*Proc. Natl. Acad. Sci. USA*
Vol. 83, pp. 3939–3943, June 1986
Genetics

# Chloramphenicol induces translation of the mRNA for a chloramphenicol-resistance gene in *Bacillus subtilis*

(*cat-86* gene/chloramphenicol acetyltransferase/promoters/rifampin/ribosomes)

ELIZABETH J. DUVALL AND PAUL S. LOVETT

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

*Communicated by Hamilton O. Smith, February 7, 1986*

**ABSTRACT** ***cat-86* is a plasmid gene specifying chloramphenicol-inducible chloramphenicol acetyltransferase activity in *Bacillus subtilis*. Inducibility has been suggested to result primarily from activation of the translation of *cat-86* mRNA by subinhibitory levels of chloramphenicol. To directly test the involvement of transcription in *cat-86* induction, the gene was transcriptionally activated with a strong promoter, resulting in the synthesis of relatively high levels of *cat-86* mRNA in uninduced cells. When RNA synthesis was blocked with rifampin (100 μg/ml), *de novo* inducibility of *cat-86* by chloramphenicol could be demonstrated for more than 30 min. These results indicate that concurrent transcription is not essential for *cat-86* induction. Accordingly, *cat-86* is one of only a few inducible bacterial genes in which the primary form of regulation is at the translational level. This form of regulation may apply to other *cat* genes of Gram-positive origin whose expression is also inducible by chloramphenicol.**

A novel type of regulation that governs mRNA translation has been suggested as the basis for the inducible expression of chloramphenicol-resistance genes (*cat* genes) that commonly occur in Gram-positive bacteria (1). *cat* genes specify CAT, an intracellular enzyme that catalyzes the acetylation of chloramphenicol and thereby eliminates the antibiotic activity of the drug. *cat* genes identified in Gram-positive bacteria are inducible with chloramphenicol, and certain of these genes are also inducible with the antibiotic amicetin (2, 3). Both inducers are known to interact with the 50S ribosomal subunit (4).

The key regulatory element for inducible *cat* genes is a pair of inverted-repeat sequences that precede the coding sequence and span the ribosome binding site (RBS) (1, 5, 6). As a consequence of these inverted repeats, *cat* transcripts are predicted to sequester the RBS in the stem of a stable RNA stem–loop. The RNA stem–loop is thought to block translation of the *cat* mRNA, because the sequestered RBS seems unavailable to base-pair with 16S rRNA (1). Induction has been proposed to result from destabilization of the RNA stem–loop, which frees the *cat* RBS, permitting translation of the mRNA (1, 5). Amicetin induction of the plasmid gene *cat-86* fails to occur in mutants of *Bacillus subtilis* whose ribosomes are insensitive to the inducer (3). Thus, ribosomes probably play an essential role in destabilizing the RNA stem–loop. A current model for *cat* induction supposes that ribosomes initiate translation of a short peptide at a site upstream of the stem–loop (6, 7). This delivers the ribosomes into the RNA stem–loop, resulting in disruption of the secondary structure. Although the role of chloramphenicol and amicetin in *cat* induction is not totally clear, it has been speculated that binding of these inducing antibiotics to ribosomes may facilitate translation of the short leader peptide or may stall translating ribosomes within the RNA stem–loop. In many respects *cat* induction seems similar to a mechanism through which erythromycin is believed to induce expression of *erm*, a gene governing inducible resistance to erythromycin in Gram-positive bacteria (8).

Analysis of *cat-86* induction has provided substantial genetic evidence suggesting that chloramphenicol may be necessary for translation of *cat-86* mRNA (summarized in ref. 9). However, since this has never been directly demonstrated, it remains unclear whether the proposed activation of translation of the mRNA by chloramphenicol can occur without concurrent transcription. It is conceivable, for example, that ribosome-mediated disruption of the RNA stem–loop might only be possible concomitant with transcription through the inverted repeats. In such a model, transcription and induction are coupled events. In an alternative model, ribosome-mediated disruption of the RNA stem–loop can occur in completed (mature) *cat* transcripts. Hence, in this model transcription and induction are separable events. To distinguish between these models, we have devised a method that permits us to test *cat-86* induction long after transcription of the gene has ceased. The results show that highly efficient induction of *cat-86* can occur without concurrent transcription.

## MATERIALS AND METHODS

**Bacteria, Plasmids, and Promoters.** *B. subtilis* strain BR151 (*trpC2 metB10 lys-3*) was used throughout. Cells were grown in penassay broth for all experiments except those involving incorporation of [$^{35}$S]methionine, in which case M9 minimal medium was used containing 0.5% glucose, 0.025% acid-hydrolyzed casein, and tryptophan and lysine at 20 μg/ml.

Two versions of the promoter cloning plasmid pPL703 (10) were used. In pPL703-Spac, the *cat-86* promoter-indicator gene was activated with the spac-I promoter (11). spac-I is a strong promoter, and the resulting maximum specific activity of the product of *cat-86*, CAT, was approximately 7 (see below for units) when the gene was induced with chloramphenicol (2 μg/ml) for 1 hr. In pPL703-P2, a restriction fragment containing promoter $P_2$ served as promoter for *cat-86*; the maximum specific activity of CAT specified by pPL703-P2 was 0.6 when gene expression was induced with chloramphenicol for 1 hr (12).

spac-I is a synthetic promoter consisting of the −35 and −10 recognition sites of an early SPO1 promoter with the *Escherichia coli lac* operator inserted 3′ of the −10 sequence (11). Transcription from spac-I in *B. subtilis* is blocked by adding rifampin to cells at 100 μg/ml. This was shown by inserting pPL703-Spac into a uracil-requiring mutant of *B. subtilis*. Two minutes after rifampin addition, incorporation of [$^{3}$H]uracil ceased. Addition of chloramphenicol (2 μg/ml)

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: CAT, chloramphenicol acetyltransferase; RBS, ribosome binding site; bp, base pair(s).

to the cells did not restore RNA synthesis, as measured by [$^3$H]uracil incorporation.

**Estimation of *cat-86* mRNA Levels.** The 387-base-pair (bp) *Bcl* I–*Hin*dIII region of *cat-86* was inserted into phage M13 mp9. Resulting single-stranded M13 phage contain the sense strand of *cat-86*. The DNA was labeled by synthesizing a radioactive complement to the viral sequences, using the Klenow fragment of DNA polymerase I and the hybridization probe primer (Bethesda Research Laboratories, catalog no. 82385A). RNA isolation and dot blot analysis were as previously described (7, 12). Decay of *cat-86* mRNA was measured by blocking cellular RNA synthesis with rifampin (100 μg/ml). RNA isolated at 2, 10, 15, and 20 min after inhibition was used for dot blot analysis. Hybridization intensities were quantitated by densitometric scanning. *cat-86* mRNA half-life measurements were made on RNA from BR151 containing pPL703-Spac or pPL703-P2. These cells were grown without chloramphenicol.

**CAT Assays.** Enzyme activity was assayed by the colorimetric procedure of Shaw (13). Protein was measured by the method of Bradford (14). CAT specific activity is expressed as μmol of chloramphenicol acetylated per min per mg of protein at 25°C.

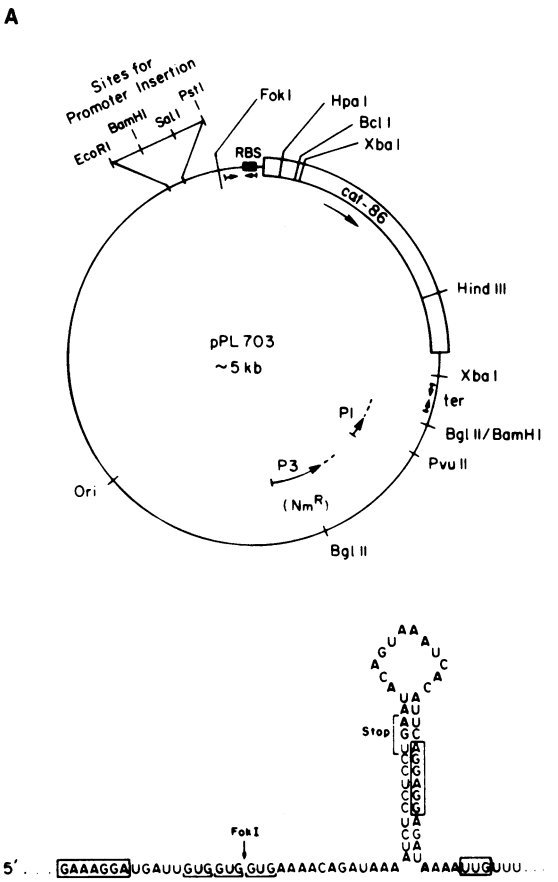
**NaDodSO$_4$/Polyacrylamide Gel Electrophoresis.** The procedure followed was that of Laemmli (15); separation gels were 12% acrylamide.

## RESULTS

**Induction of *cat-86* in Rifampin-Inhibited Cells.** *cat-86* is the indicator gene in the *B. subtilis* promoter cloning plasmid pPL703 (Fig. 1). Expression of *cat-86* in this plasmid requires the insertion of a promoter into the multicloning-site linker located 144 bp upstream from the *cat-86* coding sequence. The nucleotide sequences essential to inducible expression of *cat-86* have been shown to reside in the 144-bp region between the linker and the coding sequence (17). Accordingly, expression of *cat-86* is always chloramphenicol-inducible regardless of the promoter that is used to transcriptionally activate the gene.

Versions of *cat-86* that are activated by a strong promoter such as spac-I or by a much weaker promoter such as $P_2$ specify detectable levels of *cat-86* mRNA in *B. subtilis* cells not exposed to chloramphenicol (Fig. 1). Since spac-I directed the synthesis of about 40-fold higher levels of *cat-86* mRNA in uninduced cells than did $P_2$, we tested the ability of chloramphenicol to induce translation of the pPL703-Spac-specified RNA. Log-phase cells of BR151(pPL703-Spac) were treated with rifampin (100 μg/ml) and after 2 min, chloramphenicol was added to 2 μg/ml. The cells were then periodically assayed for CAT. The results indicate that the presence of the RNA-synthesis inhibitor permitted a normal induction of CAT activity (Fig. 2). Similar results were obtained when streptolydigin (100 μg/ml) was substituted for rifampin and when spac-I was replaced with another strong promoter, $P_4$ (12).

If chloramphenicol were inducing translation of *cat-86* mRNA, we would anticipate that the rate of decline of *cat-86* induction after inhibition of RNA synthesis might parallel the physical decay of *cat-86* mRNA. However, data in Fig. 3 show that, after rifampin inhibition, inducibility declined more slowly than the physical half-life of the mRNA, determined in separate experiments to be 8 ± 0.5 min. This result can be explained by assuming that spac-I caused the synthesis of amounts of *cat-86* mRNA far in excess of that needed to achieve induction. For example, when *cat-86* mRNA levels are very high, perhaps only a fraction of the molecules are actually complexed with chloramphenicol-bound ribosomes that are involved in destabilization of the stem–loop structure. If this explanation were correct, the longevity of *cat-86* inducibility would diminish if Spac were replaced with a weaker promoter that caused the synthesis of much lower



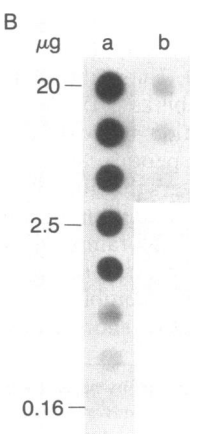




FIG. 1. (*A*) Diagram of pPL703 and the region of the *cat-86* transcript spanning the inverted repeats. *ter* is the transcription-termination site for *cat-86* (16); kb, kilobases. Boxed sequences include the *cat-86* translation initiation codon UUG, the *cat-86* RBS AGGAGG, and a potential RBS, GAAAGGA, that may be involved in initiating translation of a putative regulatory peptide beginning at a GUG codon and terminating at a UGA codon in the stem–loop (7). The complete nucleotide sequence of *cat-86* and flanking regulatory regions are in ref. 17. (*B*) Quantitation of *cat-86* mRNA in BR151 harboring pPL703-Spac (column a) or pPL703-P2 (column b) by dot blot hybridization. The two series of RNA spots each begin at 20 μg and decrease in factor-of-2 increments.

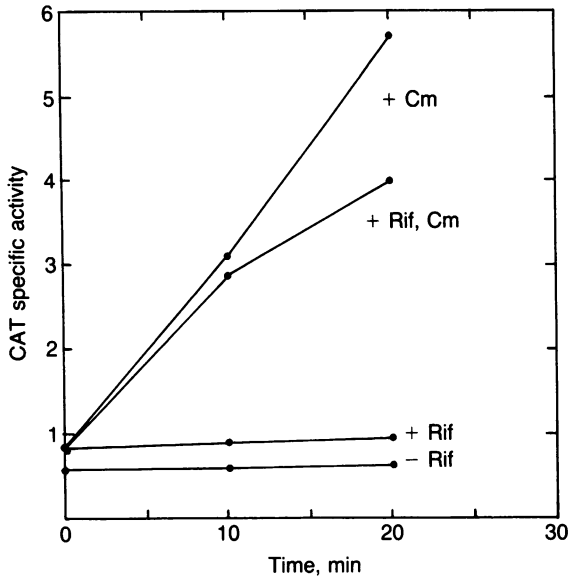



FIG. 2. Chloramphenicol induction of *cat-86* in pPL703-Spac after rifampin inhibition of host cells. Log-phase cells of BR151-(pPL703-Spac) were pretreated with rifampin at 100 μg/ml for 2 min and then induced with chloramphenicol at 2 μg/ml in the presence of rifampin (+ Rif, Cm). Controls included cells induced without rifampin (+ Cm) and uninduced cells incubated in the presence of (+ Rif) or absence (− Rif) of rifampin. CAT was assayed after cell disruption as described (1).

levels of *cat-86* mRNA. Therefore, we tested the inducibility of *cat-86* in pPL703-P2, using BR151 cells as host as outlined in Fig. 2. The result was that *cat-86* could not be detectably induced by chloramphenicol 2 min after rifampin addition, although a 5-fold induction was observed in the control cells not treated with rifampin.

*cat-86* in pPL703-P2 could be partially induced if chloramphenicol was added to host cells simultaneously with rifampin (Fig. 4). However, the subsequent decrease in *cat-86* inducibility was very rapid, appearing to decline more quickly than the physical half-life of *cat-86* mRNA. Decay of mRNA is thought to result from endonucleolytic cleavage within mRNA molecules, followed by or concurrent with exonuclease digestion (18–20). Our measurements of the physical half-life reflect the loss of hybridizable sequences due to exonuclease digestion. However, induction of an mRNA molecule can be abolished, in theory, by a single endonucleolytic break. Hence, when *cat-86* mRNA is limiting, inducibility might be expected to decline more rapidly than the loss of *cat-86* RNA sequences.

**Induction of *cat-86* After Host Protein Synthesis Has Largely Ceased due to Rifampin Inhibition.** The observation that *cat-86*, in pPL703-Spac, remained chloramphenicol-inducible long after rifampin inhibition of RNA synthesis suggested that induction was occurring in cells whose overall protein synthesis was greatly diminished due to mRNA decay. To test this, BR151 (pPL703-Spac) cells were exposed to rifampin (100 μg/ml) and samples were removed immediately (0 time), after 15 min in rifampin, and after 30 min in rifampin. Half of each sample was induced with chloramphenicol (2 μg/ml) for 15 min. Throughout the 15-min incubation, both the induced and uninduced halves of each sample were exposed to [$^{35}$S]methionine, and the presence of rifampin was maintained throughout the experiment. After the 15-min incubation, the total soluble protein was isolated from each sample and subjected to $NaDodSO_4$/PAGE and autoradiography. The results of this experiment show that when the cells are treated with rifampin and chloramphenicol simultaneously, the inducible CAT polypeptide is detected along with an array of polypeptides whose synthesis is not chloramphenicol-inducible (Fig. 5). Lysates derived from cells incubated in rifampin for 15 and 30 min prior to chloramphenicol induction contained the inducible CAT polypeptide, indicating that this protein is inducibly synthesized long after cessation of RNA synthesis. However, the numbers of non-CAT polypeptides synthesized was greatly reduced in the 15- and 30-min samples presumably because of decay of the mRNAs for these non-CAT polypeptides (Fig. 5). Certain

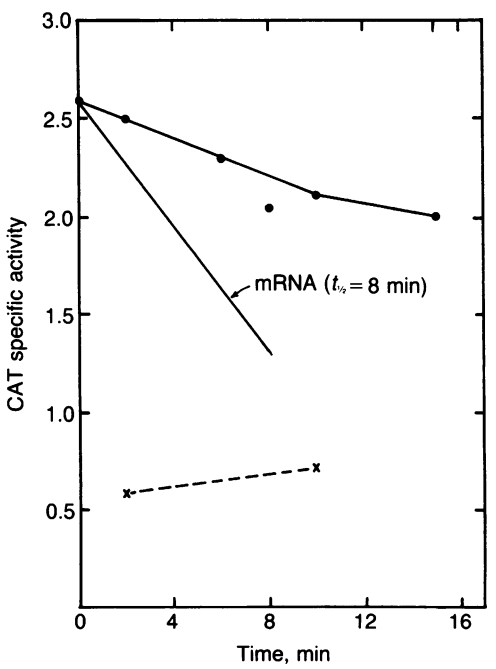



FIG. 3. Decrease in the inducibility of *cat-86* in BR151(pPL703-Spac) during rifampin inhibition. Log-phase cells were treated with rifampin (100 μg/ml) at 0 time. Samples were withdrawn immediately (0 time) and at periodic intervals up to 15 min. Each was induced with chloramphenicol (2 μg/ml) for 10 min and assayed for CAT. The broken line represents uninduced control cells incubated without rifampin. Uninduced control cells incubated with rifampin gave parallel results. The half-life ($t_{1/2}$) for *cat-86* mRNA, determined by the decrease in hybridizable sequences after rifampin inhibition of cells, was 8 ± 0.5 min. This slope is shown for comparison.

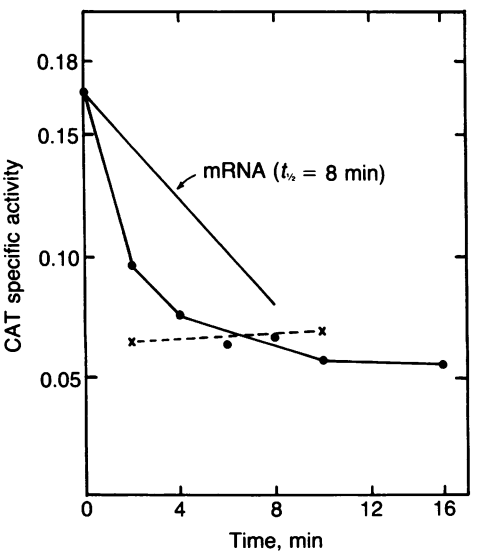



FIG. 4. Decrease in the inducibility of *cat-86* in BR151(pPL703-P2) during rifampin inhibition. Experimental details were as for Fig. 3, except that *cat-86* was activated with the $P_2$ promoter.

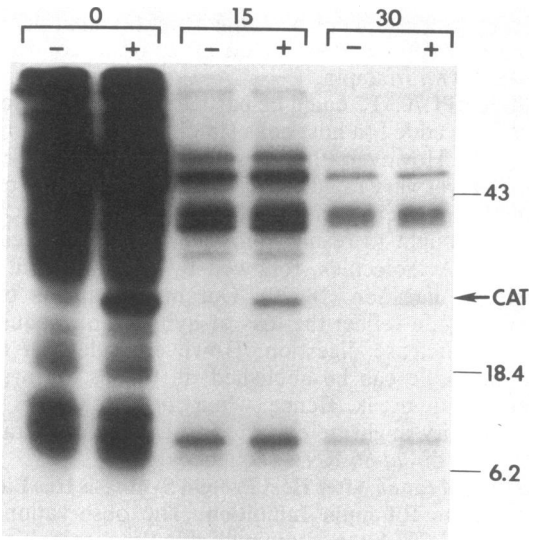



FIG. 5. Detection of a chloramphenicol-inducible polypeptide after rifampin (100 μg/ml) inhibition of BR151(pPL703-Spac). Samples were removed immediately after rifampin addition (0 time) and after 15 and 30 min incubation with the RNA synthesis inhibitor. Each sample was split. Both halves were exposed to [$^{35}$S]methionine (10 μCi/ml; 1 Ci = 37 GBq) for 15 min, one in the presence (lanes +) and one in the absence (lanes −) of chloramphenicol (2 μg/ml). Cell lysates were analyzed by NaDodSO$_4$/PAGE followed by autoradiography. Numbers at right are molecular weight markers ($M_r \times 10^{-3}$).

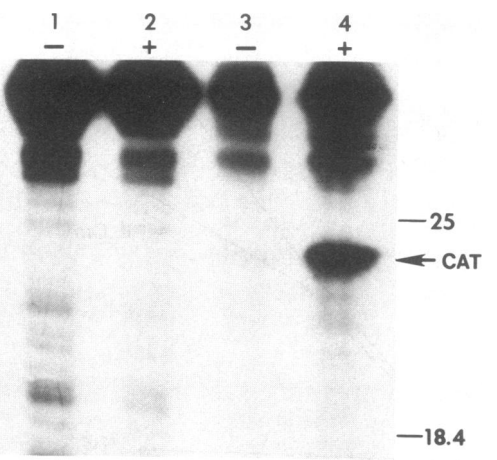



FIG. 6. Elimination of the chloramphenicol-inducible polypeptide by a deletion in *cat-86*. Log-phase BR151 cells harboring pPL703ΔXba-Spac (lanes 1 and 2) or pPL703-Spac (lanes 3 and 4) were treated with rifampin (100 μg/ml) for 15 min. [$^{35}$S]Methionine was then added to 10 μCi/ml and a portion of each culture was induced for 15 min with chloramphenicol (2 μg/ml). The cells were lysed, and the lysates were subjected to electrophoresis and autoradiography as in Fig. 5. Only the relevant region of the autoradiogram is shown.

of the non-CAT polypeptides detected in the zero-time sample were also detected in the cells that had been incubated for 30 min in rifampin. We presume these peptides are the translation products of mRNA molecules that have a long half-life or are highly abundant.

The nucleotide sequence of *cat-86* predicts the protein product will have a molecular weight of 26,000, assuming the protein is not processed after synthesis (21). However, the chloramphenicol-inducible polypeptide detected in Fig. 5 has an apparent molecular weight of 23,000, which may indicate an anomalous rate of migration of the protein in the gel system or could indicate processing of the CAT peptide after synthesis. In order to unambiguously establish that this chloramphenicol-inducible peptide was the direct product of *cat-86*, a 595-bp deletion was made in pPL703-Spac by digestion with *Xba* I and ligation. This *Xba* I deletion removes all the *cat-86* coding sequence 3′ of codon 31 (21). When this plasmid, pPL703ΔXba-Spac, was used to direct chloramphenicol-inducible protein synthesis in rifampin-blocked cells, the $M_r$ 23,000 peptide was absent (Fig. 6). Thus, the chloramphenicol-inducible peptide appears to be the product of the *cat-86* gene.

## DISCUSSION

Our results show that induction of *cat-86* by chloramphenicol results primarily from activation of translation of mRNA that is present in cells before addition of the inducer. We feel it significant that induction of *cat-86* mRNA in rifampin-treated cells could only be achieved by activating expression of the gene with a much stronger promoter than is normally found in association with *cat* genes related to *cat-86*. For example, the $P_2$ restriction fragment used in this study contains a weak promoter that is normally used to transcriptionally activate *cat-66*, a gene nearly identical to *cat-86* (12). However, $P_2$ apparently did not promote high enough levels of *cat-86* mRNA to allow detectable induction in rifampin-blocked cells. Presumably, the normal regulation of *cat-86* is the sum of several factors, including a balance between the level at which the gene product is needed within the cell and the rates of synthesis and decay of the corresponding mRNA. Use of the spac-I promoter probably produces an imbalance, allowing the testing of the translational control model. We suspect the mechanism governing chloramphenicol induction of *cat-86* is representative of the regulatory mechanism governing induction of all inducible *cat* genes that have been examined. Independent studies of the induction of the pC194 and the pUB112 *cat* genes are consistent with this interpretation (5, 6).

Our studies do not eliminate the possibility that a minor aspect of the inducible regulation of *cat-86* is at the transcriptional level. For example, Ambulos *et al.* (22) demonstrated that the RNA stem–loop that sequesters the *cat-86* RBS functions as a weak transcription-termination signal in *B. subtilis*. It remains unclear whether the termination activity of the RNA stem–loop is relieved during chloramphenicol induction. However, the published evidence (22) suggests that if chloramphenicol induction does relieve termination, the effect is minor and may not contribute significantly to the inducible regulation.

Regulation of mRNA translation is an uncommon form of gene control in prokaryotes, although other examples have been reported (8, 23–25). In the case of induction of *cat* by chloramphenicol or *erm* by erythromycin, the ribosome is clearly the cell sensor for the presence of the inducing antibiotic in the environment. When ribosomes detect low levels of an inducing antibiotic such as chloramphenicol, the drug-modified ribosomes participate in activating a gene that confers high-level chloramphenicol resistance, *cat*. Since the induction of both *cat* and *erm* primarily reflects activation of mRNA translation, perhaps this form of gene regulation provides a more rapid response to the presence of potential inhibitory antibiotics than can be achieved by transcriptional regulation.

We thank Sung So, for measuring the half-life of *cat-86* mRNA, and Dennis Henner, for generously providing the spac-I promoter. This investigation was supported by National Science Foundation Grant PCM-8202701 and National Institutes of Health Grant AI21350.

1. Duvall, E. J., Williams, D. M., Lovett, P. S., Rudolph, C., Vasantha, N. & Guyer, M. (1983) *Gene* **24**, 170–177.

2. Shaw, W. V. (1983) *Crit. Rev. Biochem.* **14,** 1–43.
3. Duvall, E. J., Mongkolsuk, S., Kim, U. J., Lovett, P. S., Henkin, T. M. & Chambliss, G. H. (1985) *J. Bacteriol.* **161,** 665–672.
4. Vazquez, D. (1979) *Mol. Biol. Biochem. Biophys.* **30,** 108–112.
5. Byeon, W.-H. & Weisblum, B. (1984) *J. Bacteriol.* **158,** 543–550.
6. Bruckner, R. & Matzura, H. (1985) *EMBO J.* **4,** 2295–2300.
7. Ambulos, N. P., Jr., Mongkolsuk, S., Kaufman, J. D. & Lovett, P. S. (1985) *J. Bacteriol.* **164,** 696–703.
8. Dubnau, D. (1982) *Crit. Rev. Biochem.* **16,** 103–132.
9. Lovett, P. S. (1985) in *Microbiology-1985*, eds. Leive, L. & Silver, S. D. (ASM, Washington, DC), pp 397–400.
10. Mongkolsuk, S., Chiang, Y.-W., Reynolds, R. G. & Lovett, P. S. (1983) *J. Bacteriol.* **155,** 1399–1406.
11. Yansura, D. G. & Henner, D. J. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 439–443.
12. Duvall, E. J., Williams, D. M., Mongkolsuk, S. & Lovett, P. S. (1984) *J. Bacteriol.* **158,** 784–790.
13. Shaw, W. V. (1975) *Methods Enzymol.* **43,** 737–755.
14. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254.
15. Laemmli, U. K. (1970) *Nature (London)* **227,** 680–685.
16. Mongkolsuk, S., Duvall, E. J. & Lovett, P. S. (1985) *Gene* **37,** 83–90.
17. Mongkolsuk, S., Ambulos, N. P., Jr., & Lovett, P. S. (1984) *J. Bacteriol.* **160,** 1–8.
18. Morse, D. E., Mosteller, R. D., Baker, R. F. & Yanofsky, C. (1969) *Nature (London)* **223,** 40–43.
19. Morkawa, N. & Imamoto, F. (1969) *Nature (London)* **223,** 37–40.
20. Guarneros, G., Montanez, C., Hernandez, T. & Court, D. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 238–242.
21. Harwood, C. R., Williams, D. M. & Lovett, P. S. (1983) *Gene* **24,** 163–169.
22. Ambulos, N. P., Jr., Mongkolsuk, S. & Lovett, P. S. (1985) *Mol. Gen. Genet.* **199,** 70–75.
23. Lemaire, G., Gold, L. & Yarus, M. (1978) *J. Mol. Biol.* **126,** 73–86.
24. Karem, J., Gold, L., Singer, B. S. & Dawson, M. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 4669–4672.
25. Miura, A., Krueger, J. H., Itoh, S., deBoer, H. & Nomura, M. (1981) *Cell* **25,** 773–779.