*Proc. Natl. Acad. Sci. USA*
Vol. 91, pp. 5612–5616, June 1994
Biochemistry

# Anti-peptidyl transferase leader peptides of attenuation-regulated chloramphenicol-resistance genes

(*cmlA*/*cat*/translation/ribosome stalling/23S rRNA)

ZHIPING GU, ROBERT HARROD, ELIZABETH J. ROGERS, AND PAUL S. LOVETT*

Department of Biological Sciences, University of Maryland Baltimore County, Catonsville, MD 21228

*Communicated by Charles Yanofsky, February 24, 1994 (received for review January 4, 1994)*

**ABSTRACT** **The chloramphenicol (Cm)-inducible *cmlA* gene of Tn*1696* specifies nonenzymatic resistance to Cm and is regulated by attenuation. The first eight codons of the leader specify a peptide that inhibits peptidyl transferase *in vitro*. Functionally similar, but less inhibitory, peptides are encoded by the leaders of Cm-inducible *cat* genes. However, the *cat* and *cmlA* coding sequences are unrelated and specify proteins of unrelated function. The inhibition of peptidyl transferase by the leader peptides is additive with that of Cm. Erythromycin competes with the inhibitory action of the peptides, and erythromycin and the peptides footprint to overlapping sites at the peptidyl transferase center of 23S rRNA. It is proposed that translation of the *cmlA* and *cat* leaders transiently pauses upon synthesis of the inhibitor peptides. The predicted site of pausing is identical to the leader site where long-term occupancy by a ribosome (ribosome stalling) will activate downstream gene expression. We therefore propose the inducer, Cm, converts a peptide-paused ribosome to the stalled state. We discuss the idea that cooperativity between leader peptide and inducer is necessary for ribosome stalling and may link the activation of a specific drug-resistance gene with a particular antibiotic.**

Translation attenuation regulates the antibiotic-inducible expression of several antibiotic-resistance genes (1–3). In this form of gene control, the antibiotic to which a gene confers resistance is also the inducer of gene expression. Gene activation results when a ribosome becomes stalled at a unique site in the leader region of the regulated transcripts (2). Stalling at this site alters the adjacent secondary structure, permitting translation of the downstream coding sequence (4–6). The antibiotic inducers for attenuation-regulated *cat* and *erm* genes, chloramphenicol (Cm) and erythromycin (Em), block translation at apparently random mRNA sites. Site specificity of ribosome stalling in the leaders is therefore not determined by the inducer but by the sequence of the leader (7).

Studies by Gu *et al.* (8) suggest the peptide product of the leader determines the site of ribosome stalling for a gene regulated by translation attenuation. The first five codons of the leader for the Cm-inducible, Cm acetyltransferase gene *cat-86* specify a peptide that inhibits peptidyl transferase (PT) *in vitro*. Truncated peptides and the reverse-mer are not inhibitory. A missense mutation in the leader that is known to block Cm induction of *cat* genes corresponds to an amino acid substitution in the pentapeptide that abolishes its anti-PT activity. The leader pentapeptides for two other regulated *cat* genes, *cat-194* and *cat-112/221*, are also inhibitors of PT and truncated versions are not (data not shown).

A ribosome occupying the induction site in the *cat-86* leader has completed translation of the first five leader codons (9, 10). It was therefore proposed that the leader pentapeptide cis inhibits PT, thereby pausing the translating ribosome at the induction site. This effect would increase the probability that Cm will encounter ribosomes in the leader that are poised for induction. Here we demonstrate that a truncated form of the leader peptide for a Cm-inducible gene that is unrelated to *cat* is also a highly effective *in vitro* inhibitor of PT (11).

## MATERIALS AND METHODS

**PT Assays.** The fragment reaction was used to measure PT activity (11). Assays were performed on ice for 1 hr as described (8); replicates varied by <5%. Unless noted, *Bacillus subtilis* 50S ribosomes, 0.1–0.2 $OD_{260}$ unit per reaction, were used as source of PT. Ribosomes were isolated (12) from logarithmic phase cells of *B. subtilis* BR151 and *Escherichia coli* DH5αF′ grown at 37°C in penassay broth and *Thermus aquaticus* ATCC 25104 grown at 70°C in Castenholz medium as modified by Noller *et al.* (13).

To quantitatively measure inhibition by a peptide, the peptide was preincubated with ribosomes for 10 min on ice as described (8). The ribosome/peptide mixture (7 μl) was then added to a reaction cocktail to initiate the reaction. Concentrations of all reactants are reported on the basis of the 50-μl aqueous portion of the reaction cocktails (8).

**Footprinting with Dimethyl Sulfate (DMS).** Methods used were taken from Christiansen *et al.* (14) substituting *B. subtilis* 70S ribosomes for *E. coli* ribosomes. Primer extensions were performed on the rRNA phenol extracted from treated ribosomes using avian myeloblastosis virus reverse transcriptase and a 20-mer primer complementary to nt 2142 through 2161 of 23S rRNA.

## RESULTS

**A Truncated Version of the *cmlA* Leader Peptide Is a PT Inhibitor.** The *cmlA* gene specifies nonenzymatic resistance to Cm in *Pseudomonas aeruginosa* and *E. coli* and encodes an apparent membrane protein that likely alters antibiotic transport (15–18). *cmlA* expression depends on Cm-inducible attenuation regulation probably comparable to that controlling the expression of *cat* genes from Gram-positive bacteria (refs. 2, 19, 20; Fig. 1). Substituting the first seven, eight, or nine codons of the *cmlA* leader for the first five codons of the *cat-86* leader demonstrated that only the first eight codons of the *cmlA* leader permitted Cm induction of the hybrid gene (E.J.R., unpublished data). This observation predicted that the *cmlA* 8-mer leader peptide, MSTSKNAD, would be inhibitory for PT (8). We observed that the eight-residue leader peptide was an effective inhibitor of PT using 50S ribosomal subunits from either *B. subtilis* or *E. coli* (Fig. 2). The reverse sequence of the 8-mer, DANKSTSM, was not

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: Cm, chloramphenicol; DMS, dimethyl sulfate; Em, erythromycin; PT, peptidyl transferase.

*To whom reprint requests should be addressed.

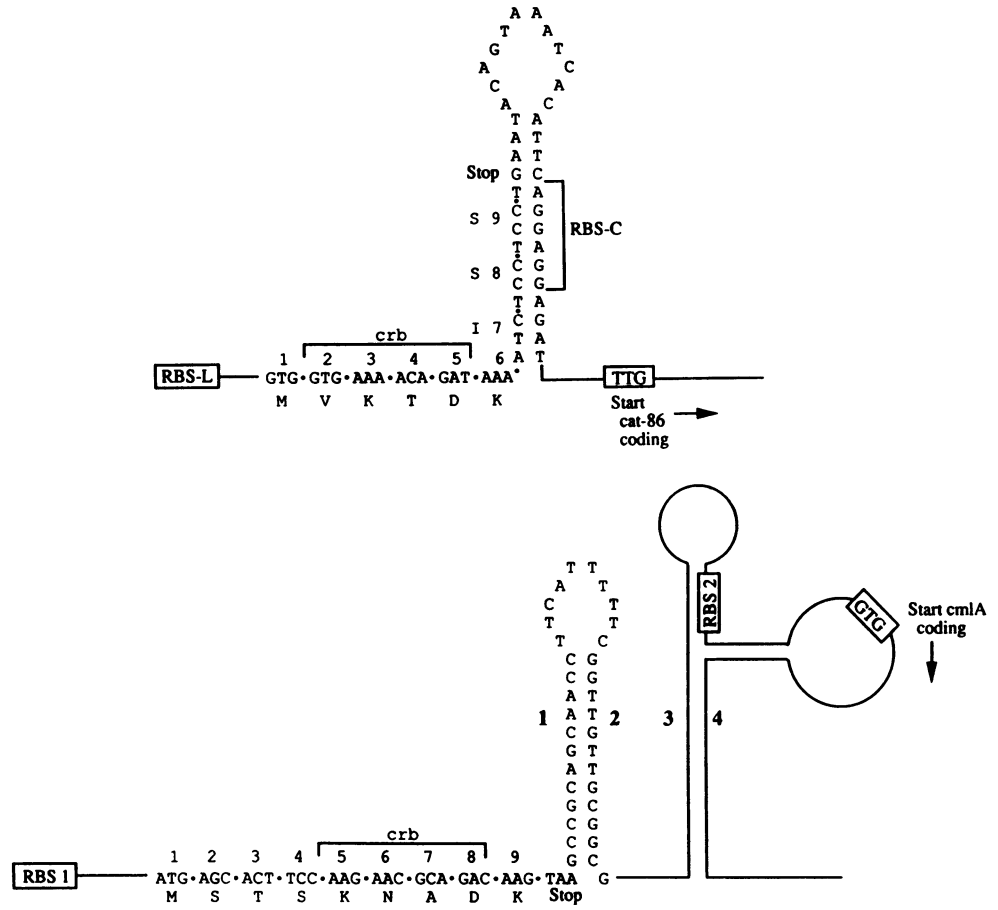


FIG. 1. The 5′ regulatory sequences in *cat-86* and *cmlA* transcripts. Both genes are induced by Cm and possess a *crb* domain in the leader. The complete sequences of the genes have been reported (20, 21).

inhibitory nor were the 6, 7-, and 9-mer *cmlA* peptides (Fig. 2). The *cmlA* 8-mer peptide was about 5-fold more inhibitory than the *cat-86* 5-mer (MVKTD) on 50S ribosome subunits from *B. subtilis* and *E. coli* (ref. 8; data not shown).

Removal of one or more amino acids from the N terminus of the 8-mer abolished anti-PT activity as did substituting Pro for Ala-7 and Gly for Asp-8 (Table 1). Equivalent changes made in the *cat-86* MVKTD sequence produced similar results (Table 1). Collectively, the data indicate that for each peptide there is a minimum size that is necessary for inhibitory activity, consisting of either eight or five residues and in neither case does the substitution of Pro for the penultimate residue or Gly for the C-terminal Asp allow the peptides to inhibit PT.

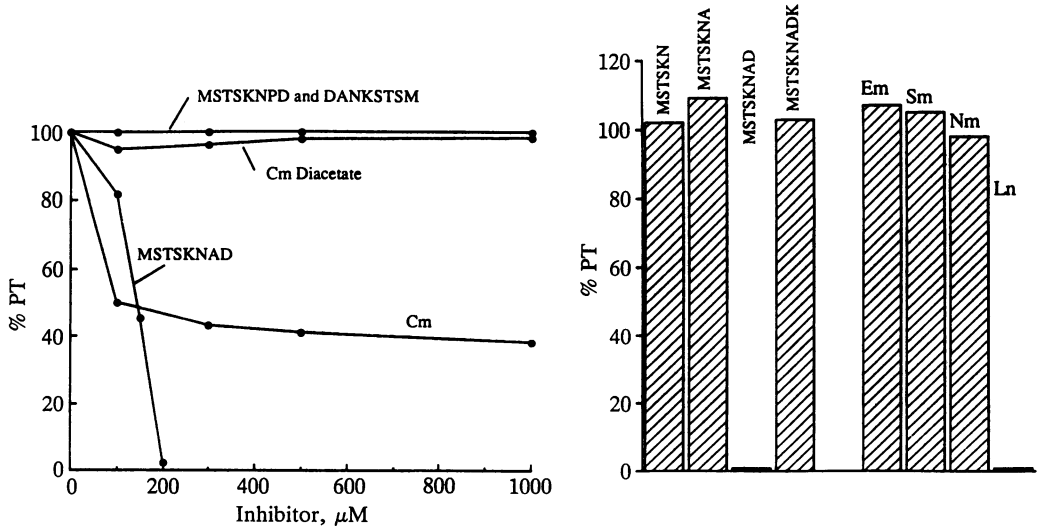


FIG. 2. PT inhibition by the *cmlA* 8-mer peptide. (*Left*) Dose–response curves of peptides and antibiotics on *B. subtilis* 50S ribosomes. Identical results were obtained using *E. coli* 50S ribosomes. (*Right*) Effects of increasing lengths of the *cmlA* leader peptide on inhibition. The 6-, 7-, and 9-mers were tested at 1000 μM and the 8-mer was tested at 200 μM. Em, streptomycin (Sm), and neomycin (Nm) were tested at 200 μM and lincomycin (Ln) was tested at 50 μM.

Table 1. Summary of the effects of variants of the 8-mer and 5-mer peptides on PT inhibition

| Peptide | % inhibition of PT |
|---|---|
| 8-mer | |
| MSTSKNAD | 98 |
| STSKNAD | 2 |
| TSKNAD | 1 |
| SKNAD | 4 |
| SSTSKNAD | 19 |
| MSTSKNAG | 0 |
| MSTSKNPD | 1 |
| MSTSKNADK | 1 |
| 5-mer | |
| MVKTD | 88 |
| VKTD | 4 |
| SVKTD | 80 |
| MVKTG | 6 |
| MVKPD | 2 |
| MVKTDK | 91 |
| MVKTDKI | 89 |
| MVKTDKISS | 89 |

Variations of the 8-mer and 5-mer peptides were tested at 250 μM and 800 μM, respectively, using *B. subtilis* 50S ribosomes. The peptide SSTSKNAD was inhibitory for PT, although the concentration required for >95% inhibition was 4-fold higher than MSTSKNAD.

Notable differences between the 8- and 5-mer peptides were also observed. Converting the *cmlA* 8-mer to a 9-mer by the C-terminal addition of Lys abolished the inhibitory activity (Fig. 2 and Table 1). Lys is the product of the ninth, and final, *cmlA* leader codon (Fig. 1). This striking change in peptide activity brought about by adding a single residue could reflect the altered charge of the peptide, or its pattern of folding. This observation may be significant to the biology of the system, since it predicts that the version of the *cmlA* leader peptide released into the cytoplasm upon complete translation of the leader will not interfere with general protein synthesis. In contrast, addition to the C terminus of the *cat-86* 5-mer those amino acids specified by the last four codons of the leader, Lys, Ile, and two Ser residues, did not alter inhibition (Table 1).

**PT Extracted by Phenol from *T. aquaticus* Ribosomes Is Inhibited by the Peptides.** The PT activity associated with the enriched rRNA that remains after phenol treatment of *T. aquaticus* ribosomes (13) was approximately as sensitive to peptide inhibition as intact 50S subunits of *E. coli* and *B. subtilis* (Fig. 3 and compare with Fig. 2). The reverse versions of the *cat* and *cmlA* peptides failed to inhibit the activity. The deproteinization method used leaves up to eight ribosomal proteins associated with the 23S rRNA (22). It therefore remains possible that the inhibitor peptides act, in part, on ribosomal protein. Intact *T. aquaticus* 50S subunits were less sensitive to peptide inhibition than the deproteinized 23S rRNA (Fig. 3). It is possible that in these ribosomes the target for peptide inhibition is masked by proteins that can be removed by phenol.

**Effects of Em and Cm on Peptide Inhibition.** Due to its sites of interaction with the 50S subunit, Em is a competitor of Cm (23). We observed that addition of Em to ribosomes prior to peptide or simultaneously with the peptide partially reversed peptide inhibition of PT (Fig. 4); competition was most effective when the antibiotic was added prior to peptide. Streptomycin did not interfere with the activity of the inhibitor peptides (Fig. 4) and this antibiotic does not compete with Cm.

PT inhibition by Cm follows two slopes (Fig. 2). At concentrations above 150 μM inhibition becomes asymptotic. Ribosomes preincubated with saturating levels of Cm

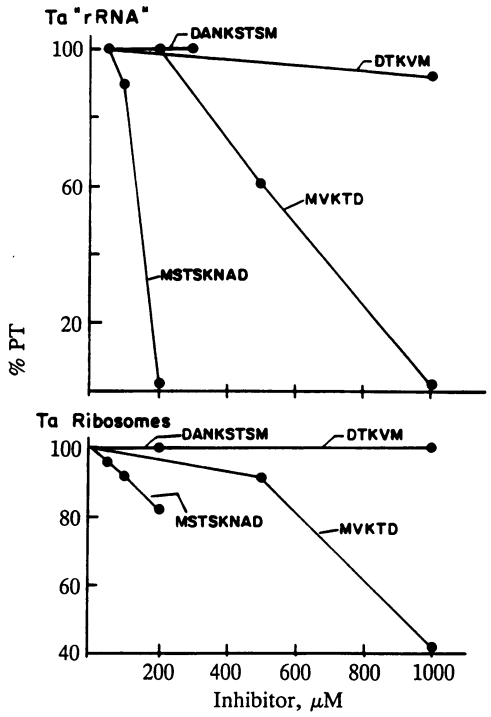


FIG. 3. PT inhibition by the *cmlA* 8-mer and the *cat-86* 5-mer peptides on deproteinized and intact 50S ribosomes of *T. aquaticus*. (*Upper*) rRNA was extracted from ribosomes as described by Noller *et al.* (13) using SDS, proteinase K, and phenol. (*Lower*) Assays performed on 50S ribosomes not deproteinized.

were exposed to increasing concentrations of 8-mer peptide. The resulting inhibition of PT was quantitatively comparable to peptide inhibition of ribosomes not preexposed to Cm (Fig. 4). These data suggest additive inhibition of PT by the *cmlA* 8-mer peptide and the inducer of the gene. Substituting the *cat-86* 5-mer for the *cmlA* 8-mer produced comparable results (data not shown).

**Footprinting Peptide Interaction with 23S rRNA by DMS Probing.** The interference of peptide inhibitory activity by Em suggested the two agents might interact with similar domains of the ribosome. Both agents were footprinted to 23S rRNA, using *B. subtilis* 70S ribosomes and DMS (14).

Exposure of ribosomes to Em diminishes DMS methylation of nt 2058 and 2059 in 23S rRNA (ref. 24; see Fig. 5) as evidenced by a reduction in reverse transcriptase drop-offs at the two preceding nucleotides, 2059 and 2060. This was confirmed for *B. subtilis* ribosomes (Fig. 6 *Upper*, compare "No inhibitor" and "Em" lanes). Exposure of ribosomes to the inhibitor peptides diminished the methylation of these same nucleotides since reverse transcriptase drop-offs at 2059 and 2060 became reduced, but not to the extent seen with Em (Fig. 6 *Upper*, "8-mer" lane; Fig. 6 *Lower*, "5-mer" lane). A footprint unique to the peptides was observed at the adjacent nt 2060, which became sensitive to DMS methylation as a consequence of prior exposure to the peptides and is disclosed by termination of reverse transcriptase at the preceding nucleotide, 2061 (Fig. 6 *Upper*, "8-mer"; Fig. 6 *Lower*, "5-mer"). Two other sites of peptide-enhanced methylation, at nt 2071 and 2077, can be seen in Fig. 6 as reverse transcriptase drop-offs at nt 2072 and 2078. The 5-mer peptide MVKTD also enhanced the methylation of nt 2071 and 2077 (data not shown). Identical footprint patterns were obtained with MKKAD and MKKSE, which are the leader 5-mer peptides encoded by *cat-194* and *cat-221/112*, respectively (refs. 26–28; data not shown). No footprints were observed when noninhibiting variants of the peptides were incubated

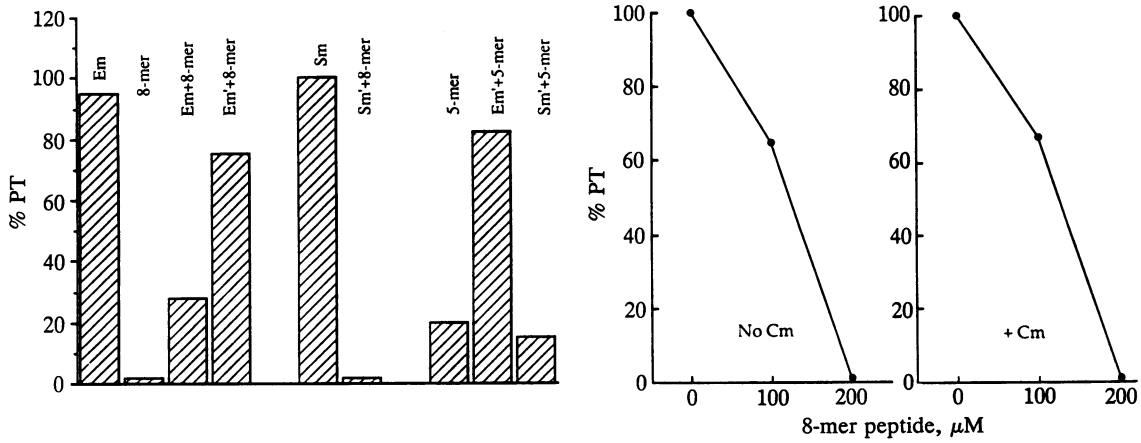


FIG. 4. Effects of Em and Cm on peptide inhibition. (*Left*) Em and streptomycin were preincubated with 50S ribosomes for 10 min. Drug–peptide combinations were added to ribosomes simultaneously (Em+8-mer) for a 10-min preincubation or drug was added to ribosomes 5 min prior to peptide and the combination was incubated for an additional 10 min prior to assay (Em'+8-mer). The 5-mer (MVKTD) was at 800 μM and the other inhibitors were at 200 μM. (*Right*) Ribosomes preexposed to 500 μM Cm for 5 min were incubated an additional 10 min with 0, 100, or 200 μM 8-mer peptide and assayed. In parallel, ribosomes were preincubated for 10 min with 0, 100, or 200 μM 8-mer and no Cm. In terms of PT activity, the 100% value for the Cm-treated ribosomes is 48% of the PT activity of ribosomes not exposed to Cm.

with ribosomes prior to DMS treatment (Fig. 6; "Rev 8-mer," "8-mer Pro," "8-mer Gly," "Rev 5-mer").

## DISCUSSION

Selection of a ribosome stall site through the inhibitory activity of a nascent leader peptide is novel among genes regulated by attenuation (29). Variations of this concept have been suggested as the basis for two other examples of translational regulation (30, 31). Peptides exit the ribosome from a site associated with the PT center (32), predicting that any regulation due to a nascent peptide will probably be focused on the 50S subunit. Our observations adequately explain a form of ribosome pausing that occurs in bacteria. It remains to be determined if our conclusions can account for examples of translational pausing, or stalling, that occur in eukaryotes (33, 34).

In systems that are known to be regulated by translation attenuation, a correlation generally exists between the nature of the drug-resistance gene and the nature of the antibiotic inducer: Cm induces only Cm-resistance genes and Em induces Em-resistance genes (1–3, 19). Site-specific ribosome stalling is key to attenuation regulation and it is on this event that we focus explanations for the action of inducer and leader peptide. One interpretation of our results is that specific ribosome stalling results from the concerted action of leader peptide and inducer on the same ribosomal activity. In itself this explanation is not sufficient to account for the ability of Cm to induce *cat* genes in cells carrying chromosomal mutations that render the protein synthetic apparatus insensitive to the antibiotic (35). Nor does it explain why *cat* genes that are induced by Cm remain induced after withdrawal of the antibiotic (35). We therefore favor a model in which cooperation between inducer and leader peptide is required for specific ribosome stalling. In this model, the capacity of an antibiotic to cooperate with a leader peptide determines its ability to induce the associated drug-resistance gene. The nature of the cooperativity is speculative. Perhaps

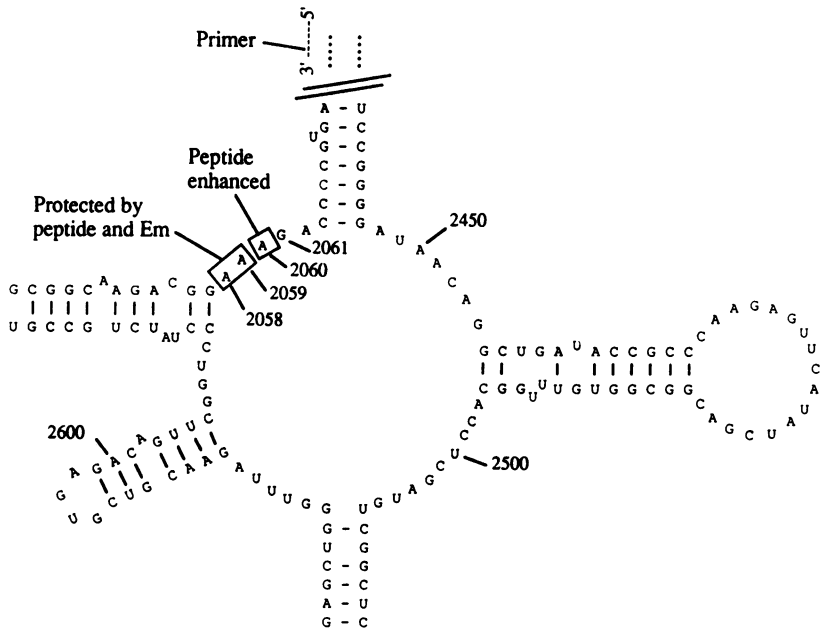


FIG. 5. PT center in 23S rRNA. Sites to which Em and the peptides footprint are indicated. Nucleotides were numbered in accordance with *E. coli* 23S rRNA (24). The annealing site for the primer used to initiate reverse transcriptase is shown.

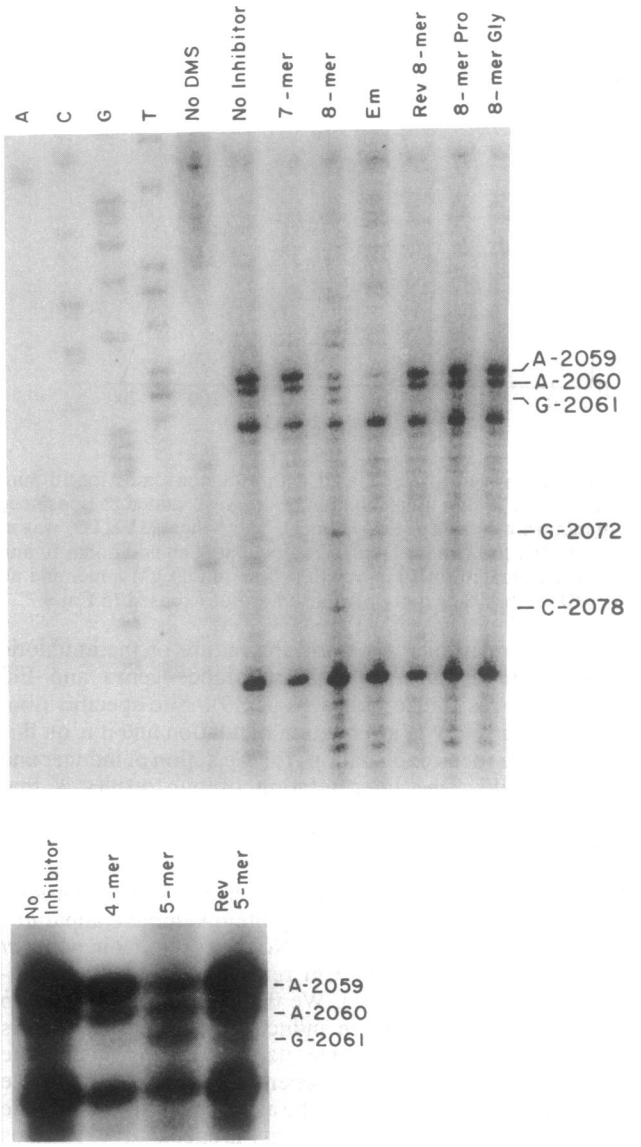


FIG. 6. Effects of Em and the peptides on reaction of 23S rRNA with DMS. (*Upper*) PhosphorImager scan of a gel showing reverse transcriptase terminations on 23S rRNA using the primer shown in Fig. 5. The 8-mer Pro and 8-mer Gly have Pro and Gly as substitutions for Ala and Asp. Peptides and Em were tested at 250 μM and 100 μM, respectively. (*Lower*) Autoradiogram showing the DMS-disclosed footprint of the *cat-86* 5-mer peptide, MVKTD, on 23S rRNA. The 4-mer, MVKT, is noninhibiting for PT (8). These peptides were tested at 500 μM. The 23S rRNA in *B. subtilis* differs slightly in size from *E. coli* 23S rRNA (25). The numbering of nucleotides used here corresponds to the *E. coli* system.

leader peptide binding to a ribosome exposes a novel Cm target, which allows the antibiotic to irreversibly damage the ribosome.

The induction of *ermC* by Em also depends on the sequence of the leader peptide (36). We therefore suggest that selection of the ribosome pause site in translation attenuation regulation is likely to be one biological function of the leader peptides.

This investigation was supported by Public Health Service Grant GM42925.

1. Dubnau, D. (1984) *Crit. Rev. Biochem.* **16,** 103–132.
2. Lovett, P. S. (1990) *J. Bacteriol.* **172,** 1–6.
3. Weisblum, B. (1983) in *Gene Function in Procaryotes*, eds. Beckwith, J., Davies, J. & Gallant, J. A. (Cold Spring Harbor Lab. Press, Plainview, NY), pp. 91–121.
4. Duvall, E. J. & Lovett, P. S. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 3939–3942.
5. Harrod, R., Gu, Z. & Lovett, P. S. (1994) *Gene* **140,** 79–83.
6. Mayford, M. & Weisblum, B. (1989) *EMBO J.* **8,** 4307–4314.
7. Rogers, E. J., Kim, U. J., Ambulos, N. P. & Lovett, P. S. (1990) *J. Bacteriol.* **172,** 110–115.
8. Gu, Z., Rogers, E. J. & Lovett, P. S. (1993) *J. Bacteriol.* **175,** 5309–5313.
9. Alexieva, Z., Duvall, E. J., Ambulos, N. P., Kim, U. J. & Lovett, P. S. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 3057–3061.
10. Gu, Z. & Lovett, P. S. (1992) *Mol. Microbiol.* **6,** 2769–2776.
11. Monro, R. E. & Marcker, K. A. (1967) *J. Mol. Biol.* **25,** 347–350.
12. Spedding, G. (1990) in *Ribosomes and Protein Synthesis: A Practical Approach*, ed. Spedding, G. (IRL, New York), pp. 1–27.
13. Noller, H. F., Hoffarth, V. & Zimnick, L. (1992) *Science* **256,** 1416–1419.
14. Christiansen, J., Egebjerg, Larsen & Garrett, R. A. (1990) in *Ribosomes and Protein Synthesis: A Practical Approach*, ed. Spedding, G. (IRL, New York), pp. 229–251.
15. Bissonnette, L., Champetier, S., Buisson, J.-P. & Roy, P. H. (1991) *J. Bacteriol.* **173,** 4493–4502.
16. Burns, J. L., Rubens, C. E., Mendelman, P. M. & Smith, A. L. (1986) *Antimicrob. Agents Chemother.* **29,** 445–450.
17. Gaffney, D. F., Cundliffe, E. & Foster, T. J. (1981) *J. Gen. Microbiol.* **125,** 113–121.
18. Rubens, C. E., McNeill, W. F. & Farrar, W. E., Jr. (1979) *J. Bacteriol.* **139,** 877–882.
19. Dorman, C. J. & Foster, T. J. (1985) *J. Bacteriol.* **161,** 147–152.
20. Stokes, H. W. & Hall, R. M. (1991) *Plasmid* **26,** 10–19.
21. Ambulos, N. P., Mongkolsuk, S., Kaufman, J. D. & Lovett, P. S. (1985) *J. Bacteriol.* **164,** 696–703.
22. Noller, H. F. (1993) *J. Bacteriol.* **175,** 5297–5300.
23. Pongs, O. (1979) in *Antibiotics: Mechanism of Action of Antibacterial Agents*, ed. Hahn, F. E. (Springer, New York), pp. 56–73.
24. Moazed, D. & Noller, H. F. (1987) *Biochimie* **69,** 879–884.
25. Green, C. J., Stewart, G. C., Hollis, M. A., Vold, B. S. & Bott, K. F. (1985) *Gene* **37,** 261–266.
26. Bruckner, R. & Matzura, H. (1985) *EMBO J.* **4,** 2295–2300.
27. Horinouchi, S. & Weisblum, B. (1982) *J. Bacteriol.* **150,** 815–825.
28. Shaw, W. V., Brenner, D. G., Le Grice, S. F. J., Skinner, S. K. & Hawkins, A. R. (1985) *FEBS Lett.* **176,** 101–106.
29. Landick, R. & Yanofsky, C. (1987) in *Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology*, eds. Neidhardt, F. C., Ingraham, J. L., Low, K. B., Magasanik, B., Schaechter, M. & Umbarger, H. E. (Am. Soc. Microbiol., Washington, DC), pp. 1302–1307.
30. Gay, D. A., Sisodia, S. S. & Cleveland, D. W. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 5763–5767.
31. Weiss, R. B., Huang, W. M. & Dunn, D. M. (1990) *Cell* **62,** 117–126.
32. Ryabova, L. A., Selivanova, O. M., Baranov, V. I., Vasiliev, V. D. & Spirin, A. S. (1988) *FEBS Lett.* **226,** 255–260.
33. Protzel, A. & Morris, A. J. (1974) *J. Biol. Chem.* **249,** 4593–4600.
34. Wolin, S. L. & Walter, P. (1988) *EMBO J.* **7,** 3559–3569.
35. Rogers, E. J., Ambulos, N. P. & Lovett, P. S. (1993) *Mol. Microbiol.* **8,** 1063–1069.
36. Mayford, M. & Weisblum, B. (1989) *J. Mol. Biol.* **206,** 69–79.