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Peptidyl Transferase Inhibition by the Nascent Leader Peptide of an Inducible *cat* Gene

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The site of ribosome stalling in the leader of *cat* transcripts is critical to induction of downstream translation. Site-specific stalling requires translation of the first five leader codons and the presence of chloramphenicol, a sequence-independent inhibitor of ribosome elongation. We demonstrate in this report that a synthetic peptide (the 5-mer) corresponding to the N-terminal five codons of the *cat-86* leader inhibits peptidyl transferase in vitro. The N-terminal 2-, 3-, and 4-mers and the reverse 5-mer (reverse amino acid sequence of the 5-mer) are virtually without effect on peptidyl transferase. A missense mutation in the *cat-86* leader that abolishes induction in vivo corresponds to an amino acid replacement in the 5-mer that completely relieves peptidyl transferase inhibition. In contrast, a missense mutation that does not interfere with in vivo induction corresponds to an amino acid replacement in the 5-mer that does not significantly alter peptidyl transferase inhibition. Our results suggest that peptidyl transferase inhibition by the nascent *cat-86* 5-mer peptide may be the primary determinant of the site of ribosome stalling in the leader. A model based on this concept can explain the site specificity of ribosome stalling as well as the response of induction to very low levels of the antibiotic inducer.

Translation attenuation, a variation of the transcription attenuation model, regulates the inducible translation of the antibiotic resistance genes *cat* and *erm* (5, 8, 13, 15). The inducers are the antibiotics to which the genes confer resistance, chloramphenicol and erythromycin, respectively. Induction results from the stalling of a ribosome at a highly specific site in the leader region of the transcripts. This stall site places the ribosome at the correct location to destabilize downstream secondary structure and activate translation of the drug resistance gene (11).

The inducers of *cat* and *erm* genes are sequence-independent inhibitors of ribosome elongation, yet induction depends on site-specific ribosome stalling. This apparent contradiction was resolved by the finding that within the *cat-86* leader there exists a signal that determines the site of ribosome stalling. This signal is specified by leader codons 2 through 5, the *crb* sequence (23), and enables chloramphenicol to stall a ribosome with its aminoacyl site at leader codon 6 (Fig. 1). In a search for the basis of the stalling specificity, we observed that *crb* is largely complementary to a sequence in 16S rRNA of *Bacillus subtilis* (22). While base pair formation between *crb* and rRNA may contribute to the specificity of stalling, four synonymous codon changes within *crb* reduced by less than half the inducibility of *cat-86* by chloramphenicol (22). Since these codon changes virtually eliminated the proposed *crb*–anti-*crb* pairing, it is evident that some other aspect of *crb* must be the primary determinant of stalling specificity during chloramphenicol induction.

Missense mutations in *crb* which should have little effect on the pairing of leader mRNA with 16S rRNA can abolish induction (4). Moreover, a change-of-frame mutation 5′ to *crb* prevents induction, whereas a similar mutation immediately 3′ to *crb* does not (23). Given these findings, it seemed likely that site-specific ribosome stalling would depend on an activity of the leader-encoded peptide. We here demonstrate that the *cat-86* nascent leader peptide inhibits a ribosome activity necessary for elongation.

MATERIALS AND METHODS

Peptidyl transferase. The fragment reaction was used to assay peptidyl transferase activity (19). Reaction mixes consisted of 60 mM Tris-HCl (pH 7.4), 400 mM KCl, 20 mM magnesium sulfate, and 1 mM neutralized puromycin in 50 μl, to which was added 25 μl of absolute ethanol. Unless specifically noted, 70S ribosomes purified from *B. subtilis* BR151 as described by Spedding (25) were added to 0.55 μM. ^{35}S-labeled *N*-formylmethionine, charged to tRNA and digested with endonuclease T1 as described by Marcker (16), was added to each reaction at 1×10^6 to 2×10^6 cpm. Analysis of these preparations by using high-voltage paper electrophoresis demonstrated that about one-half of the radioactivity was in the *N*-formylmethionine–T1 fragment, with the remainder distributed between free methionine and *N*-formylmethionine.

Peptide inhibitors were typically preincubated with the ribosomes prior to addition to the other reactants, as noted in the text. Antibiotic inhibitors such as chloramphenicol were added to reaction mixes with the ribosomes but without the preincubation step. Preincubation of ribosomes with chloramphenicol failed to increase its inhibitory effect on peptidyl transferase, whereas peptide inhibition was greatly enhanced by the preincubation step. Peptidyl transferase reaction mixes were incubated on ice for 1 h, except where noted otherwise, and reactions were terminated by addition of 50 μl of 0.3 M sodium acetate saturated with magnesium sulfate followed by 1 ml of ethyl acetate. The ethyl acetate phase was counted for radioactivity. All peptidyl transferase assays were performed in duplicate or triplicate, and the replicates varied by less than 5%. In separate experiments, the inhibitory activity of a single peptide preparation on a single ribosome preparation varied by as much as 10%. The

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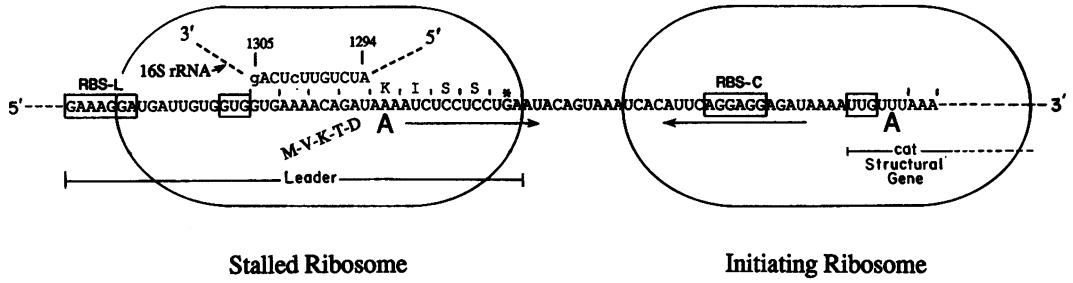

FIG. 1. Positions of the stalled and initiating ribosomes on *cat-86* mRNA during chloramphenicol induction. The aminoacyl site (A) of the stalled ribosome occupies leader codon 6 (1). MVKTD is the leader translation product whose in vivo synthesis is concomitant with delivery of the ribosome to the induction site. The 14-bp inverted repeats (horizontal arrows) are shown in linear form thought to result from stem-loop destabilization by the stalled ribosome. RBS-L and RBS-C are the leader and coding sequence ribosome binding sites. The *crb* sequence in the leader, codons 2 through 5, is shown base paired to nucleotides 1294 to 1305 of *B. subtilis* 16S rRNA. The boxed GUG and UUG are the initiation codons for the leader and coding sequences.

inhibitory activity associated with a single preparation of 5-mer peptide varied somewhat when assayed on different ribosome preparations (see text), whereas different preparations of 5-mer peptide showed similar inhibitory activities (±10%) when assayed on a single ribosome preparation.

Peptides. Peptides were synthesized by the University of Maryland Core Facility; Biosynthesis, Inc., Lewisville, Tex.; or Research Genetics, Huntsville, Ala., with the exception of the dipeptide MV, which was purchased from Sigma. As obtained, the peptides were greater than 80% homogeneous. All peptides were purified further by reverse-phase high-pressure liquid chromatography, using as the solvent 20% methanol–0.25% acetic acid in water. Purified peptides were lyophilized, and stock solutions were prepared in ribosome buffer (25). Selected peptides were analyzed either by N-terminal sequencing or by determining total amino acid composition. In each case, the results obtained were consistent with the reported amino acid sequence.

RESULTS

Induction of *cat-86* by chloramphenicol requires translation of the first five leader codons (Fig. 1) (1, 7). If the leader N-terminal pentapeptide (the 5-mer) paused the translating ribosome at the induction site, this would increase the probability that upon addition of chloramphenicol, the antibiotic would rapidly contact ribosomes poised for induction. In this model, the nascent peptide would interfere with elongation by the ribosome that has just completed its translation. Therefore, inhibition in vivo should be an intramolecular event. To test the model, it was assumed that the inhibition could be detected in a bimolecular reaction. We directed our studies to peptidyl transferase, since this activity is the site of action of several ribosomally targeted antibiotics (18).

Peptidyl transferase. Peptidyl transferase is a catalytic activity present within the large subunit of ribosomes (18). The activity has been associated with 23S rRNA and is the target for inhibition by the antibiotic chloramphenicol (18, 20). Peptidyl transferase catalyzes peptide bond formation during translation and can be quantitatively assayed by the fragment reaction which measures the in vitro transfer of *N*-formylmethionine from an endonuclease T1 fragment of tRNA to puromycin. The reaction product, *N*-formylmethionine–puromycin, phase extracts with ethyl acetate. Figures 2 and 3 show a time course for the reaction and the effects of three antibiotics on the activity. Chloramphenicol and lincomycin are inhibitors of peptidyl transferase, whereas erythromycin, which also binds to the 50S ribosome subunit, does not inhibit the reaction.

Effects of N-terminal peptides of the *cat-86* leader on peptidyl transferase. Ribosomes were preincubated for 10 min with the *cat-86* leader 4-mer MVKT and the 5-mer MVKTD. The preincubation mixes were diluted into reaction cocktails to assay for residual peptidyl transferase activity (Fig. 4). Under these conditions, the 4-mer failed to significantly inhibit the reaction, whereas the 5-mer was an effective inhibitor. To determine whether inhibition by the 5-mer was sequence specific, the five-residue peptide MVKTD was synthesized in reverse amino acid sequence, DTKVM. This reverse 5-mer had no detectable inhibitory activity toward peptidyl transferase under conditions in which the 5-mer reduced the activity by up to 93%.

The time of preincubation of ribosomes with peptide influenced the extent of inhibition by the 4- and 5-mers (Fig. 5). However, even prolonged preincubation of ribosomes with the reverse 5-mer failed to significantly inhibit peptidyl transferase. From these data, we infer that 5-mer inhibition depends on a specific sequence of amino acids, or types of amino acids, in the peptide and that this sequence is absent in the reverse orientation.

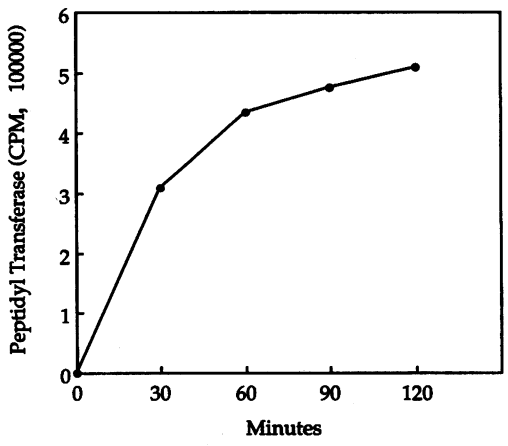

FIG. 2. Time course of the peptidyl transferase reaction. Duplicate reaction mixes were incubated on ice and sampled periodically for the conversion of puromycin to formylmethionine-puromycin by phase extraction with ethyl acetate.

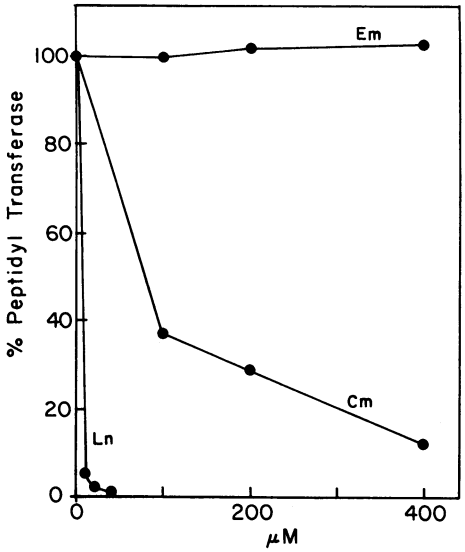

FIG. 3. Effects of chloramphenicol, lincomycin, and erythromycin on peptidyl transferase. The antibiotics were added to reaction cocktails with the ribosomes. Incubation was for 1 h on ice. The 100% value represents the radioactivity extracted from an uninhibited reaction, which was 467,000 cpm.

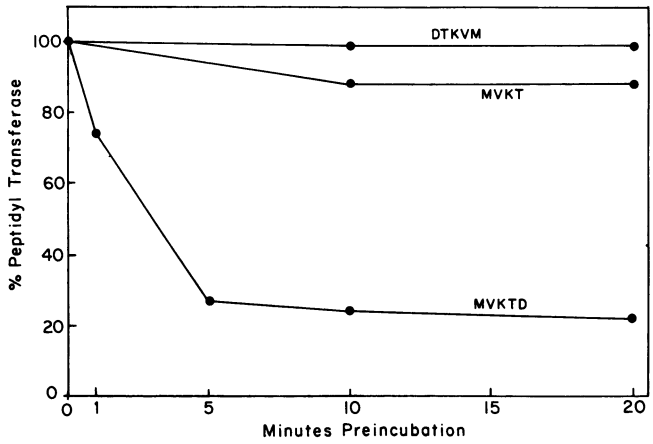

FIG. 5. Effects of varying the time of peptide-ribosome preincubation on peptide inhibition of peptidyl transferase. Experiments were performed as described in the legend to Fig. 4 with the exception that peptide-ribosome preincubation ranged from 0 to 20 min and was performed at concentrations of 14 mM peptide and 4 μM ribosomes. After preincubation, the peptide-ribosome mix was diluted into the 50-μl reaction cocktail, to which was added 25 μl of ethanol. Peptide concentration in the 50-μl reaction cocktail was 2 mM. Peptidyl transferase was assayed after a 1-h incubation of reactants on ice.

Figure 6 summarizes the results of an experiment in which truncated synthetic versions of the *cat-86* leader peptide were tested for inhibition of peptidyl transferase. No significant inhibition was observed with free methionine or with the N-terminal di-, tri-, or tetrapeptides. In contrast, the 5-mer peptide reduced peptidyl transferase activity by 88%. Pentamer inhibition of peptidyl transferase was found to vary in different experiments from 75 to 92% when the peptide was used at 2 mM. The variability correlated with the use of different ribosome preparations.

Effects of variations of the 5-mer on peptidyl transferase inhibition. Missense mutations in the *cat-86* leader can have very different effects on induction depending on the nature of

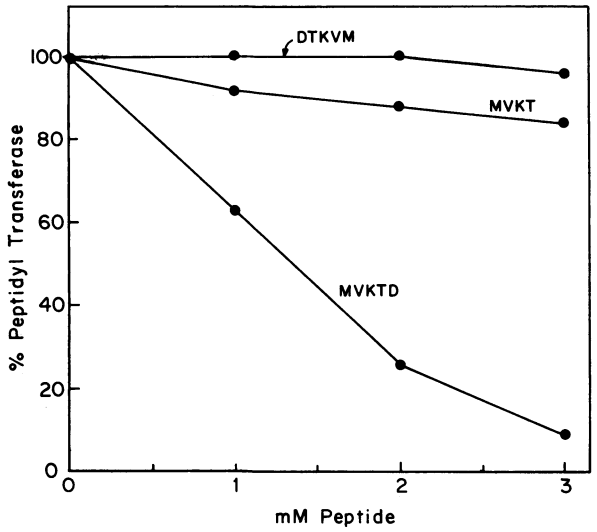

FIG. 4. Effects of peptides MVKT, MVKTD, and DTKVM on peptidyl transferase activity. Peptides were preincubated with ribosomes for 10 min. The peptide-ribosome mixes were diluted into peptidyl transferase reaction cocktails, and phase extraction was performed after a 1-h incubation on ice. Preincubation was performed in 7 μl with peptides at 7, 14, or 21 mM and ribosomes at 4 μM. The concentrations shown represent the final peptide concentrations in the 50-μl aqueous portion of the peptidyl transferase reaction cocktail.

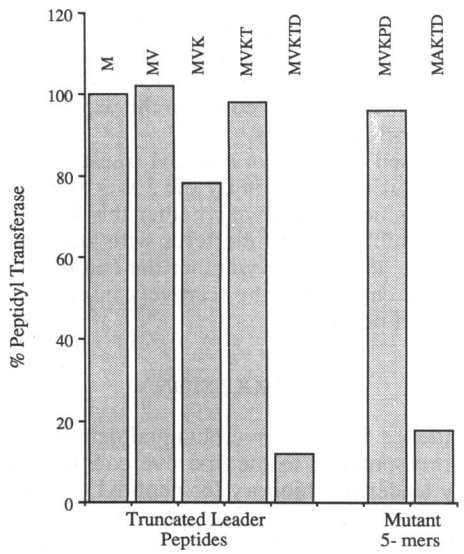

FIG. 6. Effects of peptides of the *cat-86* leader on the peptidyl transferase reaction of *B. subtilis* ribosomes. The entire *cat-86* leader is predicted by the DNA sequence to consist of nine amino acids, MVKTDKISS. Peptides or free methionine (14 mM) were preincubated with ribosomes (4 μM) in 7 μl for 10 min and then diluted into peptidyl transferase reaction cocktails to final concentrations of 2 mM and 0.55 μM, respectively. Incubation for 1 h on ice was followed by phase extraction. Mutant 5-mers correspond to peptides that would be specified by versions of the leader containing missense mutations.

TABLE 1. Peptidyl transferase of dissociated ribosomal subunits and response to the 5-mer peptide[a]

Ribosome subunit and peptide	% Activity
30S, no peptide	0
50S, no peptide	100
50S + MVKT (0.5 mM)	101
50S + MVKT (1.0 mM)	92
50S + MVKT (2.0 mM)	85
50S + MVKTD (0.5 mM)	56
50S + MVKTD (1.0 mM)	2
50S + MVKTD (2.0 mM)	0
50S + DTKVM (0.5 mM)	82
50S + DTKVM (1.0 mM)	82
50S + DTKVM (2.0 mM)	78

[a] Experiments were performed as for Fig. 4 but using 30S or 50S ribosomal subunits in place of the 70S ribosomes. The data are compiled from two separate experiments in which the incorporation of radioactivity into the uninhibited (50S) control was 250,000 and 218,000 cpm.

the mutation. Replacing leader codon 4 (ACA) with a Pro (CCA) codon abolishes induction by chloramphenicol, whereas replacement of leader codon 2 (GTG) with an Ala codon (21) does not detectably interfere with induction. Peptides with these amino acid substitutions showed different activities toward peptidyl transferase (Fig. 6). The Ala substitution had no significant effect on 5-mer inhibition, while the Pro substitution relieved essentially all inhibition.

The 50S subunit contains the target for the 5-mer peptide. Peptidyl transferase activity resides in the 50S subunit of bacterial ribosomes (19, 20). To determine whether 5-mer inhibition was due to direct interaction with the catalytic (50S) subunit or required the 30S portion, ribosomes were dissociated (25) and the 30S and 50S subunits were individually tested for peptidyl transferase and its response to the 5-mer (Table 1). All peptidyl transferase activity of the dissociated ribosomes was due to the 50S subunits, and incubation of the 50S subunits with the 5-mer peptide resulted in inhibition that was approximately twice that observed with the 70S ribosomes. However, the 4-mer and the reverse 5-mer had little effect on the peptidyl transferase activity of the 50S subunits.

The increased response of peptidyl transferase activity of the 50S subunit to inhibition by the 5-mer was consistently observed. It is not yet known whether this finding indicates that the 30S subunit itself interferes with attachment of the peptide to the ribosome or whether the conformation of the 70S ribosome causes it to be relatively insensitive to exogenously added peptide.

DISCUSSION

The evidence that we have obtained demonstrates that a peptide corresponding to the first five codons of the *cat-86* regulatory leader is inhibitory for peptidyl transferase. Peptides corresponding to the first two, three, or four leader codons show no significant inhibitory activity. Thus, when a ribosome has translated to the leader site necessary for induction, it has simultaneously synthesized an inhibitor of ribosome elongation. We presume that in vivo, the nascent 5-mer promotes stalling specificity by causing a ribosome to pause at the induction site. Conversion of the paused state to a stalled condition or perhaps to a dead-end complex (21) might be a function of chloramphenicol. This model can explain the site specificity of ribosome stalling and provides a likely explanation for the sensitivity of induction to very low levels of the antibiotic inducer. Ribosome pausing in the leader, dictated by the activity of the 5-mer, should result in ribosomes preferentially occupying the induction site. By this reasoning, ribosomes in the leader that are contacted by chloramphenicol will preferentially be those poised for induction. Our results do not eliminate the alternative explanation that the 5-mer blocks ribosome elongation only after movement of the translating ribosome is slowed by chloramphenicol. However, if this is the case, it offers no explanation for the elevated sensitivity to chloramphenicol of the induction mechanism relative to overall protein synthesis inhibition by the antibiotic. Clearly, both explanations suppose that the 5-mer acts in *cis* on the ribosome that has just completed its translation.

The activity of the 5-mer against peptidyl transferase does not in itself indicate that the peptide directly contacts the enzyme (or ribozyme [20]). Other sites of binding on the 50S component of the ribosome could result in conformational changes that indirectly alter peptidyl transferase function. Moreover, it is formally possible that the primary effect of the 5-mer in vivo results from inhibition of a ribosome activity other than peptidyl transferase, with the effect on peptidyl transferase as a peripheral consequence. However, in the absence of data to support these alternatives, we tentatively assume that the peptide is targeted to peptidyl transferase.

Previous studies of several examples of transcription attenuation offer no indication that the leader peptide has a function that is essential to the regulation (14). The role of the leader seems limited to providing a sequence that allows a ribosome to translate through a potential stall site, with stalling being dependent on the absence of a metabolite. In contrast, genes regulated by translation attenuation respond to inducers that block ribosome elongation but not in the sequence-dependent manner that is clearly necessary for the regulation. Previously reported genetic evidence (15) has argued for a function of the *cat-86* leader peptide in the regulation, and our present findings appear to identify the biological function of the peptide as the determinant of the site of ribosome stalling. Other regulated *cat* genes show similarity to *cat-86* with respect to the sequence and spatial organization of the leader (2, 3, 9, 12, 24). We therefore suspect that the observations made with the *cat-86* 5-mer peptide may apply to the leader pentapeptides of other inducible *cat* genes. Indeed, genetic studies suggest a role for the *erm* leader peptide in erythromycin-inducible regulation (6, 17), and it will be of interest to determine whether the *erm* peptides make a contribution to erythromycin-inducible regulation similar to the one that we propose for the *cat-86* leader peptide.

The proposal for ribosome regulation by a nascent peptide is not without precedent. The nascent N-terminal tetrapeptide of β-tubulin has been shown to influence the activity of the translating ribosome, determining whether the ribosome continues translation or degrades the mRNA (10). Similarly, ribosome hopping during the translation of the topoisomerase gene of bacteriophage T4 depends on a function of the nascent peptide upstream from the site of the hop (26). Observation of the effects of nascent peptides on translation may be limited to situations in which the peptide contributes to controlling gene expression in an obvious manner. Whether nascent peptides can, in general, communicate with the translating ribosome to cause subtle changes in translation rates is difficult to determine. However, the inhibition of a known ribosome-associated, catalytic activity by the *cat-86* leader peptide provides one biochemical mechanism

through which a nascent peptide can modify the function of the translating ribosome.

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