JOURNAL OF BACTERIOLOGY, Dec. 1991, p. 7881–7886
0021-9193/91/247881-06$02.00/0

Vol. 173, No. 24

Ribosome Hopping and Translational Frameshifting Are Inadequate Alternatives to Translational Attenuation in *cat-86* Regulation

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Received 24 June 1991/Accepted 9 October 1991

The induction of *cat-86* by chloramphenicol has been proposed to follow the translational attenuation model. In the absence of inducer, the *cat-86* gene is transcribed but remains phenotypically unexpressed because the transcripts sequester the ribosome binding site for the *cat* coding sequence in a stable stem-loop structure, preventing translation initiation. The translational attenuation model proposes that the natural inducer, chloramphenicol, stalls a ribosome in the leader region of *cat* transcripts, which causes localized melting of the downstream stem-loop structure, allowing initiation of translation of the *cat-86* coding sequence. Although it is established that ribosome stalling in the *cat-86* leader can induce translation of the coding sequence, several subsequent steps predicted by the model remain to be experimentally confirmed. As a consequence, the present evidence for *cat-86* regulation can also be explained by two other potential control devices, ribosome hopping and translational frameshifting. Here we describe experiments designed to determine whether the alternatives to translational attenuation regulate *cat-86*. The results obtained are inconsistent with both competing models and are consistent with predictions made by the translational attenuation model.

Translational attenuation is a gene regulatory model which is believed to accurately describe the mechanism underlying the induction of *erm* and *cat* genes by the respective antibiotics erythromycin and chloramphenicol (8, 18, 32). In appropriate host bacteria such as *Bacillus subtilis* or *Staphylococcus aureus*, *cat* and *erm* genes are induced 10-fold or more by exposure of the cells to very low concentrations of the antibiotics. In both cases, induction primarily activates translation of *cat* or *erm* transcripts rather than transcription of the respective genes. *cat* and *erm* coding sequences in mRNA are normally untranslated because the ribosome binding site is sequestered in the secondary structure and is unavailable for translation initiation. The translational attenuation model proposes that the inducing antibiotic stalls a ribosome in the leader region of the transcripts, upstream from the region of secondary structure. The stalled ribosome is thought to cause a change in the downstream secondary structure which frees the ribosome binding site and permits translation initiation of the drug resistance determinant.

Evidence in support of the translational attenuation model is extensive and has been summarized elsewhere (8, 18, 32). A review of the evidence demonstrates that it is also consistent with two forms of translational control which are fundamentally distinct from translational attenuation. In the present study, we have performed experiments with the *cat-86* gene to determine whether the alternatives, translational bypass (ribosome hopping) and translational frameshifting (6), regulate *cat-86*.

MATERIALS AND METHODS

Bacteria and plasmids. *Bacillus subtilis* BR151 (*trpC2 metB10 lys-3*) and *B. subtilis* BG2036 (38), a protease-deficient strain, were used as plasmid hosts. Plasmid pPL703 has been previously described and contains the *cat-86* gene (19). *cat-86* was activated with the P4 promoter (35). Growth media and conditions, transformation methods, and procedures for plasmid manipulations were as previously described (2). Inductions of *cat-86* were performed by growing log-phase bacteria for 2 h in broth containing 2 μg of chloramphenicol per ml.

Site-directed mutagenesis. Oligonucleotide-directed mutagenesis was performed as detailed elsewhere by using M13 vectors and the method of Taylor et al. (9, 28, 39). DNA sequencing was by the dideoxy method of Sanger et al. (25).

Purification of *cat-86*-specified protein. Previous N-terminal sequencing of the CAT-86 protein was performed on the product of a constitutively expressed version of the gene (17). To facilitate the sequencing of CAT-86 produced by the wild-type *cat-86* gene, we developed a more rapid purification scheme for the protein. The method requires less than 8 man-hours and is based on preparative isoelectric focusing. *B. subtilis* BG2036 containing the *cat-86* gene was grown to late log phase in 500 ml of 2× Penassay broth (Difco) containing 10 μg of chloramphenicol per ml. Cells were chilled and washed twice with 20 mM Tris-HCl, pH 7.8, and resuspended in 10 ml of 1× resuspension buffer (20 mM Tris-HCl [pH 7.8], 100 mM NH_4Cl, 21 mM magnesium acetate, 10 mM EDTA, 10 mM BME, 5 mM phenylmethylsulfonyl fluoride) (26). Cells were disrupted twice in a French pressure cell (16,000 lb/in^2). The lysate was centrifuged at 30,000 × *g* for 20 min, and the supernatant fraction (S-30) was centrifuged at 150,000 × *g* for 4 h. The resulting supernatant fraction (S-150) was dialyzed against distilled water.

Twenty to 25 mg of the dialyzed S-150 was applied to a Rotofor preparative isoelectric focusing cell (Bio-Rad) in a 50-ml volume containing 2% CHAPS {3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate}, 20% glycerol, and 1.6% ampholytes 3/10 (Bio-Rad). Isoelectric focusing was performed at 4°C for 4 to 5 h at 12 W of constant power; starting and ending voltages were 800 and 2,000 V, respectively. Fractions were collected and assayed for chloramphenicol acetyltransferase (CAT) and protein as previously described (23). Two CAT-containing peaks were observed (Fig. 1). Soluble CAT-86 banded at approximately pH 6.2; a more acidic band was due to precipitation of the protein. Fractions containing soluble CAT were sequentially concen-

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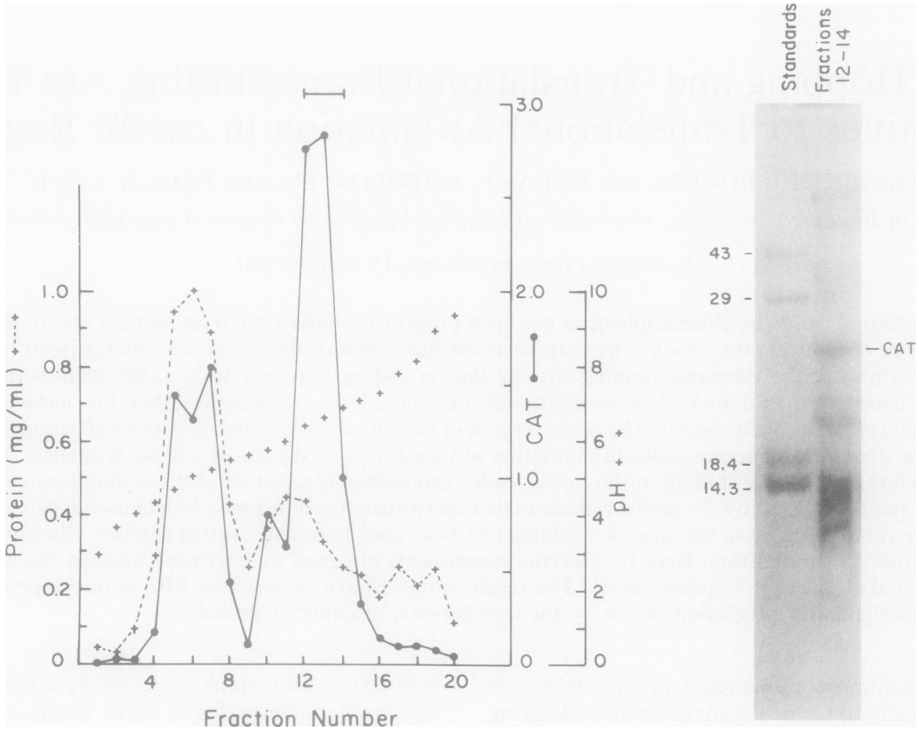

FIG. 1. Rapid purification of CAT-86 protein. CAT-86 was applied to a Rotofor cell (Bio-Rad), and isoelectric focusing was performed as described by the supplier (see also Materials and Methods). Fractions were assayed for protein, CAT activity, and pH. Soluble CAT-86, identified by the horizontal bar, was pooled, applied to a denaturing 12.5% polyacrylamide gel, and electrophoresed at room temperature. The protein bands were transferred to a polyvinylidene difluoride membrane which was stained with Coomassie blue (shown in the right portion of the figure). CAT-86 was identified by comparison with protein standards of known molecular masses, shown in kilodaltons.

trated and washed with 0.02 M Tris-HCl, pH 7.8, in an Amicon ultrafiltration cell with a PM30 membrane and Amicon microconcentrator Centricon-30. Two hundred to 300 μg of partially purified protein was loaded on a 12.5% polyacrylamide gel which had been preelectrophoresed in buffer containing 0.1 mM glutathione (oxidized form; Sigma) and allowed to stand overnight in the cold. Gels were run by using the MZE3328.IV Buffer System (22). Gels were blotted in 10 mM CAPS (3[cyclohexylamino]-1-propanesulfonic acid) (Sigma), pH 9.5, containing 0.5 mM dithiothreitol (22) in a semidry blotter (Novablot; LKB) for 1 h at 0.8 mA/cm^2, to a polyvinylidene difluoride membrane (Bio-Rad). The membrane was stained with Coomassie brilliant blue R-250 and then destained in 10% acetic acid and 50% methanol (20). After destaining, the CAT band was cut out with a razor blade and stored at −20°C. N-terminal sequencing was performed by automated Edman degradation.

Detection of CAT-86 by Western blotting (immunoblotting). Twenty-five micrograms of protein from an S-30 of BR151 cells carrying *cat-86* was subjected to electrophoresis at 150 V for 16 h in a sodium dodecyl sulfate (SDS)–12.5% polyacrylamide gel electrophoresis gel. The gel was blotted to a nitrocellulose membrane (29) in an LKB Novablot semidry blotter in the recommended buffer (39 mM glycine, 48 mM Tris, 0.0375% SDS, 20% methanol). The membrane was rinsed for 5 min in TBST (10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.05% Tween 20) and was blocked for 30 min in 1% bovine serum albumin in TBST. CAT polyclonal antibody was diluted 1,000-fold in TBST and was incubated with the membrane at room temperature for 30 min. The membrane was washed thrice with TBST. Goat anti-rabbit alkaline phosphatase immunoglobulin G (Promega) was diluted 7,500-fold in TBST and incubated with the membrane for 30 min. The membrane was washed as described above and then incubated with the color reagent (200 μl of nitroblue tetrazolium, 100 μl of 5-bromo-4-chloro-3-indolyl phosphate in 30 ml of 100 mM Tris-HCl [pH 9.5], 100 mM NaCl, 5 mM $MgCl_2$). After 15 min, the reaction was stopped by rinsing the filter with distilled water.

RESULTS

Translational bypass. Gene 60 of bacteriophage T4 is the only documented example of a novel variation of translation termed translational bypass, or ribosome hopping. Transcripts of gene 60 specify topoisomerase. The protein sequence of topoisomerase revealed that 50 nucleotides of the transcript are not translated (16). These 50 untranslated nucleotides are internal in the coding sequence. The available evidence (34) suggests that a ribosome can hop over the untranslated region carrying the upstream nascent peptide and continue uninterrupted translation to the end of the coding sequence. This extraordinary event depends on the nascent upstream peptide, a region of RNA secondary structure at the ribosome hop site, and appropriately positioned takeoff and landing codons which apparently must be identical (34). Comparison of the relevant region of gene 60 transcripts with the regulatory region 5′ to *cat-86* shows apparent structural similarities (compare Fig. 2 with Fig. 7 of reference 34).

A ribosome that is active in *cat-86* induction is stalled in the regulatory leader, with its peptidyl site at leader codon 5

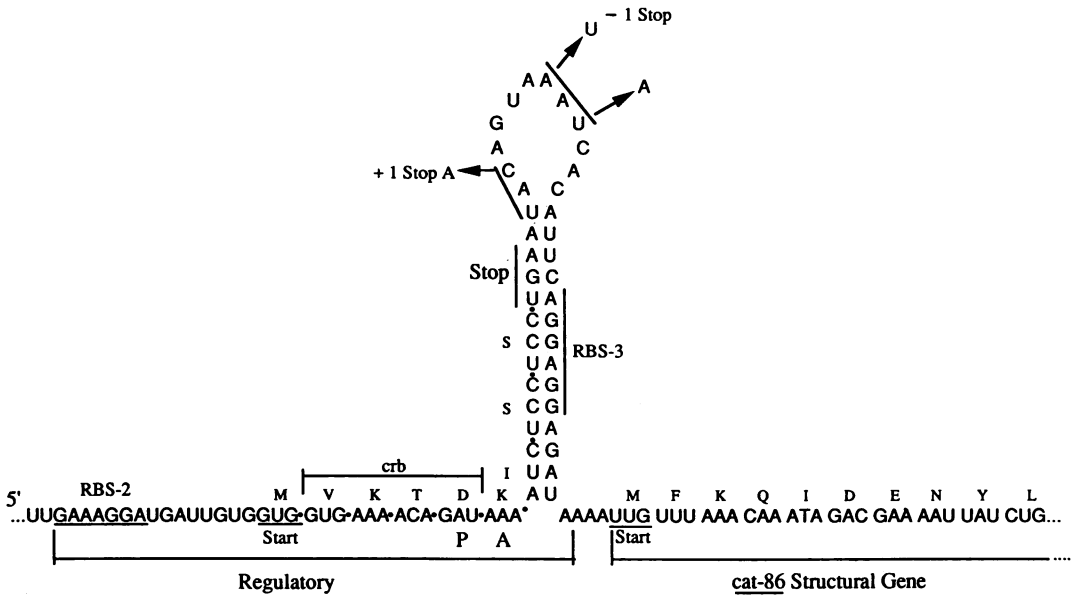

FIG. 2. Sequence in mRNA of the *cat-86* regulatory region and the first 10 codons of the *cat-86* coding sequence. RBS-2 and RBS-3 are the ribosome binding sites for the regulatory leader and the *cat-86* structural gene, respectively. The initiation codons for the leader, GUG, and the coding sequence, UUG, are underlined. The designation *crb* indicates the four leader codons essential to drug induction of *cat-86* (24). Shown are the mutations made by site-directed mutagenesis in the stem-loop region which place a stop codon (TAA) in the +1 or −1 frame of the leader reading frame.

(Fig. 2) (1). It is conceivable that leader codon 5 (Asp) serves as a takeoff codon. A possible landing codon (Asp) can be identified 59 nucleotides downstream within the *cat-86* coding sequence (4) (Fig. 2). We performed two experiments with *cat-86* to determine whether the translational bypass system might apply to the drug-inducible regulation seen with this gene. First, if ribosome hopping were to occur from leader codon 5 to the next downstream Asp codon, which is codon 6 of the structural gene (2), then induction should not depend on translation initiation at the start codon for the *cat-86* structural gene, TTG. Accordingly, the TTG codon was changed to AAA, a codon that does not serve for translation initiation in *B. subtilis* (5). This mutation decreased inducible expression of *cat-86* by more than 99%, suggesting that translation initiation at TTG was necessary for inducible expression of *cat-86*.

Second, we determined where translation of the induced *cat-86* gene product initiates. In the translational bypass model, the upstream nascent peptide constitutes the N-terminal sequence of the protein product of gene 60 (34). In the *cat-86* system, the equivalent to the nascent peptide could be the leader peptide. If a form of ribosome hopping regulated *cat-86*, one would anticipate that the mature *cat-86* protein product resulting from induction would contain N-terminal amino acids corresponding to the first five leader codons. However, N-terminal sequencing of the *cat-86*-specified protein resulting from induction with chloramphenicol demonstrated that the first 10 amino acids corresponded to the first 10 codons of the *cat-86* structural gene and the initiation codon TTG-specified Met (Fig. 2). Thus, the data strongly suggest that the translational bypass model, as presently conceived, does not apply to *cat-86* regulation.

+1 Translational frameshifting. Inspection of the regulatory region of *cat-86* shows that translation from the regulatory leader through the RNA secondary structure is blocked by intervening stop codons in both the leader frame (2 stops; TGA and TAA) and the −1 frame (one stop; TAA) (Fig. 2). However, the +1 frame is open between the leader and the *cat-86* ribosome binding site (RBS-3). Inspection of the regions of secondary structure that regulate other inducible *cat* genes also shows that the +1 frame is open between the leader and the *cat* coding sequence (7, 15, 27). It is possible that a shift in reading frame in the leader, perhaps brought about by the inducer, could allow translation initiated in the leader to continue to RBS-3. In this model, we would expect that reinitiation would occur at RBS-3, since continued translation in the +1 frame is both out of frame with the *cat* coding sequence and terminated by an ochre codon early in the coding sequence (Fig. 2). To test this idea, we first changed the reading frame of the regulatory region by mutagenesis. A nucleotide, T, was deleted from codon 2′ of the leader sequence upstream from *crb* in a version of *cat-86* designated Mut FRM (24) (Fig. 3). This +1 frame change resulted in constitutive *cat* expression (specific activity of 6.8). The N-terminal sequence of the resulting protein demonstrated that translation initiation was occurring at the normal *cat-86* start codon, TTG, and that the first 10 amino acids, including Met specified by the TTG initiation codon, were those predicted from the sequence of the *cat-86* structural gene (Fig. 2). Thus, a +1 change of reading frame 5′ to the region of RNA secondary structure appears to allow a ribosome to initiate translation at RBS-3.

To determine whether chloramphenicol induction of wild-type *cat-86* depended on a +1 translational frameshift in the leader, we introduced a translation stop codon (TAA) in the +1 frame at the 5′ end of the loop (Fig. 2). This mutated form of *cat-86* was, however, fully inducible with chloramphenicol (induced and uninduced specific activities were 7.2 and 0.51).

−1 Translational frameshifting. The regulatory region of *cat-86* is remarkably similar to sequences that stimulate −1 frameshifting in *Escherichia coli* (for examples, see refer-

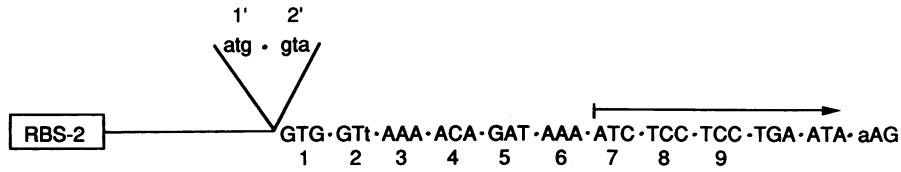

FIG. 3. Leader mutations to produce a +1 frameshift. Two additional codons, designated 1′ and 2′, were added to the 5′ end of the leader coding sequence, and leader codon 2 was changed from GUG to GUU. This version of *cat-86*, designated Mut FRM, is inducible with chloramphenicol (24). The mutation at codon 2 removes a UGA stop codon from the +1 frame of the leader coding sequence, and the addition of codons 1′ and 2′ allows deletion of a nucleotide from the 2′ codon without interfering with the *crb* sequence.

ences 30 and 31). Moreover, the single stop codon (TAA) in the −1 frame of the leader overlaps leader codons 5 and 6, . . .ACA.GAT.AAA. . ., and is therefore at the 3′ end of the leader sequence essential to induction, *crb* (Fig. 2) (24). After leader codon 6, translation in the −1 frame through the stem-loop to the *cat-86* structural gene is unimpeded (Fig. 2). Further, a −1 frameshift mutation between the leader and the *cat-86* coding sequence is predicted to fuse the leader peptide to the peptide encoded by the *cat-86* structural gene, on the basis of the DNA sequence of the region. It is evident that induction of *cat-86* does not result from a fusion of the leader peptide with the structural gene for the following reason. The N terminus of CAT-86 protein resulting from induction begins with Met specified by the initiation codon TTG. Moreover, if CAT-86 were synthesized as a fusion protein with subsequent processing to remove N-terminal amino acids, the amino acid specified by the *cat-86* start codon TTG would be Leu; TTG specifies Met when TTG is used as an initiation codon and Leu when used as an internal codon. Since the start codon for *cat-86*, TTG, specifies Met, CAT-86 protein cannot be synthesized as a fusion protein with subsequent removal of the predicted 21 N-terminal amino acids.

It is conceivable that stalling a ribosome at *crb* results in a subsequent −1 frameshift which would allow the ribosome to continue translation to RBS-3. Reinitiation of translation at RBS-3 would produce authentic CAT-86 protein. To test this notion, we added a single nucleotide, G, immediately 3′ to leader codon 6 (Fig. 2). This produced a −1 change of frame 3′ to *crb*. The result was constitutive *cat-86* expression (specific activity of 5.1). Western blot analysis of the resulting protein showed that two forms of CAT-86 protein were synthesized by the mutant gene (Fig. 4). The faster-migrating form of the CAT-86 protein comigrated with authentic CAT-86 protein, and we presume that this form corresponds to the wild-type version of the protein. The more slowly migrating (larger) peptide specified by the mutant gene is thought to be the fusion peptide, which should contain 21 additional amino acids at the N terminus. Since both peptides are produced by cells grown without inducer, we suspect that a ribosome translating through the stem-loop can reinitiate at RBS-3, as was previously shown with the +1 frameshift mutation. By this reasoning, a ribosome that reinitates at RBS-3 will produce the smaller peptide, while a ribosome that continues uninterrupted translation through RBS-3 will produce the larger, fusion peptide.

Two observations suggested that it was unlikely that *cat-86* regulation was due to a −1 translational frameshift in the leader. First, the −1 frameshift mutation described above resulted in the production of primarily the fusion peptide (Fig. 4). However, the protein produced by drug induction of the wild-type gene was shown to initiate at the authentic *cat-86* start codon, TTG, and therefore could not be the fusion peptide. Secondly, *cat-194* and *cat-221* genes contain stop codons in the −1 frame in the region of the stem-loop (15, 27). Thus, *cat-86* is unique in lacking −1 frame stop codons in the stem-loop region, yet we have supposed that the mechanisms regulating the three genes are similar if not identical. Nonetheless, we introduced a stop codon (TAA) in the −1 frame of the loop region of an otherwise wild-type *cat-86* gene (Fig. 2). This mutation did not alter the inducibility of the gene (induced and uninduced specific activities were 6.4 and 0.48). Thus, the data suggest that neither −1 nor +1 translational frameshifting is an integral component of the *cat-86* regulatory mechanism.

DISCUSSION

The translational attenuation regulatory model is widely regarded to be an explanation of the events that modulate induction of *cat* and *erm* genes. All tests of the model performed with *cat-86* have produced results that are consistent with the model (18). Translational attenuation is an extremely complex control device, and many aspects of the model remain to be adequately tested. The available data confirm those portions of the model that are most amenable to experimental analysis. Thus, it is established that the RNA secondary structure in *cat* transcripts blocks translation of the *cat* coding sequence (3, 4, 13) and that induction is due to activation of the translation of the transcripts (10). Further, induction of the gene can result from stalling a ribosome at a discrete location in the leader region of *cat* transcripts (1). However, because other predictions of the

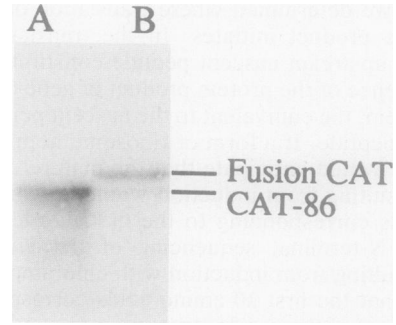

FIG. 4. Western blot demonstrating CAT-86 proteins resulting from a −1 frameshift in the leader. Lane A contains wild-type CAT-86 protein specified by *cat-86C2* (17). Lane B contains the proteins specified by a version of *cat-86* with an inserted G residue immediately after leader codon 6 (−1 frameshift). The −1 frameshift mutation places the leader and structural gene coding sequences in the same frame. Hence, the upper CAT protein band seen in lane B is believed to be the fusion protein.

model remain to be experimentally established, it is prudent to consider alternative regulatory models and to devise tests that distinguish between the models. Ribosome hopping and translational frameshifting are variations of the basic translation scheme which conceivably could be modified to regulate *cat* and *erm* (21, 33); previous studies of both genes have produced results which do not eliminate any of the models from consideration. In the present study, we performed experiments to determine whether *cat-86* might be regulated by ribosome hopping or translational frameshifting. Our results suggest that neither of these alternative models appears to accurately describe *cat-86* regulation. The data do not demonstrate that *cat-86* is regulated by the translational attenuation model but rather eliminate from consideration two highly plausible alternatives.

Translational attenuation, a variation of the transcriptional attenuation model, has been invoked to explain only the inducible regulation of two gene classes, *cat* and *erm* (7, 11, 12, 14), although the model may also apply to *tet* genes, which occur naturally in gram-positive bacteria. However, the regulatory principle of translational attenuation seems very similar to the regulation of translation of transcripts of the *mom* gene of bacteriophage Mu (36, 37). The *mom* gene ribosome binding site and initiation codon are sequestered in the secondary structure. The secondary structure is relieved not by ribosome stalling but by the binding of the regulatory protein Com to a sequence immediately 5′ to the secondary structure. Thus, the principle of blocking translation by sequestering a ribosome binding site in RNA duplexes has now been seen in several systems (21, 33). A unique aspect of translational attenuation is the means that has evolved to destabilize the secondary structure in response to a ribosomally targeted antibiotic.

Ribosome hopping, translational frameshifting, and translational attenuation are valuable devices for dissecting ribosome function. For example, the sites of ribosome stalling in *cat* and *erm* leaders have been shown to contain regions of complementarity with 16S rRNA from gram-positive bacteria (23). This may indicate a role for mRNA-rRNA pairing in the regulation. Clearly, rRNA-mRNA pairing could be fundamental to a variety of ribosome-mediated regulatory schemes, such as frameshifting and hopping. The results of the present study limit the roles that 16S rRNA may potentially play in *cat* regulation.

ACKNOWLEDGMENTS

N. Vasantha is thanked for generously providing rabbit anti-CAT-86 antibody.

This investigation was supported by Public Health Service grant GM42925 from the National Institutes of Health and grant DMB-8802124 from the National Science Foundation.

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