Analysis of the Regulatory Sequences Needed for Induction of the Chloramphenicol Acetyltransferase Gene *cat-86* by Chloramphenicol and Amicetin

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Induction of the chloramphenicol acetyltransferase gene *cat-86* in *Bacillus subtilis* results from the activation of translation of *cat-86* mRNA. The inducers, chloramphenicol and amicetin, are thought to enable ribosomes to destabilize a stem-loop structure in *cat-86* mRNA that sequesters the ribosome binding site for the *cat-86* coding sequence, designated RBS-3. The region of *cat-86* mRNA which is 5' to the stem-loop contained two additional ribosome binding sites, RBS-1 and RBS-2, located 84 and 56 nucleotides, respectively, upstream from RBS-3. RBS-1 and RBS-2 were each followed by a potential translation initiation codon and a short open reading frame. *Bal* 31-generated deletions into the 5' end of the regulatory region that removed RBS-1 but did not enter RBS-2 caused a fourfold decrease in the uninduced and chloramphenicol-induced level of *cat-86* expression and a more than 10-fold reduction in the amicetin-induced level of expression. Deletions that removed both RBS-1 and RBS-2 but did not enter the stem-loop abolished both chloramphenicol- and amicetin-inducible expression. These data indicate that RBS-2 and sequences 3' to RBS-2 are minimally essential to chloramphenicol induction. However, the presence of RBS-1 in the mRNA elevated the maximum level of expression obtained during chloramphenicol induction. These studies also demonstrate that induction of *cat-86* by amicetin is highly dependent on RBS-1. To determine whether a correlation existed between RBS-1 and amicetin inducibility, we examined the sequence of the regulatory regions for two natural variants of *cat-86*, *cat-66* and *cat-57*, which are chloramphenicol inducible but are very poorly induced by amicetin. Both contained nucleotide sequence differences from *cat-86* in the vicinity of RBS-1 that would prevent translation of the leader peptide associated with RBS-1 in *cat-86*. In contrast, the regulatory regions for the three genes were virtually identical in the vicinity of RBS-2. These data indicate that efficient induction by amicetin requires sequences that are not essential for induction by chloramphenicol.

Chloramphenicol acetyltransferase (CAT) in gram-positive bacteria is specified by genes designated *cat*, whose expression is induced by chloramphenicol (Cm) (23). One such gene, *cat-86*, was cloned from *Bacillus pumilus* DNA into *Bacillus subtilis* by using plasmid pUB110 as a vector (28). Subsequent replacement of the natural *cat-86* promoter with a short multicloning site linker generated the promoter-cloning plasmid pPL703 (17) (Fig. 1). By use of pPL703 it has been shown that the sequences necessary for the Cm-inducible expression of *cat-86* are independent of both the promoter that is selected to activate the gene and the *cat-86* coding sequence (16). Accordingly, the inducible regulation of *cat-86* results from sequences located within a 144-base-pair (bp) regulatory region that intervenes between the coding sequence and the upstream site of promoter insertion.

The nucleotide sequence of the 144-bp regulatory region demonstrates that the ribosome binding site for the *cat-86* coding sequence, hereafter designated RBS-3, is within one member of a pair of inverted repeat sequences (7). Consequently, *cat-86* mRNA is predicted to sequester RBS-3 within a stable stem-loop structure, where the RBS is apparently unavailable to base-pair with 16S rRNA. This predicted secondary structure of the mRNA in the vicinity of the RBS has been found to be characteristic of all the inducible *cat* genes that have been examined (5, 7, 8, 10, 24).

The importance of the RNA stem-loop in Cm induction is suggested by the analysis of constitutively expressed variants of three inducible *cat* genes. In each case, the constitutive phenotype correlates with a deletion that removes all or a major portion of the upstream member of the inverted repeats (1, 3, 5, 20). These deletions prevent the formation of the stem-loop in *cat* mRNA that normally sequesters RBS-3. Based on these observations, it has been suggested that the induction of *cat-86* and other *cat* genes of gram-positive bacterial origin results from destabilization of the stem-loop in mRNA (11). This would free the RBS for the *cat* coding sequence, allowing translation of the mRNA. This proposed form of regulation is due to control exerted at the level of mRNA translation; consistent with this model, efficient induction of *cat-86* can be demonstrated in rifampin-inhibited *B. subtilis* cells (5a). Thus, the predominant regulatory event in induction of *cat* gene expression modulates mRNA translation.

To determine whether sequences 5' to the RNA stem-loop were involved in the inducible expression of *cat-86*, Ambulos et al. (2) deleted 85 bp from the 5' end of the 144-bp regulatory region. This deletion, which ended 15 bp upstream from the stem-loop, prevented induction of *cat-86* with both Cm and the alternative inducer amicetin (6). To more precisely define those portions of the regulatory region which are essential to Cm and amicetin induction, we tested the inducibility of versions of *cat-86* containing deletions of various lengths into the regulatory region.

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FIG. 1. Diagram of the promoter-cloning plasmid pPL703. This plasmid consists of a 1,250-bp *Pst*I-*Bgl*II fragment of *B. pumilus* NCIB8600 DNA inserted between the *Eco*RI and *Bam*HI sites of pUB110 by use of a 21-bp *Eco*RI-*Pst*I fragment from M13mp7 (17, 28). The promoterless gene *cat-86* resides within the 1,250-bp fragment, and the gene is followed by an efficient transcription termination signal, designated *ter* (18). The pUB110 portion of pPL703 provides an origin of replication and a neomycin resistance gene (*Neo*^r). *cat-86* specifies CAT when the gene is transcriptionally activated by inserting a promoter into any of four unique restriction sites 5' to *cat-86*: *Eco*RI, *Bam*HI, *Sal*I, and *Pst*I. RBS-1, RBS-2, and RBS-3 designate the approximate locations of ribosome binding sites identified by their homology with 16S rRNA.

MATERIALS AND METHODS

Bacteria and plasmids. *B. subtilis* BR151 (*trpC2 metB10 lys-3*) was used throughout. All plasmids used are shown in Table 1. Inducibility of *cat-86* was tested in plasmid pPL703 (Fig. 1) and deletion derivatives which were constructed by *Bal* 31 digestion as shown below. In such plasmids, *cat-86* lacked a promoter. The promoter inserted into all such plasmids was SpacII. SpacII consists of -10 and -35 recognition sequences that are identical to those of an early SPO1 promoter, and inserted 3' to the -10 sequence is the *lac* operator (29). SpacII was derived from the Spac promoter described by Yansura and Henner (29) by cutting Spac with *Eco*RV and *Hind*III and inserting this 147-bp fragment between the *Eco*RV and *Hind*III sites in the linker in pIC-20R (14). The SpacII promoter fragment was then excised from pIC-20R as a 167-bp *Eco*RI-*Pst*I fragment and inserted into pPL703 and derivative plasmids. SpacII was used in these studies because it lacks the ribosome binding site present in Spac (29). We chose to eliminate the ribosome binding site to avoid potential difficulties that might arise in interpreting whether or not an effect was the result of translation initiating in the promoter.

The growth media used and the transformation methods have been described previously (12). Plasmid isolation and restriction mapping of plasmids were done as described elsewhere (8, 13).

***Bal* 31 digestion into the *cat-86* regulatory region.** Plasmid

pPL703 (Fig. 1) was linearized with *Eco*RI and digested at 30°C with a mixture of slow and fast *Bal* 31 (International Biotechnology, Inc.) at a ratio of 1 U of enzyme per 5 µg of DNA. After 5 min the reaction was terminated and the digested ends were repaired with T4 DNA polymerase (13). The plasmid was cut with *Xba*I, and the digestion products were resolved by electrophoresis through 8% polyacrylamide gels. The rate of *Bal* 31 digestion was sufficiently heterogeneous that the *Eco*RI-*Xba*I fragment had been digested into a range of size classes. Fragments estimated to have undergone deletions ranging from 10 to 100 bp were electroeluted from the gel. These were inserted between the *Hinc*II and *Xba*I sites of pUC18. The size range of the inserts was then judged by electrophoresis of digestion products of the pUC18 derivatives. Selected fragments were recloned in M13mp18 between *Eco*RI and *Hind*III, and these were sequenced by the Sanger method (21). After sequencing, five of the digested fragments were reinserted in pPL703 as *Pst*I-*Hpa*I fragments.

***B. pumilus cat* genes which are poorly amicetin inducible.** *cat-86* was previously shown to be inducible by the ribosomally targeted antibiotic amicetin as well as by Cm (6). Certain *cat* genes which are closely related to *cat-86*, as evidenced by hybridization studies and DNA sequencing, were found to be inducible by Cm but were poorly induced by amicetin. One of these genes is *cat-66*, which originated in *B. pumilus* ATCC 6631 (Fig. 2). The cloning and partial sequencing of *cat-66* has been reported (8). *cat-66* is in plasmid pPL503. *cat-57* originated in *B. pumilus* NRS576; it is Cm inducible but very weakly induced by amicetin (see below). *cat-57* was cloned into BR151 on a 2.7-kilobase (kb) *Eco*RI fragment by using pUB110 as a vector, yielding pPL536. The restriction pattern of relevant regions of the two cloned fragments is shown in Fig. 2. The 425-bp *Eco*RI-*Xba*I fragment from pPL536 was cloned into M13mp10 and M13mp11 and sequenced by the Sanger method (21) in both directions.

Enzyme assays. CAT was assayed by the colorimetric method of Shaw (22), and protein was measured by the Bradford procedure (4). CAT specific activity in expressed as micromoles of Cm acetylated per minute per milligram of protein.

Induction of *cat-86*. Strain BR151 cells carrying pPL703 derivatives in which *cat-86* was activated by a promoter were grown at 37°C to mid-log phase in Penassay broth (Difco Laboratories). The cells were diluted and regrown

TABLE 1. Plasmids

Designation	Properties	Reference
pPL703	Promoter-cloning plasmid (Fig. 1)	17
pPL703Δ17, Δ39, Δ48, Δ60, Δ82	pPL703 derivatives containing deletions into the <i>cat-86</i> regulatory region (Fig. 3)	This paper
pPL503	pUB110 containing a 1.3-kb <i>Eco</i> RI fragment spanning the <i>cat-66</i> gene	8
pPL536	pUB110 containing a 2.7-kb <i>Eco</i> RI fragment spanning the <i>cat-57</i> gene	This paper
pC194	Plasmid originating in <i>Staphylococcus aureus</i> containing a Cm-inducible <i>cat</i> gene	10
pC221	Plasmid originating in <i>S. aureus</i> containing a Cm-inducible <i>cat</i> gene	24

FIG. 2. Restriction pattern 5' to the coding sequences for *cat-57* and *cat-66*. *EcoRI* fragments spanning *cat-66* and *cat-57* were cloned from *B. pumilus* ATCC 6631 and NRS576, respectively. The location of the *cat* coding sequence within the cloned fragments was determined by hybridization with *cat-86*. The region 5' to the coding sequence and the first 90 bp of the coding sequence were sequenced. (*PstI*) refers to the position in the regulatory region for *cat-57* where a sequence occurs that differs from the *PstI* recognition sequence by two base substitutions. The regulatory regions for *cat-66* and *cat-86* contain a *PstI* site at this location. Numbers represent base pairs.

thrice, and Cm (2 µg/ml), amicetin (0.5 µg/ml), or bamicetin (0.5 µg/ml) was added. Incubation was continued, and samples were periodically removed for analysis of CAT and protein.

RESULTS

***cat-86* inducibility by the amicetin family of antibiotics.** Expression of *cat-86* can be induced in *B. subtilis* by either amicetin or Cm (6). Since two Cm-inducible *cat* genes of

staphylococcal origin were found not to be induced by amicetin, we were interested in determining whether induction of *cat-86* is a unique property of amicetin or whether closely related antibiotics also functioned as inducers of this gene. Amicetin is a 4-amino-hexose pyrimidine nucleoside that inhibits protein synthesis in bacteria and is a member of a related group of antibiotics which includes bamicetin, plicacetin, and oxamicetin (Fig. 3) (25, 27). Each of these drugs except oxamicetin was tested for induction of the chloramphenicol-inducible gene *cat-86*. Amicetin and

Chloramphenicol

Amicetin Family

FIG. 3. Structures of Cm, amicetin, bamicetin, and plicacetin. Redrawn from Vazquez (27).

FIG. 4. Nucleotide sequence of the regulatory region preceding *cat-86* in pPL703 and five deletion mutants. Bold horizontal arrows depict the inverted repeats. RBS-1 is followed by an ATG codon and an open reading frame (leader 1) that would deliver initiated ribosomes to RBS-2. RBS-2 is followed by three GTC codons and an open reading frame (leader 2) that would deliver translating ribosomes into the inverted repeats. GTG is shown to specify the amino acid valine. However, when GTG serves as an initiator codon, the amino acid inserted is methionine (15). RBS-3 is followed by the translation start codon TTG and an open reading frame that is the *cat-86* coding sequence (2).

bamicetin were effective inducers, whereas plicaceticin had no detectable inducing activity (Table 2). The MIC of plicaceticin for *B. subtilis* BR151, 2 µg/ml, was the same as that of bamicetin and amicetin. This indicates that plicaceticin is capable of entering *B. subtilis*, and therefore the inability of plicaceticin to induce *cat-86* is due to a functional difference between this drug and the related compounds amicetin and bamicetin. Structurally, plicaceticin differs from bamicetin only in the moiety designated R2 in Fig. 3. We presume that this group plays a role in the inducing action of both amicetin and bamicetin.

Mutants of strain BR151 selected for resistance to amicetin fail to support *cat-86* induction by amicetin (6). One such mutation, *ami-1*, was shown to alter the structure of the 50S ribosomal protein BL12a (6). As might be expected, the *ami-1* mutation also conferred resistance to bamicetin, and bamicetin was unable to induce *cat-86* in BR151 *ami-1* (data not shown).

The *cat* genes on plasmid pC194 and plasmid pC221 were not inducible with either bamicetin or amicetin, although both were chloramphenicol inducible. The MIC of amicetin for BR151 carrying either pC194 or pC221 was 2 µg/ml; therefore the presence of these plasmids does not block cell sensitivity to amicetin.

Effect of *Bal* 31-generated deletions into the *cat-86* regulatory region on induction by chloramphenicol and amicetin. Induction of *cat-86* by either amicetin or chloramphenicol

requires sequences within a 144-bp transcribed regulatory region that immediately precedes the coding sequence. A previous study revealed that a deletion, ΔA, which removed 85 bp from the 5' end of this regulatory region blocked induction (2). Since this deletion did not enter the inverted repeats, it was evident that sequences upstream from the inverted repeats are essential to induction by both Cm and amicetin. To identify the sequences 5' to the inverted repeats which are essential for induction, *Bal* 31 was used to generate a series of deletions which extended into the 5' end of the regulatory region. Five of the deletants were tested for induction by Cm. The deletions designated Δ17 and Δ39 removed 17 and 39 bp from the 5' end of the regulatory region, respectively, and these had no discernible effect on *cat-86* induction (Fig. 4 and 5). A deletion that removed 48 bp, Δ48, resulted in a nearly fourfold decrease in the uninduced and Cm-induced level of expression of *cat-86* (Fig. 5). Clearly, however, the Δ48 mutation did not block induction of *cat-86* by Cm (Fig. 5). The Δ60 mutation was similar to Δ48 in that *cat-86* remained Cm inducible. The Δ82 mutation resulted in complete loss of inducibility of *cat-86* by Cm (Fig. 5).

The same deletion mutants analyzed for induction by Cm were also analyzed for induction by amicetin. The Δ17 and the Δ39 mutations had no detectable effect on amicetin induction, whereas both the Δ48 and Δ60 mutations decreased the amicetin-induced level of *cat-86* expression 10-fold (Fig. 6). This very low level induction by amicetin was not, however, seen with the Δ82 mutant (Fig. 6).

Natural variants of *cat-86* which are poorly induced by amicetin. The foregoing studies show that the endpoints of the Δ39 and Δ48 deletions flank a sequence that plays an important role in the induced and uninduced level of *cat-86* expression. Moreover, this sequence appears to be much more important for amicetin to function as an efficient inducer of *cat-86* expression than for induction by Cm. The 9-bp sequence that intervenes between the endpoints of the Δ39 and Δ48 deletions contains RBS-1, which is followed by a potential translation initiation codon, ATG, and an open reading frame (Fig. 4). Translation initiating from RBS-1 should result in the synthesis of leader peptide 1 and would deliver translating ribosomes to RBS-2. If translation of

TABLE 2. Induction of *cat-86* in pPL703-SpaciI with CM and members of the amicetin family of antibiotics^a

Treatment (µg/ml)	CAT sp. act. ^b
Uninduced	0.6
Cm (2)	5.9
Amicetin (0.5)	4.7
Bamicetin (0.5)	5.0
Plicaceticin (0.5)	0.7

^a Strain BR151 (pPL703-SpaciI) was incubated for 2 h at 37°C with no antibiotic (uninduced) or with the various antibiotics at the concentrations noted. Cells were harvested, lysed, and assayed for CAT and total protein.

^b Expressed as micromoles of Cm acetylated per minute per milligram of protein.

FIG. 5. Cm induction of derivatives of *cat-86* containing deletions into the regulatory region. pPL703 and the five deletion derivatives were activated with the SpacII promoter fragment and inserted into strain BR151. Cells containing the plasmids were grown to mid-log phase and diluted 1,000-fold. This was repeated three times before Cm was added at 2 μ g/ml. After Cm addition, induced cells were periodically withdrawn and assayed for CAT. Induction curves of pPL703-SpacII, pPL703 Δ 17-SpacII, and pPL703 Δ 39-SpacII were similar, and therefore only the last is shown.

FIG. 6. Amicetin induction of derivatives of *cat-86* containing deletions into the regulatory region. Experimental details are the same as for Fig. 5, except the inducer was amicetin at 0.5 μ g/ml.

leader 1 were important primarily to amicetin induction, variants of *cat-86* which are Cm inducible but poorly induced by amicetin might contain alterations that perturb translation of leader 1. *cat-66* and *cat-57* are two such genes (Fig. 2). Both are closely related to *cat-86*, and both are very poorly induced by amicetin. Induction of *cat-66* with amicetin or bamicetin produced only a twofold increase of CAT levels in 2 h, whereas *cat-57* was not detectably induced with either amicetin or bamicetin (Table 3). The promoter associated with *cat-66* is much weaker than the Spac promoter (5a). Similarly, the low CAT level specified by *cat-57* during Cm induction also suggested that its promoter was weak. Accordingly, we inserted the Spac promoter fragment at the *Eco*RI site in pPL536 which is 5' to the coding sequence (Fig. 2). This resulted in a significant increase in the Cm-induced CAT level, and this allowed detection of a slight induction with amicetin and bamicetin (Table 3). By contrast neither *cat-66* nor *cat-57* was detectably induced with plicaceticin. Our interpretation of these data is that the regulatory regions associated with *cat-66* and *cat-57* seem to allow very weak, but detectable, induction by amicetin.

The nucleotide sequences of the regulatory regions for *cat-57* and *cat-66* show differences from the *cat-86* regulatory region at locations which are important for leader 1

synthesis. The region in *cat-86* corresponding to the sequence between Δ 39 and Δ 48 was absent from *cat-57* (Fig. 7). Thus, the *cat-57* regulatory region lacks RBS-1. Furthermore, the ATG codon 3' to the endpoint of the Δ 48 deletion in *cat-86* was absent from the *cat-57* sequence due to a base substitution. The sequence corresponding to RBS-1 in *cat-86* was present in the *cat-66* regulatory region (Fig. 7). However, the ATG codon that is 3' to RBS-1 in *cat-86* was absent from the corresponding region in *cat-66* due to a base substitution. These results are consistent with our hypothesis that translation of leader 1 may be necessary for efficient induction by amicetin.

TABLE 3. Induction of *cat-66* and *cat-57* with amicetin, bamicetin, and chloramphenicol^a

Treatment (μ g/ml)	CAT sp. act. ^b		
	<i>cat-66</i>	<i>cat-57</i>	<i>cat-57</i> ^c
Uninduced	0.06	0.02	0.67
Cm (2)	0.39	0.18	5.6
Amicetin (0.5)	0.12	0.01	1.02
Bamicetin (0.5)	0.13	0.01	1.1
Plicaceticin (0.5)	0.07	0.02	0.71

^a Strain BR151 cells harboring pPL503 (*cat-66*) or pPL536 (*cat-57*) were induced for 2 h at 37°C with the antibiotics at the final concentrations noted. Cells were lysed and assayed for CAT and total protein.

^b Expressed as micromoles of Cm acetylated per minute per milligram of protein.

^c Assays were performed on BR151 harboring a derivative of pPL536 in which the Spac promoter fragment was inserted at an *Eco*RI site 5' to the *cat-57* coding sequence.

FIG. 7. Comparison of the 144-bp regulatory region preceding *cat-86* with the regulatory regions preceding *cat-57* and *cat-66*. *cat-57* and *cat-66* are closely related to *cat-86* but are poorly inducible with amicetin. Comparisons are between *cat-86* and *cat-57* (top) and *cat-86* and *cat-66* (bottom). Mismatches are designated by displacing bases above and below the lines. The *cat-86* regulatory sequence is the same as that shown in Fig. 4.

DISCUSSION

Cm induction of *cat* gene expression is the result of a regulatory mechanism that controls the translation of *cat* mRNA. A stem-loop in the mRNA sequesters the RBS for each Cm-inducible *cat* gene that has been studied, and constitutively expressed variants result from mutations that free the RBS from the sequestered state. These mutations are generally due to deletions that remove one of the inverted repeats that specify the stem-loop in the mRNA. In one instance, a constitutively expressed mutant resulted from a duplication event in which a 20-bp sequence spanning the RBS was tandemly repeated (2). This provided the *cat-86* gene with a novel regulatory arrangement immediately 5' to the coding sequence; the mRNA contains a stem-loop with a sequestered RBS followed by an RBS that is predicted not to be sequestered in the secondary structure. This duplication mutation offers the only direct genetic evidence that the constitutive phenotype is due to the availability of a nonsequestered RBS rather than to elimination of the stem-loop (2).

Duvall et al. (7) proposed that in vivo destabilization of the stem-loop in *cat* mRNA occurs during induction. This was suggested to result from a modification in ribosomal function as a consequence of the inducing antibiotic binding to the

50S subunit. Evidence implicating the ribosome as a mediator of induction has been obtained for induction of the *cat-86* gene by the ribosomally targeted antibiotic amicetin (6). We suspect that induction by Cm also involves the ribosome, but this remains untested.

To determine whether sequences upstream from the stem-loop in *cat-86* mRNA were involved in the induction process, we constructed a series of deletion mutations into the 144-bp *cat-86* regulatory region. We then tested the effect of each deletion on *cat-86* induction by Cm and amicetin to determine whether removal of a specific domain within the regulatory region correlated with loss of inducibility by one or both antibiotics. The Δ39 mutation had no effect on induction by either antibiotic, whereas the Δ48 mutation, as well as the Δ60 mutation, resulted in a fourfold decrease in the uninduced and Cm-induced CAT levels. Both the Δ48 and the Δ60 mutations had a more severe effect on amicetin induction, reducing the amicetin-induced CAT level more than 10-fold. The endpoints of the Δ39 and Δ48 deletions therefore flank a sequence, 5'-GAATGGACA, that is clearly important to the level of *cat-86* expression in both the induced and uninduced states, and this sequence seems to be more important for amicetin to function as an inducer than for induction by Cm. Within this sequence is a relatively weak ribosome binding site, designated RBS-1 (Fig. 8). Eight

16S rRNA	3' UCUUCCUCC . . .	
RBS-3	5' AGGAGG . . .	$\Delta G = -16.6$
RBS-2	5' . GAAAGGA . . .	$\Delta G = -14$
RBS-1	5' . GAAUGGA . . .	$\Delta G = -8.6$

FIG. 8. Comparison of the region of homology between 16S rRNA and RBS-3, RBS-2, and RBS-1. The ΔG values shown were calculated as described in reference 26.

base pairs 3' to RBS-1 is an ATG codon, followed by an open reading frame of six additional codons. If RBS-1 were used for translation initiation, leader peptide 1 would be translated and would deliver translating ribosomes to RBS-2.

In contrast to the $\Delta 48$ and $\Delta 60$ deletions, the $\Delta 82$ deletion eliminated induction of *cat-86* by both Cm and ampicillin. Thus, the sequence between the endpoints of the $\Delta 60$ and $\Delta 82$ deletions is essential for Cm induction and for the slight induction effect of ampicillin seen in the $\Delta 48$ and $\Delta 60$ mutants. The segment deleted in constructing $\Delta 82$ includes RBS-2 (Fig. 8). RBS-2 is followed by three GTG codons which may function as translation initiation codons (15) and an open reading frame that would allow the synthesis of leader peptide 2. Ribosomes translating leader peptide 2 would be delivered to the RNA stem-loop, which could, in theory, disrupt the RNA secondary structure. Translation from RBS-2 has been suggested to be the crucial step in destabilizing the RNA stem-loop (2, 5). Since induction only occurs in the presence of Cm or ampicillin, these drugs may functionally alter ribosomes which could facilitate translation of leader 2 or cause the ribosomes to stall at a specific site during translation of this leader (2, 5).

One explanation of the results of the deletion studies is that translation from RBS-2 is directly involved in induction, whereas translation from RBS-1 only increases the probability of ribosomal loading at RBS-2. The removal of RBS-1 severely depressed the ability of ampicillin to induce *cat-86*; hence, ampicillin may be a poor inducer except when the ribosomal density at RBS-2 is high. Translation from RBS-1 does appear to be important for ampicillin induction, since the *cat-57* and *cat-66* genes, which are very poorly induced by ampicillin, contain nucleotide sequence variations that would prevent leader 1 synthesis.

The effect of deletions into the *cat-86* regulatory region on Cm induction were generally similar to the results obtained by Bruckner and Matzura (5) in a deletion analysis of the regulatory region for the pUB112 *cat* gene. The pUB112 *cat* gene is preceded by two ribosome binding sites, designated SD-1 and SD-2, which are analogous to the *cat-86* RBS-2 and RBS-3 sites, respectively. Deletions which removed SD-1 (equivalent to RBS-2) blocked induction, indicating the participation of this sequence in the induction events.

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