Chloramphenicol-Induced Translation of *cat-86* mRNA Requires Two *cis*-Acting Regulatory Regions

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Sequences essential to the chloramphenicol-inducible expression of *cat-86*, a chloramphenicol acetyltransferase gene, reside in a 144-base pair (bp) regulatory region that intervenes between the *cat-86* coding sequence and its promoter. A key regulatory element within the 144-bp segment consists of a pair of inverted-repeat sequences that immediately precede the *cat-86* coding region and span the ribosome-binding site for the gene. Because of the location of the inverted repeats, *cat-86* transcripts are predicted to sequester the ribosome-binding site in a stable RNA stem-loop structure which should block translation of *cat-86* mRNA. Chloramphenicol induction of gene expression is believed to result from ribosome-mediated destabilization of the RNA stem-loop structure, which frees the *cat-86* ribosome-binding site, thereby allowing translation. In this study we demonstrated that deletion of 85 bp from the 5' end of the 144-bp regulatory region abolishes inducible expression of *cat-86*, although the gene is transcribed. This deletion leaves intact both the inverted repeats and the *cat-86* coding sequence, and the deletion mutation is not complementable. Therefore, inducible regulation of *cat-86* requires the inverted repeats plus an upstream, *cis*-acting regulatory region. The *cis*-acting region is believed to control translation of *cat-86* mRNA by its essential participation in chloramphenicol-induced opening of the RNA stem-loop. *cat-86* deleted for the 85-bp regulatory region and therefore virtually unexpressed was used to select for mutations that restore expression to the gene. An analysis of one mutant plasmid showed that the *cat-86* gene is constitutively expressed and that this results from a duplication of the DNA sequence that spans the ribosome-binding site. The duplication provides *cat-86* with two ribosome-binding sites. One of these sites is predicted to be sequestered in an RNA stem-loop, and the other is not involved in RNA secondary structure.

Bacterial resistance to the antibiotic chloramphenicol is commonly due to the presence of the enzyme chloramphenicol acetyltransferase (CAT) (23). CATs from a variety of bacteria have been shown to catalyze the acetylation of chloramphenicol, rendering the drug biologically inactive (22). CAT is an intracellular, multimeric enzyme consisting of a single protein subunit (23). The gene for the subunit protein is designated *cat*. As a rule, *cat* genes which occur naturally in gram-negative bacteria are expressed constitutively, whereas *cat* genes which have been identified in gram-positive bacteria are typically inducible with chloramphenicol (22).

The inducibility of five *cat* genes from gram-positive bacteria has been shown to correlate with a unique nucleotide sequence arrangement immediately 5' to the *cat* coding region (7, 8, 11, 24; R. Brückner and H. Matzura, EMBO J., in press). In each case the *cat* ribosome-binding site in DNA is within one member of a pair of inverted-repeat sequences. Therefore, transcripts of these *cat* genes are predicted to sequester the ribosome-binding site in the stem of a stable RNA stem-loop structure. The apparent consequence is that *cat* mRNA is not translated because the ribosome-binding site is unavailable to base pair with 16S rRNA. This initial observation led to the proposal by Duvall et al. (7) that chloramphenicol induction may be due to destabilization of the RNA stem-loop structure and *cat* induction might result primarily from a form of posttranscriptional regulation. Evidence in support of this general model has been provided through a study of the inducibility of two similar, but nonidentical *cat* genes, the *cat-86* gene cloned from *Bacillus pumilus* and the *cat* gene that is resident on *Staphylococcus*

aureus plasmid pC194. Constitutively expressed variants of both genes result from deletions that extend into the upstream member of the inverted repeats (1, 19). These deletions prevent the formation of the RNA stem-loop that would normally sequester the *cat* ribosome-binding site. Moreover, the regulatory signals essential to the inducibility of *cat-86* have been shown to reside within a 144-base pair (bp) transcribed region that intervenes between *cat-86* and its promoter (14). This regulatory region spans the inverted repeats.

If *cat* induction results only from an increase in the efficiency of translation of *cat* mRNA, uninduced cells would be expected to contain detectable *cat* mRNA levels, and these levels would probably not increase during induction. In the case of the pC194 *cat* gene, Byeon and Weisblum (5) detected a basal level of the corresponding mRNA in uninduced cells, and no increase was observed during induction. This suggests an absence of transcriptional regulation in the induction of pC194 *cat*. In contrast, the level of *cat-86* mRNA that is present in uninduced cells has been shown to increase about twofold during induction (2, 8). This increase is significantly less than the 10- to 15-fold increase in the specific activity of CAT that occurs during *cat-86* induction, indicating that the increase in mRNA level is not a major factor governing induction. However, the increase in *cat-86* mRNA does appear to be due to a function of the RNA stem-loop. The *cat-86* RNA stem-loop acts as a weak transcription termination signal in vitro and in vivo (2). Termination in vivo is partially relieved during induction. Therefore, destabilization of the *cat-86* RNA stem-loop seems to primarily increase the translation efficiency of the mRNA and also appears to increase the level of the mRNA.

Operating hypotheses to explain *cat* induction propose

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TABLE 1. Plasmids

Plasmid	Relevant properties	Reference
pPL703	Promoter-cloning plasmid ^a	15
pPL708	pPL703 + 284-bp <i>Eco</i> RI fragment from SPO2 containing a promoter	7
pPL703-Spac	pPL703 + the Spac promoter	25
pPL703ΔA	pPL703 deleted for 85-bp regulatory component A	This paper
pPL708ΔA	pPL708 deleted for 85-bp regulatory component A	This paper
pPL703ΔA-Spac	pPL703-Spac deleted for 85-bp regulatory component A	This paper
pPL703ΔAC1	pPL703ΔA containing a 20-bp duplication that spans the <i>cat-86</i> ribosome-binding site	This paper
p5'αB10Δ1	Constitutively expressed <i>cat-86</i> variant in which the gene is activated by the amylase promoter	19

^a See Fig. 1.

that the inducing antibiotic binds to ribosomes, enabling the particles to perform a function related to destabilization of the RNA stem-loop. Although it is clear that ribosomes are involved in induction (6; Ambulos, manuscript in preparation), it is not known whether the ribosomes interact directly with the RNA stem-loop or whether mRNA sequences upstream from the RNA stem-loop are involved. For example, we previously identified a sequence (5' GAAAGGA) 26 bp upstream from the *cat-86* RNA stem-loop which, in theory, shares extensive homology with the 3' end of 16S rRNA (14, 18). If this sequence or other upstream sequences were involved in the ribosome-mediated induction of *cat-86*, deletions to sites immediately 5' to the inverted repeats should block induction. In this study we demonstrated the effect on induction of a deletion of the *cat-86* regulatory region that extends to a site 15 bp upstream from the inverted repeats.

MATERIALS AND METHODS

Bacteria and plasmids. The strains of *Bacillus subtilis* used were strain BR151 (*trpC2 lys-3 metB10*) and strain BGSC 1A422 (*recE4 leuB6*) from the *Bacillus* Genetic Stock Center, Ohio State University. *Escherichia coli* JM103 was the plating host used for M13 cloning vectors (13). All of the growth media used have been described previously (8). The plasmids used are shown in Table 1. The procedures used for plasmid isolation, restriction enzyme mapping, and agarose gel electrophoresis have been described previously (8). M13 and pUC vectors were purchased from Bethesda Research Laboratories, Inc.

Construction of pPL708ΔA and pPL703ΔA. The 637-bp *Eco*RI-*Hind*III fragment from pPL703 (Fig. 1 and 2) had previously been inserted into pSP65 (2). The insertion contained two *Fok*I-sensitive sites. One was located between the *Eco*RI and *Xba*I sites in the insertion. The pSP65 derivative containing the insertion was digested with *Fok*I, gap filled with DNA polymerase I, and digested to completion with *Xba*I. The resulting 155-bp fragment had one blunt end (corresponding to the *Fok*I cleavage site at position -59 in Fig. 2) and one *Xba*I end, and this fragment was electroeluted from a 1% agarose gel and cloned between the

*Hinc*II and *Xba*I sites of pUC18. In pUC18 the *Hinc*II site is immediately preceded by a *Pst*I site, and the cloned fragment contained a *Bcl*II site adjacent to the *Xba*I end (Fig. 2). Although the *Bcl*II site is methylated in strain JM103 and therefore cannot be cut with *Bcl*II, this site can be cleaved with *Sau*3A. Therefore, the cloned fragment was cut from the pUC18 vector as a *Pst*I-*Sau*3A fragment. This fragment was used to replace the *Pst*I-*Bcl*II fragment in pPL708, yielding pPL708ΔA. pPL703ΔA was derived from pPL708ΔA by digesting the plasmid with *Eco*RI and ligation. This removed the 284-bp promoter fragment (21). To confirm the construction, the *Pst*I-*Hind*III fragment from pPL708ΔA was cloned into M13mp11, and the fragment was sequenced in one direction from the *Pst*I site to the *Xba*I site at codon 31 of *cat-86*. This sequence was identical to the predicted sequence. To assure that the manipulations had not altered the *cat-86* coding sequence, the *Xba*I fragment (595 bp) was cut from pPL708ΔA, and this fragment was used to replace the 595-bp *Xba*I fragment in pPL708. In the resulting plasmid *cat-86* was inducibly expressed.

Enzyme assays and DNA sequencing. CAT was assayed by

FIG. 1. Diagram of promoter-cloning plasmid pPL703. This plasmid consists of a 1,250-bp *Pst*I-*Bgl*II DNA fragment spanning *cat-86*, which was previously cloned from *Bacillus pumilus* NCIB 8600, inserted between the *Eco*RI and *Bam*HI sites of pUB110 by using a 21-bp *Eco*RI-*Pst*I synthetic linker. The complete nucleotide sequence of the 1,250-bp DNA is shown in Fig. 2. The large arrows labeled P1 and P3 are vector transcripts reported by J. F. Curran and C. R. Stewart (Virology, in press) and Brückner et al. (4). These transcripts do not enter the *cat-86* coding sequence (S. Mongkolsuk, E. J. Duvall, and P. S. Lovett, Gene, in press). The short inverted arrows indicate inverted repeats that precede and follow *cat-86*. The inverted repeats that follow *cat-86* are designated ter, since they act as efficient transcription termination sites in *B. subtilis* and *E. coli* (Mongkolsuk et al., in press). The 144 bp of DNA between the *Pst*I site and the translation initiation codon for *cat-86* contains the signals for chloramphenicol and ampicillin inducibility (6). The *Fok*I cleavage site divides the 144-bp regulatory region into components A and B. Nm^r indicates the vector gene that confers neomycin resistance. RBS, Ribosome binding site; kb, kilobase.

Detection of *cat-86* RNA by the dot blot procedure. RNA was isolated from plasmid-containing strain BR151 cells as previously described (16) and spotted onto nitrocellulose strips at concentrations of 5, 10, and 20 µg per spot. The probe was prepared by cloning the 387-bp *Bcl*I-*Hind*III region from within the *cat-86* coding sequence into M13mp9 and synthesizing a radioactive complement to the M13 sequences in the single-stranded phage by using the hybridization probe primer.

Construction and properties of a noninducible variant of *cat-86*. *cat-86* is the indicator gene in *B. subtilis* promoter-cloning plasmid pPL703 (Fig. 1). This gene encodes CAT. When *cat-86* is transcriptionally activated by inserting various promoters into the multicloning site linker in pPL703, the resulting expression of the gene is invariably chloramphenicol inducible. In two cases it has been demonstrated that the transcription start site for *cat-86* mRNA is within the inserted promoter-containing restriction fragment (8, 16). Therefore, induction appears not to involve regulation of transcription initiation. Chloramphenicol induction also does not require the *cat-86* gene product. This was most effectively demonstrated by deleting *cat-86* coding sequences 3'

TABLE 2. Chloramphenicol resistance and CAT levels specified by derivatives of pPL703^a

Plasmid	Promoter for <i>cat-86</i>	Chloramphenicol MIC (μg/ml)	CAT sp (μmol/min per mg) with the following inducers:		
			None	Chloramphenicol (2 μg/ml)	Amicetin (0.5 μg/ml)
pPL703ΔA	Absent	2	0.01	0.01	0.01
pPL703	Absent	5	0.02	0.04	0.05
pPL708ΔA	Present	10	0.08	0.08	0.08
pPL708	Present	>50	0.26	2.74	2.53

^a Strain BR151 harboring the plasmids was grown for 18 h in Pennassay broth (Difco Laboratories) containing the following concentrations of chloramphenicol: 0, 1, 2, 5, 10, 20, 30, and 50 μg/ml. The lowest concentration preventing detectable growth was the MIC. CAT specific activity was determined by incubating plasmid-containing strain BR151 cells for 2 h in drug-free broth or broth containing 2 μg of chloramphenicol per ml or 0.5 μg of amicetin per ml.

to codon 2 and replacing the deleted region with an in-frame *lacZ* fusion (14). The resulting plasmid specified chloramphenicol-inducible β-galactosidase. Therefore, plasmid sequences which are essential to inducibility reside exclusively within a 144-bp regulatory region (designated -1 to -144 in Fig. 2) that intervenes between the site of the promoter insertion in pPL703 and the *cat-86* coding sequence (14) (Fig. 2). A previously identified *cis*-acting regulatory element within this 144-bp sequence is proximal to the *cat-86* coding region (7). This element consists of two 14-bp inverted-repeat sequences which are separated by 12 bp. The inverted repeat which is most proximal to *cat-86* spans the ribosome-binding site for the gene. Therefore, transcripts of *cat-86* are predicted to sequester the ribosome-binding site in RNA secondary structure (7). It is believed that induction of *cat-86* involves destabilization of the RNA secondary structure through the action of host ribosomes which are functionally altered by binding either of the two inducers of *cat-86* (chloramphenicol and amicetin) (6, 7). To determine whether sequences upstream from the inverted repeats were involved in the inducible expression of *cat-86*, 85 bp was deleted from the 5' end of the 144-bp regulatory region by taking advantage of a *FokI* cleavage site at this location. This deletion, which we designated ΔA, extends to a site that is 15 bp upstream from the inverted repeats (Fig. 2). Therefore, both the inverted repeats and the *cat-86* coding sequence are not structurally affected by the deletion.

Plasmids pPL703ΔA and pPL708ΔA both contain the 85-bp deletion, and these plasmids differ only in the presence or absence of an inserted promoter for *cat-86*. pPL703ΔA lacks the promoter, and pPL708ΔA contains the 284-bp *EcoRI* promoter-containing fragment from phage SPO2 (21). pPL703 and pPL708 are the corresponding nondeleted forms of these plasmids. To examine the effects of the 85-bp deletion on *cat-86* expression, the four plasmids were transformed into *B. subtilis* strain BR151, and the MICs of chloramphenicol were determined. The MIC determined for pPL708 was >50 μg of antibiotic per ml, whereas the MIC determined for pPL708ΔA was 10 μg/ml (Table 2). Therefore, the 85-bp deletion greatly reduced *cat-86* expression. However, the MIC determined for pPL708ΔA was somewhat greater than the MICs determined for promoter-deficient plasmids pPL703 and pPL703ΔA (Table 2). To measure the effect of the deletion more precisely, the CAT levels specified by the plasmids were determined (Table 2).

The CAT specific activity specified by pPL708 in uninduced cells was about 0.26 μmol/min per mg, and this value increased 10-fold when the cells were grown for 2 h in the presence of subinhibitory levels of either inducer (chloramphenicol or amicetin). By comparison, the CAT specific activity specified by pPL708ΔA in uninduced cells was only 0.08 μmol/min per mg, and this value did not detectably increase during 2 h of growth in the presence of either chloramphenicol or amicetin. In agreement with the MIC studies, the CAT level specified by pPL708ΔA was somewhat greater than the CAT level specified by promoter-deficient plasmids pPL703 and pPL703ΔA (Table 1).

The lack of *cat-86* expression in pPL708ΔA could have resulted if the 85-bp deletion prevented induction by removing sequences that are essential to destabilization of the RNA stem-loop. Alternatively, the deletion might unexpectedly block transcripts from entering the *cat-86* coding sequence. To test this possibility, RNA from strain BR151 harboring pPL703ΔA, pPL708ΔA, and pPL708 was spotted onto nitrocellulose strips. To this we hybridized a radioactive probe consisting of an internal sequence from within *cat-86* in an M13 vector. No hybridization was detected to the pPL703ΔA-specified RNA, as predicted since the *cat-86* gene lacks a promoter (Fig. 3). However, comparable hybridization intensities were obtained with RNAs specified by pPL708ΔA and pPL708 (Fig. 3). Therefore, the lack of expression of *cat-86* in pPL708ΔA was judged to be due to an inability of the gene to be induced, and we believe that induction primarily results from an increase in the efficiency of translation of *cat-86* mRNA.

The inability of *cat-86* in pPL708ΔA to be inducibly expressed greatly diminished the ability of the plasmid to confer chloramphenicol resistance on *B. subtilis*, but did not abolish the chloramphenicol resistance trait. For example, log-phase cells of strain BR151 harboring nondeleted parent plasmid pPL708, a plasmid which specifies both chloramphenicol resistance and neomycin resistance, exhibited an efficiency of plating of about 1 on agar containing either neomycin (10 μg/ml) or chloramphenicol (10 μg/ml), and full colony size on these media was attained after about 24 h of incubation. The promoter-deficient plasmid pPL703 exhibited an efficiency of plating of 1 on neomycin-containing agar and an efficiency of plating of less than 10⁻⁸ on chloramphenicol agar. By comparison, cells harboring pPL708ΔA plated at an efficiency of only 0.1 on agar containing 10 μg of chloramphenicol per ml. However, individual colonies were not visually detected until after 30 h of incubation, and

FIG. 3. Dot blot analysis of RNAs from strain BR151 containing pPL703ΔA, pPL708ΔA, and pPL708. RNAs were isolated from cells grown without chloramphenicol and were spotted at concentrations of 5, 10, and 20 μg per spot.

development of full colony size required about 48 h of incubation. Representative cells containing pPL708ΔA that grew to colonies during prolonged incubation in the presence of 10 μg of chloramphenicol per ml were not mutants, since the *cat-86* gene remained noninducible and was weakly expressed. These observations are consistent with our interpretation that the *cat-86* coding sequence in pPL708ΔA is structurally intact and is very weakly expressed, but the expression is not enhanced by exposure to chloramphenicol.

Noninducibility of *cat-86* deleted for component A was independent of the promoter chosen to transcriptionally activate the gene. The promoter used to transcriptionally activate *cat-86* in pPL708 and pPL708ΔA is within a 284-bp *EcoRI* fragment (fragment P4) derived from phage SPO2 (21). It was possible that the loss of inducible expression of *cat-86* in pPL708ΔA was due to an unanticipated interaction between sequences in promoter fragment P4 and *cat-86*. Such a hypothetical interaction might be made possible by deletion of the intervening component A. This was tested by replacing promoter fragment P4 with the Spac promoter (26). Spac is a strong promoter and consists of a sequence nearly identical to an early SPO1 phage promoter, except that the *E. coli lac* operator sequences are inserted 3' to the -10 recognition site (26). When this promoter was placed upstream from *cat-86* in pPL703, expression of the gene was inducible with both chloramphenicol and ampicillin (Table 3). Moreover, the level of expression of the gene in the uninduced and induced states reflected the strength of the promoter. When Spac was inserted upstream from *cat-86* in pPL703ΔA, expression of the gene was not inducible (Table 3). Thus, the loss of inducible expression of *cat-86* is likely due only to deletion of component A.

Noninducibility of *cat-86* in pPL708ΔA was not complementable. If deletion of component A removed a coding sequence whose product was essential to induction, the mutation should have been complemented by constructing a cell containing pPL708ΔA and a second plasmid harboring a transcribed, intact *cat-86* regulatory region. To accomplish this, the 595-bp *XbaI* fragment was deleted from pPL708 (Fig. 1). This deletion extended from codon 31 of *cat-86* to a site 3' to the *cat-86* coding sequence (Fig. 2). This *XbaI*-deleted plasmid was joined to the *XbaI* site of pE194 in both of the two possible orientations, and the fusion plasmids were transformed into strain BGSC 1A422 (*recE4*) harboring pPL708ΔA. The *recE4* mutation has been shown previously to block recombination between homologous plasmids in the same cell (12). These attempts at complementation failed to permit *cat-86* induction with either chloramphenicol or ampicillin (data not shown).

The same pE194 derivatives of pPL708 used in the experiment described above were introduced into strain BGSC 1A422 cells harboring p5'αB10Δ1. This plasmid contains a spontaneously arising, constitutively expressed variant of *cat-86*, and the gene is transcriptionally activated by the *B. subtilis* amylase promoter (19). The mutation conferring the constitutive phenotype is a deletion that removes the majority of the upstream member of the pair of inverted repeats that normally precedes *cat-86*. These attempts to complement the deletion mutation did not restore chloramphenicol inducibility to the *cat-86* gene in p5'αB10Δ1. Therefore, the regulatory sequences deleted from pPL708ΔA, which resulted in noninducibility, and the sequences deleted from p5'αB10Δ1 that resulted in the constitutive phenotype were both *cis*-acting.

Duplication mutation that restored expression to *cat-86*ΔA. If deletion of component A removed sequences which were

TABLE 3. Induction of *cat-86* expression in pPL703 and pPL703ΔA when the gene was activated by the Spac promoter

Plasmid	CAT sp act (μmol/min per mg) ^a		
	Uninduced	With chloramphenicol	With ampicillin
pPL703-Spac	0.6	5.8	4.9
pPL703ΔA-Spac	0.38	0.4	0.46

^a Strain BR151 harboring the plasmids was grown for 2 h in Penassay broth containing 2 μg of chloramphenicol per ml or 0.5 μg of ampicillin per ml. The cells were lysed and assayed for CAT specific activity.

needed to effect destabilization of the RNA stem-loop, we anticipated that secondary mutations which restored *cat-86* expression would bypass the induction signals. For example, it has been shown that deletions which extend into the upstream member of the inverted repeats result in constitutive, high-level expression of both the pC194 *cat* gene and *cat-86* (1, 19). Therefore, we isolated several mutants of pPL708ΔA in which *cat-86* was strongly expressed. This was accomplished by isolating rare colonies of strain BR151(pPL708ΔA) that grew on agar containing 80 μg of chloramphenicol per ml in 24 h. The CAT specific activity in cultures derived from three such colonies was about 10 μmol/min per mg, and this level of expression was independent of the presence of chloramphenicol during growth. Hence, *cat-86* was constitutively expressed. The constitutive expression of *cat-86* cotransformed with the plasmids isolated from each of the three cultures and was therefore due to a plasmid mutation. Preliminary screening of the three mutant plasmids indicated that each contained a small insertion rather than the expected deletion. One of these mutant plasmids, designated pPL708ΔAC1, was examined in detail.

pPL708ΔAC1 specifies the same CAT level (10 U/mg of protein) in strain BR151 cells grown in the presence and absence of 2 μg of chloramphenicol per ml. Removal of the 284-bp promoter-containing *EcoRI* fragment from the plasmid yielded pPL703ΔAC1, and this plasmid no longer conferred chloramphenicol resistance. Therefore, the dependence of *cat-86* expression on an inserted promoter was the same in pPL708ΔAC1 as it was in pPL708. Furthermore, insertion of a different promoter into pPL703ΔAC1, such as promoter P2 or the Spac promoter, activated constitutive expression of the *cat-86* gene, indicating that the constitutively expressed phenotype was not due to a promoter mutation. The location of the insertion in pPL708ΔAC1 was determined by digesting the plasmid with restriction enzymes in pairwise combinations and electrophoresing the products in agarose or acrylamide gels in parallel with similarly digested pPL708ΔA. An insertion about 20 bp long was detected in the *EcoRI*-*BclI* region, and this fragment spanned the *cat-86* ribosome-binding site. To identify the nature of the insertion, the *BamHI*-*HindIII* fragment from pPL708ΔAC1 was cloned into M13mp11, and the insertion was sequenced from the *BamHI* site to the *BclI* site. This sequence demonstrated the presence of a 20-nucleotide duplication which spanned the ribosome-binding site (Fig. 4); this duplication repeated the sequence from -17 to +3 (Fig. 2). Hence, the duplication provides *cat-86* with two ribosome-binding sites separated by 11 bp. We presume that the ribosome-binding site immediately preceding the coding sequence is not involved in RNA secondary structure; this ribosome-binding site is presumably used for translation initiation of *cat-86* mRNA specified by pPL708ΔAC1.

FIG. 4. Twenty-base pair sequence in pPL703 and pPL703ΔΔ that is duplicated in pPL703ΔAC1. The horizontal arrows indicate the inverted repeats, and the sequences in boxes are the *cat-86* ribosome-binding site (AGGAGG) and the *cat-86* translation initiation codon TTG. Small letters show the sequence flanking the 20-bp sequence (see Fig. 2). RBS, Ribosome-binding site.

DISCUSSION

The inverted-repeat sequences that span the ribosome-binding site for chloramphenicol-inducible *cat* genes constitute a *cis*-acting regulatory element. Transcripts that span the inverted repeats are predicted to sequester the *cat* ribosome-binding site in RNA secondary structure (Fig. 5). This secondary structure blocks the translation of *cat* mRNA, and we infer that chloramphenicol induction results from disruption of the RNA secondary structure. We and other workers have proposed that chloramphenicol induction is due to the binding of chloramphenicol to ribosomes, enabling the ribosomes to perform a function related to disruption of the RNA stem-loop. For example, Byeon and Weisblum (5) suggested that chloramphenicol binding to the 50S ribosomal subunit may uncover sequences in 23S rRNA that are nearly identical to a ribosome-binding site. Such sequences in rRNA may, in theory, compete with the formation of the RNA stem-loop, thereby freeing the *cat* ribosome-binding site. Although evidence supporting this mechanism is lacking, there is strong evidence implicating ribosomes in *cat-86* induction. *cat-86* expression can be induced by either chloramphenicol or ampicillin (6). Both of these compounds are antibiotics that are known to bind to the 50S ribosomal subunit. If the antibiotics must interact with the ribosomes to induce *cat-86*, we predict that chloramphenicol- or ampicillin-resistant mutants of *B. subtilis* that exhibit alterations in the ribosomal target for these drugs should not permit *cat-86* to be induced by the corresponding drug. This has been demonstrated for ampicillin (6). Ampicillin-resistant ribosomal mutants of *B. subtilis* prevent ampicillin from serving as an inducer of *cat-86*, although in these mutants chloramphenicol remains an effective inducer of *cat-86* expression. The converse experiment in which chloramphenicol-resistant ribosomal mutants of *B. subtilis* are tested for *cat-86* inducibility has not been performed because chloramphenicol-resistant ribosomal mutants of *B. subtilis* 168 have not been isolated (6).

In order for ribosomes to destabilize the RNA stem-loop, it is necessary for the ribosomes to bind to *cat* mRNA at sites either 5' to the RNA stem-loop or at the stem-loop. Ribosome attachment to mRNA is mediated by the association of 16S rRNA in the 30S ribosomal subunit with complementary sequences in mRNA (i.e., the ribosome-binding site) (9). Ribosome-binding sites that function in translation initiation in gram-positive bacteria exhibit extensive homology with the 3' end of 16S rRNA (17, 18). No

strong ribosome-binding site exists in the immediate vicinity of the RNA stem-loop, except for the sequestered ribosome-binding site which is presumably unavailable for ribosome attachment. However, we previously noted that the sequence 5' GAAAGGA occurs 26 bp upstream from the *cat-86* inverted repeats (14) (Fig. 2). This sequence exhibits a calculated (theoretical) ΔG for binding of -14 kcal/mol to the 3' end of *B. subtilis* 16S rRNA, 3' UCUUCCUCC... (25). Such a sequence could potentially serve as a site of ribosome attachment to *cat-86* mRNA (18). Similar sites of potential ribosome attachment can be identified upstream from the inverted repeats preceding each of the other inducible *cat* genes that have been examined, and these sites have recently also been described by Brückner and Matzura (in press). The 85-bp ΔA deletion into the *cat-86* regulatory region removes the upstream site of potential ribosome attachment, 5' GAAAGGA, as well as other upstream sequences. However, we strongly suspect that the loss of chloramphenicol-inducible expression of *cat-86* associated with this deletion is due to the removal of the upstream ribosome binding site, 5' GAAAGGA. This is suggested by two observations. First, *Bal* 31 digestion has been used to remove 60 bp from the 5' end of the 144-bp *cat-86* regulatory

FIG. 5. Possible regulatory elements in *cat-86* mRNA upstream from the coding sequence. The potential upstream ribosome-binding site, 5' GAAAGGA, is followed by three GUG codons that may serve for translation initiation. Translation initiated at any of these GUG codons would terminate at the UGA codon in the RNA stem-loop. The AGGAGG and UUG sequences in boxes are the ribosome-binding site and the translation initiation codon for the *cat-86* coding sequence. The sequence upstream from the arrow labeled FokI are deleted in *cat-86*ΔΔ.

region, and this deletion, which ended 5' to GAAAGGA, had no effect on chloramphenicol induction (Ambulos, unpublished data). Second, in a recent study by Brückner and Matzura (in press), *Bal* 31-generated deletions that ended 5' to the upstream ribosome-binding site for the inducible *cat* on pUB112 did not alter inducibility, whereas deletions that removed this site blocked inducibility.

Brückner and Matzura (in press) have suggested that the upstream ribosome-binding site identified 5' to the inverted repeats for *cat-86*, *cat-66*, and *cat* genes on plasmids pC221, pC194, and pUB112 may allow translation of a short peptide that terminates in the left leg of the inverted repeats. For example, if ribosomes bind to the upstream ribosome-binding site for *cat-86*, 5' GAAAGGA, and initiate translation from one of the three downstream GUG codons, the ribosomes would, in theory, translate to the UGA codon in the RNA stem-loop, which may cause destabilization (Fig. 5). However, induced translation of *cat* mRNA depends on a step requiring chloramphenicol. Accordingly, it has been suggested (Brückner and Matzura, in press) that chloramphenicol bound to ribosomes may cause their "stalling" in the inverted repeats during translation of the short peptide. An alternative interpretation of these observations is that utilization of the upstream ribosome-binding site for translation initiation may be facilitated by chloramphenicol. Each of the upstream ribosome-binding sites is atypical. For example, the proposed upstream ribosome-binding site for *cat-86* is 5' GAAAGGA, while the ribosome-binding site for the *cat-86* coding sequence is 5' AGGAGG. Utilization of 5' GAAAGGA for translation initiation seems to require bases in 16S rRNA that are not directly involved in utilizing 5' AGGAGG. Perhaps the participation of the bases at the extreme 3' end of 16S rRNA in translation initiation is made possible by a conformational alteration in the ribosomes resulting from chloramphenicol bound to the 50S subunit.

The present study provides the most direct evidence to date that induction of *cat-86* expression is largely the result of increased efficiency of translation of mRNA. The ΔA mutation, which blocks inducible expression of *cat-86*, did not block transcription of the gene, although the level of phenotypic expression of *cat-86\Delta A* was only very slightly greater than that of a promoterless version of the gene which was not detectably transcribed. Furthermore, the mutation that restored expression to *cat-86\Delta A* resulted from a duplication event that provided the gene with a ribosome-binding site that was not involved in RNA secondary structure.

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