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The Leader Peptides of Attenuation-Regulated Chloramphenicol Resistance Genes Inhibit Translational Termination

JOHN G. MOFFAT,[1] WARREN P. TATE,[1] AND PAUL S. LOVETT[2]*

Department of Biochemistry and Centre for Gene Research, University of Otago, Dunedin, New Zealand,[1] and Department of Biological Sciences, University of Maryland, Baltimore County, Catonsville, Maryland 21228[2]

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Placing a translation stop codon at the ribosomal pause site in the leader of the attenuation-regulated *cat-86* gene activates *cat* expression in the absence of the inducer, chloramphenicol. Genetic experiments have shown that this phenomenon depends on the amino acid sequence of the leader-encoded peptide and could readily be explained if the peptide was an inhibitor of translation termination. Here we demonstrate that the *cat-86* leader pentapeptide is an in vitro inhibitor of translation termination in addition to its previously described anti-peptidyltransferase activity.

Translation attenuation is used to regulate several antibiotic resistance genes, including *cat* and *erm* (2, 6, 17). Induction occurs when a ribosome that is translating the leader stalls at a specific site (1). Stalling requires an inducer, which is typically the antibiotic to which the regulated gene confers resistance, and a specific sequence of amino acids in the leader-encoded peptide (7, 11–13). The leader peptide encoded by the *cat-86* gene has been shown to be the selector of the site of ribosome stalling. The inducer, chloramphenicol, possibly stabilizes the peptide-paused ribosome at the induction site, enabling the ribosome to cause localized alterations in RNA secondary structure.

The mechanism that allows the *cat* leader peptide to pause a ribosome has recently been identified. This peptide is an inhibitor of peptidyltransferase, a ribosome-catalyzed activity that forms peptide bonds (3–5). Hence, when a ribosome has translated to the leader induction site, i.e., the aminoacyl site at leader codon 6, that ribosome has simultaneously synthesized a pentapeptide inhibitor of translation.

Replacing *cat-86* leader codon 6 (Lys; AAA) with any of the three translation stop codons causes a sixfold increase in uninduced *cat* expression (14). This phenomenon has been termed autoinduction. Autoinduction is observed only when the amino acid sequence of the leader pentapeptide is the same as that which supports chloramphenicol induction of *cat* expression, arguing that autoinduction requires a function of the peptide (14). Genetic studies of autoinduction have suggested a model in which the leader-encoded peptide is expected to be an inhibitor of translation termination (Fig. 1).

Translation termination requires a stop codon and a decoding molecule, which is polypeptide chain release factor (RF). The RF protein binds to the ribosome in response to a stop codon in the ribosomal A site and modifies the activity of the peptidyltransferase center so that the P site peptidyl-tRNA linkage is hydrolyzed rather than transferred to an incoming aminoacyl-tRNA (8, 15). Nascent peptides could interfere with termination by modifying the RF binding site, which includes components on the 30S and 50S subunits, or preventing RF from appropriately modifying the peptidyltransferase center. Lastly, nascent peptide could directly inhibit the peptidyl-tRNA hydrolysis reaction.

To test these possibilities, two peptidyltransferase-inhibiting peptides, MVKTD and MKNTD (4, 5), were assayed for their effects on polypeptide chain release. Just as f[^{3}H]Met-tRNA is a model for peptide bond formation (9, 10), so the release of free f[^{3}H]Met from f[^{3}H]Met-tRNA is an in vitro model for termination (16). The termination reaction requires 70S subunits and RF, which switches peptidyltransferase from bond-forming activity to hydrolysis activity in the presence of either a stop codon trinucleotide or ethanol. *Escherichia coli* ribosomes (5 pmol) were preincubated at 4°C for 10 min with one of the inhibitor peptides or reverse-mers prior to the addition of f[^{3}H]Met-tRNA (2.5 pmol), ethanol to 20%, and either puromycin (20 μM) or *E. coli* RF-1 protein (5 μg/ml) in a reaction volume of 50 μl. Reactions containing puromycin were incubated for 30 min at 4°C and extracted with ethyl acetate to assay for peptidyltransferase activity. Reactions containing RF-1 were incubated for 60 min at 4°C and extracted to assay for RF-mediated termination (16). Under these reaction conditions, MVKTD and MKNTD were effective inhibitors of peptidyltransferase as well as RF-mediated termination (Fig. 2). Reverse-mers failed to significantly inhibit either reaction (Fig. 2).

To test the effect of peptides on the binding of f[^{3}H]Met-tRNA to the ribosomal P site, mixtures of ribosome, peptide, and f[^{3}H]Met-tRNA prepared as described above were incubated without the addition of RF or puromycin; complex formation was assayed by radioactivity retained on glass fiber filters. Under conditions in which peptide bond formation and peptide release were nearly abolished by MVKTD and MKNTD (Fig. 2), the formation of the substrate complex was

TABLE 1. Effects of MVKTD and MKNTD peptides on the binding of f[^{3}H]Met-tRNA to *E. coli* 70S ribosomes

Peptide	% f[^{3}H]Met-tRNA bound ± SD[a]
MVKTD	74 ± 9
DTKVM	105 ± 15
MKNTD	48 ± 15
DTNKM	91 ± 14

[a] Data are averages of at least three separate determinations.

* Corresponding author.

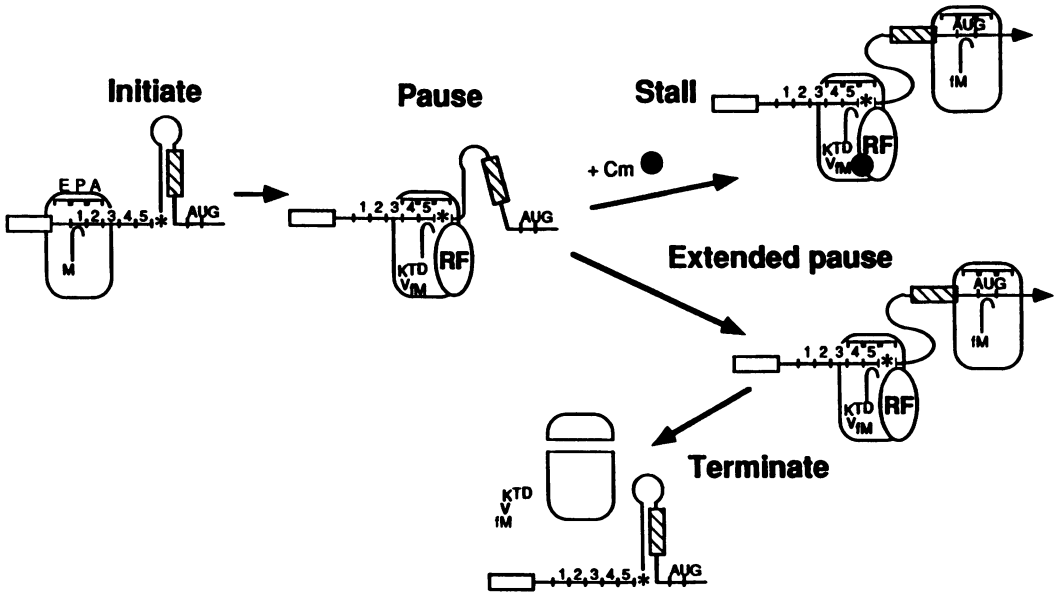

FIG. 1. Model for the role of the *cat-86* leader pentapeptide in the autoinduction of expression. It is proposed that the nascent 5-mer peptide MVKTD causes a translational pause at leader codon 6 by transiently *cis* inhibiting the peptidyltransferase component of the translating ribosome. The addition of chloramphenicol (3) is believed to convert the paused ribosome to a stalled condition. When leader codon 6 is a translation stop codon (13), the termination that normally occurs at this codon is converted to an extended pause because of the antitermination properties of MVKTD. In both cases, activation of *cat* expression occurs when the ribosome occupying leader codon 6 destabilizes the adjacent stem-loop structure, allowing translation initiation of the *cat* coding sequence. Leader codons 1 through 5 are sequentially numbered. E, P, and A, the exit, peptidyl, and aminoacyl sites of the ribosome, respectively. *, position of the introduced stop codon at leader codon 6.

only partially inhibited (Table 1). MVKTD inhibited substrate complex formation by 26%, and inhibition by MKNTD was 52%.

Stop codon-induced RF binding to the ribosome, the first step in the termination reaction, can also be assayed separately by the incorporation of ^{3}H-labelled stop codon trinucleotide into a ribosomal complex (16). A 10-min preincubation of ribosomes (12 pmol) with 2 or 5 mM peptides had no effect on RF-2-dependent binding of ^{3}H-UGA to ribosomes as measured by the retention of radioactivity on glass fiber filters.

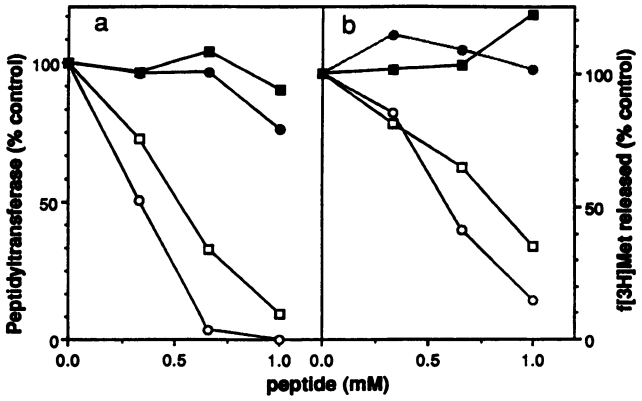

FIG. 2. Effects of MVKTD (open squares) and MKNTD (open circles) on peptide bond formation and peptide release. Peptides were preincubated with ribosomes and then diluted into reaction cocktails containing either puromycin (a) or RF-1 (b). Concentrations are final peptide concentrations in 50-μl reaction cocktails. DTKVM, solid squares; DTNKM, solid circles.

Therefore, peptide inhibition of translation termination cannot be fully accounted for by peptide inhibition of peptidyl-tRNA binding to the ribosomal P site nor by an effect on RF stop codon recognition and binding. Therefore, we suggest that MVKTD and MKNTD negatively affect RF-dependent peptidyl-tRNA hydrolysis.

Our results are consistent with a model for autoinduction (Fig. 1) in which nascent peptide interferes with termination by interacting with the peptidyltransferase center, resulting in a significantly longer pause than the one a ribosome normally makes at a stop codon. It is conceivable that similar ribosomal target sites are involved in peptide inhibition of peptidyltransferase and polypeptide release.

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