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Peptide inhibitors of peptidyltransferase alter the conformation of domains IV and V of large subunit rRNA: A model for nascent peptide control of translation

(chloramphenicol/RNase footprinting/ribosomes/translation attenuation regulation)

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ABSTRACT **Peptides of 5 and 8 residues encoded by the leaders of attenuation regulated chloramphenicol-resistance genes inhibit the peptidyltransferase of microorganisms from the three kingdoms. Therefore, the ribosomal target for the peptides is likely to be a conserved structure and/or sequence. The inhibitor peptides "footprint" to nucleotides of domain V in large subunit rRNA when peptide–ribosome complexes are probed with dimethyl sulfate. Accordingly, rRNA was examined as a candidate for the site of peptide binding. Inhibitor peptides MVKTD and MSTSKNAD were mixed with rRNA phenol-extracted from *Escherichia coli* ribosomes. The conformation of the RNA was then probed by limited digestion with nucleases that cleave at single-stranded (T1 endonuclease) and double-stranded (V1 endonuclease) sites. Both peptides selectively altered the susceptibility of domains IV and V of 23S rRNA to digestion by T1 endonuclease. Peptide effects on cleavage by V1 nuclease were observed only in domain V. The T1 nuclease susceptibility of domain V of *in vitro*-transcribed 23S rRNA was also altered by the peptides, demonstrating that peptide binding to the rRNA is independent of ribosomal protein. We propose the peptides MVKTD and MSTSKNAD perturb peptidyltransferase center catalytic activities by altering the conformation of domains IV and V of 23S rRNA. These findings provide a general mechanism through which nascent peptides may cis-regulate the catalytic activities of translating ribosomes.**

Inducible chloramphenicol (Cm)-resistance genes *cat* and *cmlA* are regulated by translation attenuation (1–4). In this form of translational control, the inducer Cm stalls a ribosome in a leader sequence located at the 5′ end of the regulated transcripts. Stalling causes a localized change in the secondary structure of the mRNA and exposes a normally sequestered ribosome binding site, allowing translation of the resistance determinant (5, 6).

Translation of the *cat* coding sequence results when a ribosome becomes stalled in the leader with its amino acyl site at leader codon 6 (7). This site of ribosome stalling is selected by the five codons that precede leader codon 6 (8). These five codons specify a 5-mer peptide, MVKTD, that is an *in vitro* inhibitor of peptidyltransferase (PT; refs. 9 and 10) and translation termination (11). PT inhibition *in vitro* requires relatively high peptide/ribosome ratios, and it has been suggested (9, 10) that inhibition *in vivo* depends on peptide synthesis at its target. The activation of translation of the *cmlA* resistance determinant also requires ribosome stalling, but at leader codon 9 (2). In the *cmlA* example, the eight codons upstream from the site of ribosome stalling specify an 8-mer peptide, MSTSKNAD, that inhibits PT (2). Both the *cat*-encoded 5-mer peptide and the *cmlA*-encoded 8-mer peptide inhibit PT of bacterial, yeast, and Archea ribosomes (12), implying the target for the inhibitor peptides is a ribosomal component that is conserved across kingdoms.

Studies by Noller *et al.* (13) indicate that PT activity may be determined by large subunit rRNA, perhaps influenced by specific ribosomal proteins. Here we demonstrate that the PT inhibitory leader peptides alter the conformation of domains IV and V of large subunit rRNA, which are located at the PT center. These observations suggest a general model through which nascent peptides may regulate translation.

MATERIALS AND METHODS

Source of rRNA. Unless noted, 23S rRNA was obtained by phenol extraction of 70S ribosomes isolated from *Escherichia coli* DH5αF′ (14); a freshly extracted rRNA preparation was used for each experiment. *In vitro*-transcribed 23S rRNA was prepared from a clone provided by J. Ofengand (15).

Oligonucleotides and Peptides. DNA oligonucleotides were synthesized that prime DNA synthesis within different domains of 23S rRNA of *E. coli* (16). Peptides were synthesized by Research Genetics (Huntsville, AL), Biosynthesis (Lewisville, TX), or on an Applied Biosystems model 432 peptide synthesizer. Each peptide was purified by reverse-phase HPLC as described (10).

Nuclease Probing of RNA Secondary Structure. T1 endonuclease (Sigma) and V1 endonuclease (Pharmacia) (17, 18) were diluted in water to a concentration empirically found to produce a single or very few cleavages per RNA molecule during incubation. In a typical experiment, 5 μg of total rRNA, consisting of 0.165 μM 23S rRNA, 0.165 μM 16S rRNA, and 0.165 μM 5S rRNA, was incubated for 10 min on ice with 1 mM peptide in 7 μl of ribosome buffer (10 mM Tris·HCl, pH 7.5/10 mM magnesium acetate/60 mM ammonium chloride/3 mM 2-mercaptoethanol; ref. 14). The ratio of peptide molecules to 23S rRNA molecules was ≈6000:1; in experiments (not shown), we have obtained comparable footprinting results with peptide/23S rRNA ratios of 4000:1. Dilute T1 or V1 nuclease and $MgCl_2$ to 10 mM were added in 13 μl of water; for V1 cleavage the 13-μl mixture contained 200 mM NaCl. The 20-μl digestion mixture was incubated an additional 10 min on ice. The RNA was then extracted with phenol/chloroform, precipitated with ethanol, and resuspended in the above buffer supplemented with 0.75 M ammonium acetate. After a second ethanol precipitation, the rRNA was resuspended in buffer appropriate for reverse transcriptase (RT) and primers were annealed to allow avian myeloblastosis virus RT mapping.

RESULTS

Inhibitor Peptides Alter the Susceptibility of Domains IV and V of Bacterial 23S rRNA to Cleavage by T1 Nuclease. T1

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Abbreviations: PT, peptidyltransferase; RT, reverse transcriptase; Cm, chloramphenicol.

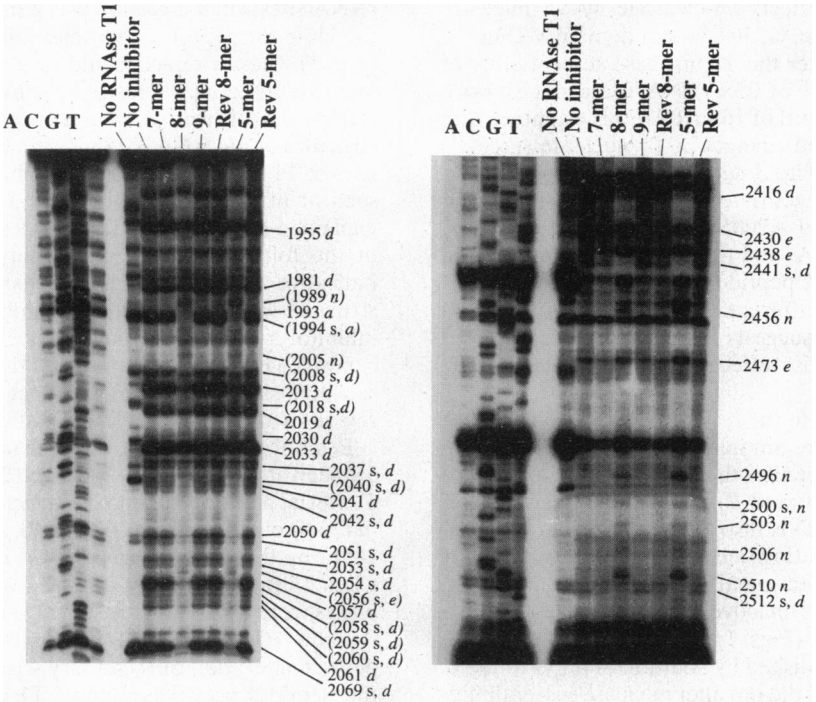

FIG. 1. Effects of MSTSKNAD and MVKTD peptides on T1 nuclease probing of domains IV and V of *E. coli* 23S rRNA. RT was used to map sites of termination in rRNA that had been incubated with inhibitor 5- and 8-mer peptides, control peptides [7-mer, 9-mer, reverse (Rev) 8-mer, and Rev 5-mer], or no peptide (No inhibitor) and digested with T1 endonuclease. RT mapping data in the NO RNase T1 lane were obtained with rRNA that had not been exposed to peptides or to T1 nuclease. (*Left*) Primer L-EC1 (complementary to nt 2110–2129) was used to prime RT. (*Right*) Primer L-EC2, complementary to nt 2538–2559, was used to prime RT. Certain sites of RT termination differ among lanes labeled No inhibitor, 8-mer, and 5-mer. *d*, Nucleotides at which termination is decreased relative to the No inhibitor lane; *e*, enhanced termination; *a*, absence of termination; *n*, new site of termination as a consequence of the inhibitor peptides; *s*, change in an RT termination site between the No RNase T1 lane and the lanes containing RNA exposed to the 5- and 8-mers. Such changes presumably measure the direct or indirect effects of the inhibitor peptides on secondary structure of the rRNA (see text). The sequencing ladders (lanes A, C, G, and T) were generated by using RT. Therefore, the nucleotides shown in the sequencing ladder are the complements of the nucleotide in the rRNA; i.e., a C residue in the sequencing ladder is a G residue in the RNA. Parentheses indicate sites at which the designated change was observed only with particular rRNA preparations.

nuclease cleaves single-stranded RNA on the 3′ side of guanosine residues (17) and V1 nuclease preferentially cleaves duplexed regions of RNA (18). The susceptibility of sites within rRNA to these nucleases, therefore, provides an indication of secondary structure. Each site cleaved by the nucleases is a termination point for DNA synthesis by RT. Therefore, sites of nuclease-dependent termination of reverse transcription mark the locations of nuclease-susceptible sites. Additionally, RT has a probability of terminating at hairpin structures in uncleaved rRNA, which provides a further measure of secondary structure (19, 20). It should therefore be possible to determine whether the PT inhibitor peptides alter the secondary structure of rRNA.

Incubation of phenol-extracted rRNA with the PT inhibitor peptides MVKTD and MSTSKNAD altered the T1 nuclease susceptibility of several sites within domains IV and V of 23S

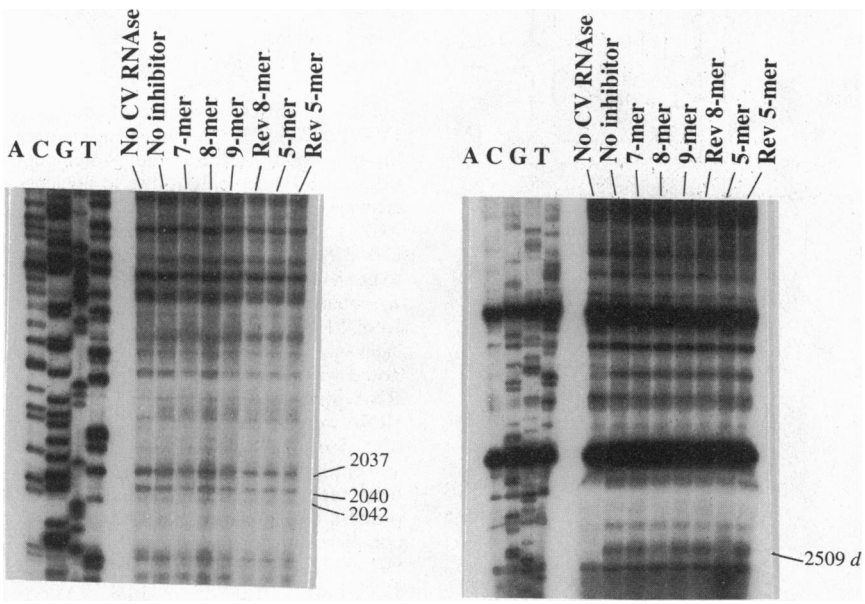

FIG. 2. Effects of MSTSKNAD and MVKTD on V1 nuclease probing of domains IV and V of *E. coli* 23S rRNA. Experimental details are as in Fig. 1 except that V1 nuclease was used. (*Left*) L-EC1 was primer. (*Right*) L-EC2 was primer. The nt 2037, 2040, and 2042 are noted (*Left*) as sites of secondary structure RT terminations that are unaffected by the peptides during V1 cleavage but are diminished during T1 cleavage (Fig. 1; see text). V1 RNase = CV RNase.

rRNA (Fig. 1). Peptide effects on cleavage by V1 nuclease were limited to a single site, G2509, within domain V (Fig. 2). Both peptides failed to alter the T1 nuclease susceptibility of domains I, II, III, and VI of 23S rRNA (data not shown). Additionally, the 3′ one-third of 16S rRNA was examined and showed no peptide-induced changes in T1 nuclease susceptibility (data not shown). Fig. 3 summarizes peptide-induced changes in nuclease cleavages detected in 23S rRNA. Two of the changes, at G1989 and A2005, were observed only with particular batches of rRNA, and these are bracketed in Figs. 1 and 3. The variability of peptide effects on specific nucleotides, probably due to different levels of protein removal during phenol extraction, suggests that although the peptides bind to rRNA, their precise effects could be influenced by ribosomal proteins.

Peptides corresponding to the reverse amino acid sequence of the inhibitor peptides are not inhibitory for PT (2, 10), and the reverse peptides did not alter the nuclease susceptibility of domain IV or V (Figs. 1 and 2). The anti-PT activity of MVKTD and MSTSKNAD is also eliminated when the peptides are shortened by omitting one amino acid from the N or C terminus (2, 10). A C-terminal truncation of the 8-mer, designated the 7-mer, was inactive in altering the nuclease susceptibility of the rRNA (Figs. 1 and 2). Lastly, the anti-PT activity of the 8-mer is abolished by addition of a Lys to the C terminus (2) and this 9-mer did not alter nuclease susceptibility of the rRNA (Figs. 1 and 2).

Changes resulting from the interaction of inhibitor peptides with rRNA fall into three general classes. Class 1 effects describe the loss or reduction of nuclease cleavage at specific rRNA sites when digestion was performed in the presence of the inhibitor peptides; examples are seen at nt 1993 and 2013 (Fig. 3). Class 1 effects could result from peptide masking of nuclease susceptible sites, a measure of peptide binding to the rRNA, and/or from peptide-induced changes in the secondary structure of the RNA. Class 2 effects denote new or enhanced sites of T1 cleavage due to the inhibitor peptides; examples are seen at nt 2496 and 2430. Class 2 effects are most directly explained by proposing the peptides introduce subtle changes in the folding of domains V and IV. Additional evidence indicative of peptide-induced changes in rRNA secondary structure was observed in the vicinity of nt 2509 and 2510. The inhibitor peptides partially protected this region from V1 cleavage (Fig. 2) and also caused the appearance of a new T1 sensitive site (Fig. 1), arguing that the peptides may open a region of the RNA that is normally duplexed.

Examples of a third effect of the inhibitor peptides on rRNA are seen at nt 2037, 2040, 2042, and 2056 (Fig. 1). In these class 3 effects, a site of secondary-structure-dependent RT termination is eliminated (nt 2037, 2040, and 2042) or enhanced (nt 2056) by the inhibitor peptides (Fig. 1) during T1 cleavage. Class 3 effects were not detected when peptide–rRNA complexes were digested with V1 nuclease (see Fig. 2), suggesting that the identified class 3 changes are not directly due to peptide alteration of secondary structure. Rather, we suspect the peptides provoke a site of T1 cleavage elsewhere in the RNA that alters the pattern of folding.

T1 Nuclease Susceptibility of *in Vitro*-Transcribed 23S rRNA Is Altered by the Peptides. 23S rRNA phenol-extracted from *Thermus aquaticus* ribosomes contains a minor fraction

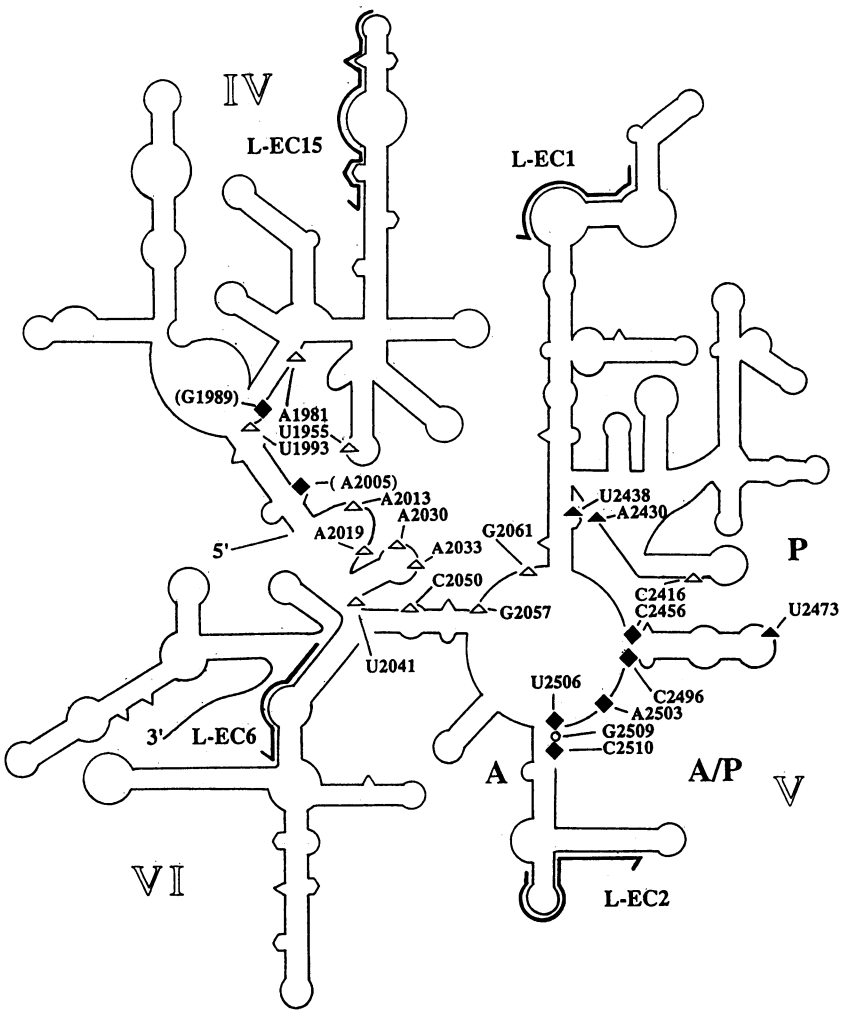

FIG. 3. Stick diagram of domains IV–VI of 23S rRNA summarizing changes brought about by the 5- and 8-mer peptides on RT termination due to nuclease cleavage. Primers shown are L-EC1, L-EC2, and L-EC6 (complementary to nt 270–2789), and L-EC15 (complementary to nt 1842–1870). ◆, New T1 cleavage site; ▲, enhanced T1 cleavage; △, protection from T1 cleavage; ○, protection from V1 cleavage. Parentheses indicate the change noted was observed only with particular RNA preparations. Domains I–III of the rRNA were also examined and showed no effects of the peptides on T1 nuclease susceptibility nor on nuclease-independent secondary structure changes. The primers used to map these regions were complementary to nt 177–192, 444–476, 901–924, 1128–1141, 1368–1389, and 1555–1568.

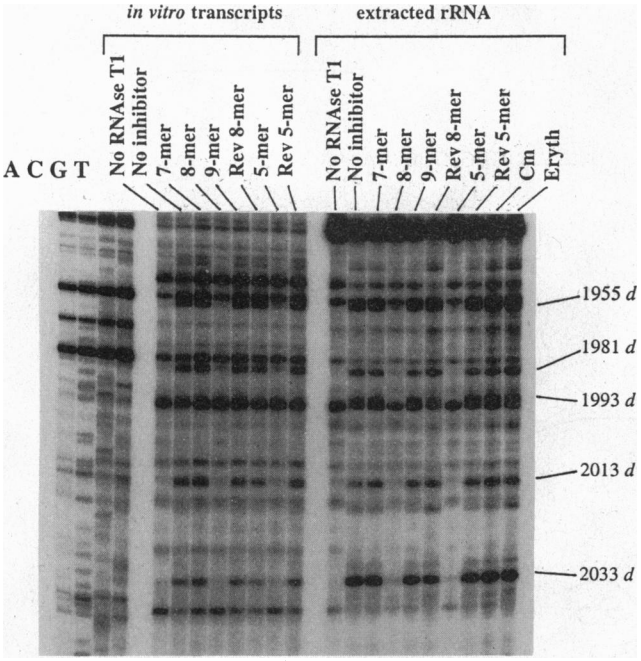

FIG. 4. Effect of the 5- and 8-mer peptides on T1 nuclease susceptibility of *in vitro*-prepared 23S rRNA and rRNA extracted from ribosomes. 23S rRNA was transcribed *in vitro* and purified as described (15). Other experimental details are as in Fig. 1. The primer was L-EC1. Since the same total quantity of *in vivo* and *in vitro* RNA (5 μg) was used, the ratio of peptide/23S rRNA is ≈4000:1 for the *in vitro* RNA and 6000:1 for the *in vivo* RNA. Cm and erythromycin (Eryth) were used at 500 μM. Neither antibiotic had a detectable effect on the T1 nuclease susceptibility of the rRNA. Nucleotides noted in the figure are those clearly altered in the *in vitro* rRNA.

of associated ribosomal protein (13). Conceivably, the binding of the inhibitor peptides to phenol-extracted rRNA could require an associated phenol-resistant ribosomal protein. We therefore examined peptide effects on the T1 nuclease susceptibility in *in vitro*-transcribed *E. coli* 23S rRNA (15). Both the 5- and 8-mer peptides modified the nuclease susceptibility of sites within domain V of the *in vitro*-transcribed RNA and the control peptides did not (Fig. 4). The modifications were comparable to those produced by the peptides on rRNA extracted from ribosomes. Differences in nuclease susceptibility between peptide-free *in vivo* and *in vitro* rRNA are perhaps due to the conditions under which the RNAs fold: *in vitro*-prepared rRNA contains unmodified nucleotides (15) and folds independent of bacterial proteins.

Reversing Peptide Effects on rRNA. To test the reversibility of peptide effects on rRNA, *E. coli* rRNA was incubated with MVKTD or MSTSKNAD for 10 min and a portion was taken to confirm by T1 endonuclease that the peptides had altered the cleavage of domain V. The remainder of the rRNA–peptide mixture was precipitated with 95% ice-cold ethanol, resuspended in ribosome buffer containing 0.75 M ammonium acetate, and reprecipitated. The rRNA product was then taken for nuclease probing. RT mapping using L-EC1 as primer demonstrated that removal from the rRNA of the inhibitor peptides restored the T1 nuclease susceptibility seen with rRNA not previously exposed to the peptides (data not shown).

DISCUSSION

Peptide-bond formation during translation and peptide release during translation termination take place in the vicinity of the ribosome's PT center (21, 22). The PT center is thought to form the most interior portion of an exit tunnel through which nascent peptides pass as they leave the ribosome (23). The ribosomal P site is adjacent to the PT center and P-site-bound tRNA (the peptidyl-tRNA) is joined at its 3′ terminus to the 5- and 8-mer peptides *in vivo*. Therefore, *in vivo* the nascent *cat*- and *cmlA*-encoded leader peptides extend from their attachment to peptidyl-tRNA into the PT center.

We suggest a mechanism through which the PT inhibitor peptides may bring about translational pausing (Fig. 5). In our model, synthesis of the 5- and 8-mer peptides *in vivo* produces a concomitant alteration of the secondary structure of PT center rRNA within the translating ribosome. The alteration eliminates PT center catalytic activities that include both peptide bond formation (2, 10, 12) and translation termination (11, 22, 24). The peptide-induced changes in rRNA secondary structure are reversible and may be analogous to allosteric changes in proteins due to small effector molecules (25).

Dissociation of the nascent inhibitor peptide from the rRNA should permit the paused ribosome to resume translation of the leader. Since resumption of translation drives the inhibitor peptide sequence away from its rRNA target (Fig. 5), the peptides function as pause signals for translation rather than signals for translation termination.

Peptides as Cis-Regulators of Ribosome Function. *cat*- and *cmlA*-encoded leader peptides bind to domains IV and V of *E. coli* and yeast rRNA (unpublished data). Neither peptide is obviously related to known examples of RNA binding proteins (26, 27). Therefore, the PT inhibitor peptides represent a specific class of controlling element for RNA. Nascent peptide effects on ribosomes could offer an explanation for other examples of translational control (28–33), although it is not yet known whether these examples of putative regulatory peptides are capable of binding to rRNA. While it is likely that ribosome function could be altered by peptide binding to ribosomal protein, the rRNA binding properties of the *cat*- and *cmlA*-encoded leader peptides and the apparent rRNA targets of several antibiotics (34) argue that the interaction of small molecules with rRNA may be the preferred mechanism to regulate selected activities within the ribosome.

Origin of the rRNA Binding Peptides. The rRNA binding property of the leader peptides is suggestive of ribosomal proteins, and perhaps sequences specifying the PT inhibitor peptides might have arisen through the modification of the coding regions for ribosomal protein domains that interact with rRNA. The inhibitor peptides are short and amino acid replacements that allow PT inhibition may significantly change the peptides from putative parental proteins. Consequently, computer homology searches to delineate ancestral relationships may be difficult.

Evidence for a Second Function of the Leader Peptides. Induction of *cat* and *cmlA* requires Cm, whereas the anti-PT activity of the leader peptides and their binding to rRNA occurs in the absence of the antibiotic. Accordingly, there must be two different steps in translation attenuation regulation: selection of the ribosomal stall site by the nascent peptide and an inducer-dependent effect. *cmlA* and most *cat* genes are induced by Cm but not by other ribosomally targeting antibiotics. In contrast, *erm* genes are regulated by translation attenuation and are induced by erythromycin but not Cm, indicating specificity of the antibiotic inducer (35, 36). One gene, *cat-86*, is unusual since it is inducible not only by Cm but also by two non-Cm antibiotics, erythromycin and amicetin (37, 38). Amicetin, which interacts with the PT center (39, 40), is of particular interest because selected missense mutations in the *cat-86* leader abolish induction by amicetin but have no effect on induction by Cm (41). Based on these observations, we speculate that the *cat*- and *cmlA*-encoded leader peptides may also cooperate with the antibiotic inducer to influence a ribosomal event essential for induction.

We thank J. Ofengand for the cloned *E. coli* 23S rRNA gene and B.

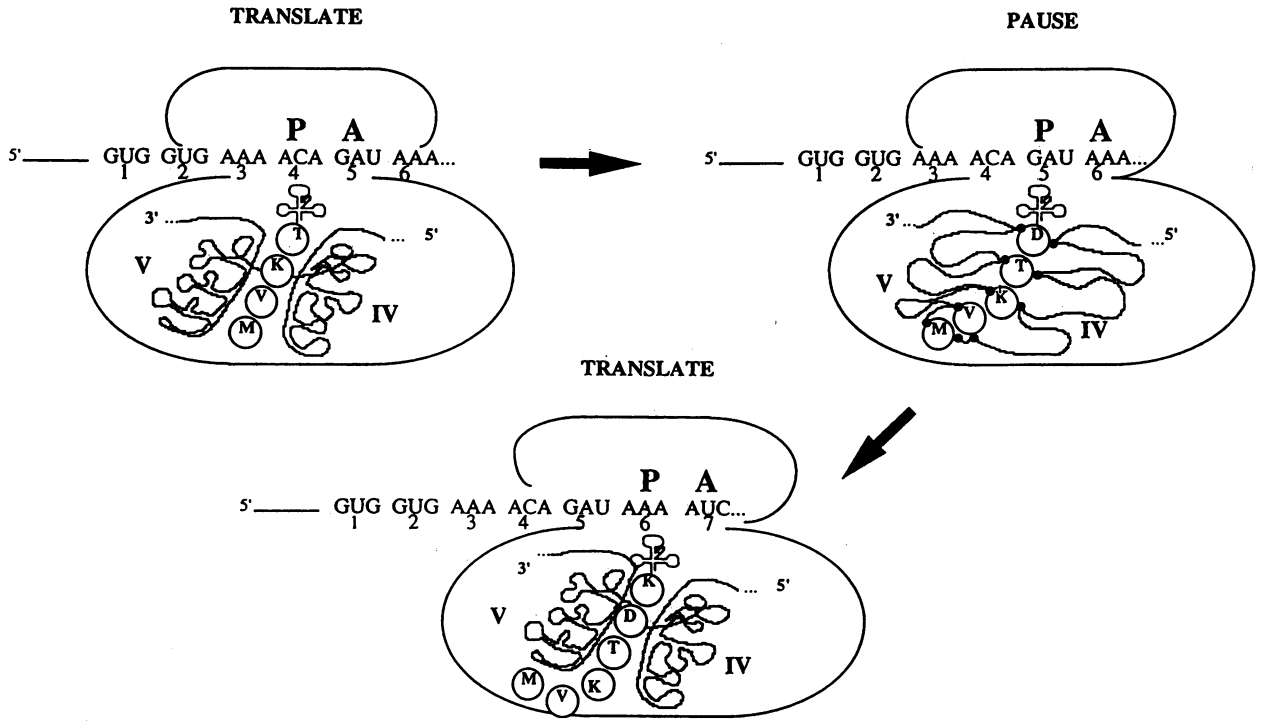

FIG. 5. Model for peptide-induced ribosome pausing during *cat*-encoded leader translation. A ribosome that has translated the first 5 codons of the *cat*-encoded leader peptide pauses due to loss of PT activity. PT inhibition is caused by a change in conformation of 23S rRNA resulting from the interaction of the nascent pentapeptide with the rRNA. The interaction between rRNA and peptide is reversible. Dissociation of the peptide from the rRNA restores its conformation and PT activity. Translation promptly resumes and thus drives the inhibitor pentapeptide away from target sequences in the rRNA.

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