosome complexes when reactions contain 20% ethanol, we conclude that cloacin DF13 inhibits formation of the termination codon recognition complex. Thus, cleavage of the 3'-OH 49-nucleotide sequence of the 16 S rRNA perturbs the codon-directed binding of RF to ribosomes.

The termination of proteins involves two separable partial reactions, terminator codon recognition and peptidyl-tRNA hydrolysis. These reactions require soluble release factors acting in concert with ribosomal functions. The Escherichia coli release factors are protein nucleases which bind to ribosomes with specific codons (RF1, UAA or UAG, and RF2, UAA, or UGA) (1). The mechanism of terminator codon recognition is therefore of particular interest since all other codons are recognized by tRNA species. The details of RF interaction with ribosomal proteins during codon recognition have been studied using antibiotic inhibitors (2), antibodies with specificity for ribosomal proteins (3) and recently by chemical cross-linkage using bifunctional chemical agents (2). We report here that a specific nuclease cleavage of the 16 S rRNA will eliminate RF ribosomal binding. The bacteriocin, cloacin DF13 (cloacin) nuclease, specifically cleaves the 16 S rRNA 49 nucleotides from the 3' OH terminus (4). This nuclease, in its purified state, has no additional nuclease activity and has been used as a specific probe for the study of functions related to the 30 S ribosomal subunit (5, 6). The nucleotide sequence remains in complex with the ribosome under standard conditions (7). NMR studies of the nucleotide fragment reveals no evidence of intramolecular Watson-Crick base pairing under normal physiological conditions (8).

The effect of varying concentrations of cloacin on in vitro peptide chain termination is shown in Fig. 1. The UGA-directed RF2 release of f13HMet from f13HMet-tRNA A U G·ribosome complexes is markedly inhibited by pretreatment of the ribosomal complexes with cloacin. This inactivation curve is similar to that previously reported (4) for cloacin inhibition of protein synthesis and cleavage of 16 S rRNA. The cloacin inhibition of peptide chain termination is observed with RF1 and RF2 and their corresponding terminator codons (Table I). Although the cloacin used in these studies is apparently homogeneous and free of extraneous nuclease activity (5, 6), we examined for cloacin inactivation of the trinucleotide codons.

Trinucleotides were preincubated with cloacin under conditions used in Table I for the ribosomal complexes after which reactions were initiated by addition of untreated ribosomal complexes and RF1 or RF2. Cloacin had little or no effect on the trinucleotides and thus cloacin inhibits peptide chain termination as expected by its action on the ribosomal complexes. Examination of the RNA components associated with these ribosomes following cloacin treatment revealed the appearance of a nucleotide fragment equal in size to the 49-nucleotide fragment previously reported (4) for cloacin. There was no evidence of tRNA or 5 S RNA degradation.

In an effort to define the cloacin site of action, we have examined the partial reactions of peptide chain termination, codon recognition, and peptidyl-tRNA hydrolysis. Release factors interact with ribosomes independent of codon recognition, and peptidyl-tRNA hydrolysis. This partial reaction is not affected by streptomycin or tetracycline, known inhibitors of codon recognition events. Cloacin similarly has no effect on the RF-dependent release of f13HMet or...
The recognition of terminator codons is known to involve the protein release factors RF1 and RF2. These proteins bind to ribosomes with codon specificity (9). Caporcelli and Klein (15) used methods of equilibrium dialysis to study RF codon recognition in the absence of ribosomes. Correlation between oligonucleotide binding and RF codon specificity was observed but was not strict. Collectively, these studies support the concept that protein RF molecules recognize terminator codons directly. Definitive proof of this codon recognition mechanism requires identification of the RF "anticodon." Only preliminary data on RF1 and RF2 structures are available presently. RF1 has a molecular weight of 44,000 to 49,000, while that of RF2 is 47,000 to 50,000 (16). RF1 is free of phosphate and RF2 is free of nucleotides (15). Recently RF1 and RF2 have been found to have common immunologic determinants and thus possess some structural similarities (17).

An alternate model for termination recognition has been described by Shine and Dalgarno (18). They determined the nucleotide of the 3' terminus of 16 S rRNA to be G-G-A-U-C-A-G-C-C-C-C-U-U-A-A, and proposed the 3' terminus of 16 S rRNA may be responsible for codon recognition. They proposed that UAAn could recognize the terminator codons UAA and UAG and that UCA could pair with UCA.

While these reports do not replicate the earlier data on terminator codon recognition or establish a role for their involvement in chain termination, they raise the possibility of an alternate mechanism for terminator codon recognition. Additional information relevant to a base pairing mechanism for termination has been derived from the study of analogue termination trinucleotide codons (19). These and other codon recognition studies indicate RF codon interactions closely resemble the specificities of Watson-Crick base pairing (U exclusive of C, A, or G; and A exclusive of U, C, or G) and also wobble base pairing (A is equivalent to G). The nucleotide sequence of 16 S rRNA from other bacterial species have now been examined (20) and differ from Escherichia coli with respect to their 3'-OH sequences (e.g. Bacillus subtilis, C-U-U-U-C-U).

In studies not shown here, we have examined the codon recognition properties of RF1 and RF2 from E. coli and B. subtilis on their respective and heterologous ribosomes. We observed no qualitative differences to suggest ribosome specificity regarding the terminator codon recognition event.

In these and other studies, we are now probing the ribo-

### Table I

| Reaction additions | Control | Cloacin-treated |
|-------------------|---------|-----------------|
| RF1               | 1.60    | 0.22            |
| RF1 + UAG         | 4.21    | 0.02            |
| RF1 + UAA         | 4.06    | 0.09            |
| RF2               | 2.99    | 0.04            |

**Cloacin inhibition of peptide chain termination**

Cloacin treatment of ribosomal intermediates at 0.29 μg of cloacin/A260 ribosomes and codon-directed RF release of [3H]Met were performed as described in Fig. 1.

### Table II

**Codon-independent RF release of [3H]methionine**

Codon-independent RF release of [3H]Met was accomplished by omitting the terminator trinucleotide, initiating the reaction by the addition of ethanol to a concentration of 20% (v/v), and incubating at 4° for 30 min. Cloacin pretreatment of ribosomal complexes was achieved as in Table I.

### Table III

**Cloacin inhibition of termination codon recognition**

Formation of terminator codon recognition complex was determined by measurement of ribosomal bound [3H]UAA as described by Scolnick and Caskey (12). The [3H]UAA binding assay was incubated for 15 min at 4° and contained in 0.05 ml: 0.10 M Tris-acetate, pH 7.2; 0.02 M magnesium acetate; 0.10 mM ammonium acetate; 17.8 pmol of [3H]UAA, 9.02 Ci/mmol; 20% in ethanol; 1.15 A260 units of *Escherichia coli* B ribosomes; and 35.7 μg of RF1 or 17.8 μg of RF2 preparations, as indicated. The terminator codon recognition complex was collected on nitrocellulose filter (HA Millipore filter, 0.45 μm pore size) as in the tRNA codon recognition of Nirenberg and Leder (13). *E. coli* B ribosomes were pretreated in the presence or absence of cloacin at a level of 0.29 μg/A260 ribosome for 5 min at 37° prior to incubation at 4°.

| Reaction additions | Control ribosomes | Cloacin-treated ribosomes |
|-------------------|-------------------|--------------------------|
| RF1               | 1.60              | 0.22                     |
| RF2               | 1.60              | 0.31                     |

**DISCUSSION**

The formation of terminator codon recognition complexes as shown in Table III. Cloacin cleavage of the ribosome markedly interferes with the mechanism of RF binding to ribosomes in response to codons.

In order to investigate the specificity of cloacin, we have studied aa-tRNA ribosomal binding using poly(U)-directed ribosomal binding of [3H]Phe-tRNA. Cloacin inhibited this reaction in the presence and absence of EF-Tu and GTP (data not shown, and Refs. 13 and 14). These studies indicate cloacin cleavage of the 30 S ribosomal subunit inhibits both RF and aa-tRNA codon-directed ribosomal binding.
somal requirements for peptide chain termination. Cloacin provides a means of nuclease cleavage at a specific site in the E. coli 16 S rRNA structure. Since fMet-tRNA·AUG·ribosomal complexes remained stable and puromycin-reactive following cloacin treatment, we were able to examine the effect of such cleavage on peptide chain termination. Cloacin cleavage of the 49-nucleotide 3′-OH terminus of 16 S rRNA (8) rendered the ribosomes inactive to both codon-directed RF release of fMet and formation of RF·[3H]UAA·ribosome complexes. The RF-dependent release of fMet in reactions containing 20% ethanol was not affected. Since this latter reaction is a measure of the 50 S ribosome's peptidyltransferase participation in the release of nascent chains at peptide chain termination (11), we conclude that cloacin has affected the recognition of termination codons and not inactivated the ribosome to all RF-dependent partial reactions. Others have found cloacin-treated ribosomes to be capable of forming fMet-tRNA·AUG·ribosome complexes (14) which are reactive with puromycin and a second aa-tRNA (4). The inability of cloacin-treated ribosomes to form codon recognition complexes is not unique for RF since aa-tRNA will not bind to these ribosomes in response to synthetic poly- and oligonucleotides. In separate studies which used antibodies directed to specific ribosomal proteins, we have found other ribosomal components which affect both RF and aa-tRNA binding to ribosomes (L7/L12, S9, S11, S2, and S3) (3).

These studies indicate the 3′-terminus of 16 S rRNA, when perturbated by cloacin nicking, renders the ribosome inactive to codon recognition complex formation. Since both aa-tRNA and RF binding are affected, we can assume they have similar ribosomal requirements at the A site which differs from those of fMet-tRNA binding to the P site. It is unclear at this time if the perturbated component(s) critical for this complex formation are rRNA, proteins, or both. It is clear that this 3.2% fraction of the 16 S rRNA affects critical functions of the ribosome such as binding of RF to ribosomes by terminator codons. Further studies are needed to test directly the model of terminator codon recognition suggested by Shine and Dal-garno (18). The preponderence of experimental data still favor the RF as the recognition molecule.

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