Site in the cat-86 Regulatory Leader That Permits Amicetin to Induce Expression of the Gene

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Expression of the plasmid gene cat-86 is induced in Bacillus subtilis by two antibiotics, chloramphenicol and the nucleoside antibiotic amicetin. We proposed that induction by either drug causes the destabilization of a stem-loop structure in cat-86 mRNA that sequesters the ribosome-binding site for the cat coding sequence. The destabilization event frees the ribosome-binding site, permitting the initiation of translation of cat-86 mRNA. cat-86 induction is due to the stalling of a ribosome in a leader region of cat-86 mRNA, which is located 5′ to the RNA stem-loop structure. A stalled ribosome that is active in cat-86 induction has its aminoacyl site occupied by leader codon 6. To test the hypothesis that a leader site 5′ to codon 6 permits a ribosome to stall in the presence of an inducing antibiotic, we inserted an extra codon between leader codons 5 and 6. This insertion blocked induction, which was then restored by the deletion of leader codon 6. Thus, induction seems to require the maintenance of a precise spatial relationship between an upstream leader site(s) and leader codon 6. Mutations in the ribosome-binding site for the cat-86 leader, RBS-2, which decreased its strength of binding to 16S rRNA, prevented induction. In contrast, mutations that significantly altered the sequence of RBS-2 but increased its strength of binding to 16S rRNA did not block induction by either chloramphenicol or amicetin. We therefore suspected that the proposed leader site that permitted drug-mediated stalling was located between RBS-2 and leader codon 6. This region of the cat-86 leader contains an eight-nucleotide sequence (conserved region I) that is largely conserved among all known cat leaders. The codon immediately 5′ to conserved region I differs, however, between amicetin-inducible and amicetin-noninducible cat genes. In amicetin-inducible cat genes such as cat-86, the codon 5′ to conserved region I is a valine codon, GTG. The same codon in amicetin-noninducible cat genes is a lysine codon, either AAA or AAG. When the GTG codon immediately 5′ to conserved region I in cat-86 was changed to AAA, amicetin was no longer active in cat-86 induction, but chloramphenicol induction was unaffected by the mutation. The potential role of the GTG codon in amicetin induction is discussed.

The plasmid gene cat-86 specifies chloramphenicol acetyltransferase (CAT) in Bacillus subtilis (14). Expression of the gene is induced by two structurally distinct antibiotics, chloramphenicol and amicetin (10). Both inducers are bacteriostatic agents which inhibit protein synthesis in bacteria by binding to the large subunit (50S) of ribosomes (6, 27). Several other antibiotics which also inhibit protein synthesis in bacteria fail to induce cat-86 expression (10). Therefore, induction of cat-86 by chloramphenol and amicetin is probably due to a unique aspect of the function or structure of these drugs.

CAT specified by cat-86 inactivates the antibiotic activity of chloramphenicol but does not inactivate amicetin (10). Amicetin is therefore a gratuitous inducer of cat-86. However, certain cat genes whose expression is induced by chloramphenicol are not induced by amicetin (10). Therefore, although we suspect that both antibiotics activate cat-86 by fundamentally similar processes, at least one aspect of the action of the two drugs as inducers must differ.

Based on our current understanding of the events necessary for activation of cat-86 expression, we have proposed a general model for the role of the inducers (1, 8). Inducers that act through a ribosome intermediary are believed to cause the destabilization of a stem-loop structure in cat-86 mRNA that sequesters the ribosome-binding site for the gene (9, 14). We have suggested that a region located 5′ to the RNA stem-loop structure contains a sequence which causes a ribosome to stall when the inducing antibiotic is bound to the ribosome. The stall sequence for amicetin, chloramphenicol, or both must block the transit of the ribosome at the proper position in the leader, to allow the stalled ribosome to mask sequences in the RNA stem-loop structure and cause stem-loop structure destabilization (1, 8). In the present study we present evidence which is consistent with the existence of a stall site(s) in the cat-86 leader. We further identify a region at the 5′ end of the cat-86 regulatory leader that is essential for amicetin to induce cat-86 but that is not needed for chloramphenicol induction of the gene.

MATERIALS AND METHODS

Bacteria and plasmids. B. subtilis BR151 (trpC2 metB10 lys-3) was used as the plasmid host. The plasmids used were pPL703 (Fig. 1) (17) and mutant derivatives of that plasmid. pPL703 contains the promoter-deficient gene cat-86. Transcriptional activation of cat-86 was accomplished by inserting the P4 (17) promoter-containing fragment into the multi-cloning site linker located 144 base pairs (bp) 5′ to the cat-86 coding sequence. Methods for plasmid manipulations and induction of cat-86 expression by chloramphenicol (2 μg/ml) and amicetin (0.5 μg/ml) were as described previously (8, 15).

CAT assays. CAT was assayed at 25°C by the colorimetric procedure described by Shaw (23). Protein was measured by the method of Bradford (4). The specific activity of CAT is
reported as the micromoles of chloramphenicol acetylated per minute per milligram of protein.

Site-directed mutagenesis. Oligonucleotide-directed mutagenesis was performed as described previously (8) following the general methods of Taylor et al. (25) and Zoller and Smith (29). DNA sequencing was done with M13 vectors by the dideoxy procedure of Sanger et al. (22).

Amicetin inducibility of cat genes. Cat genes which are on the Staphylococcus aureus plasmids pC194, pC221, and pUB112 are not detectably induced by amicetin in B. subtilis; but each is induced by chloramphenicol. Cat genes cat-86, cat-66, and cat-57 were each cloned from the DNA of separate strains of Bacillus pumilus, and each was amicetin inducible and chloramphenicol inducible in B. subtilis and B. pumilus. Among the three cat genes cloned from B. pumilus strains, cat-86 responds to amicetin as an inducer more extensively than does cat-66 or cat-57 (1a). This appears to be due to the fact that the regulatory region 5' to cat-86 contains two short open reading frames which code for leader peptides previously designated as leaders 1 and 2. Leader 2 is now referred to as the regulatory leader, since it is minimally essential to drug induction in all cat genes examined (Fig. 1). Cat-66 and cat-57 lack a functional leader 1, which, although not essential to drug induction, has been found to increase the stability of the mRNA about threefold (N. P. Ambulos, unpublished data). Thus, the extent of induction of cat-86 by amicetin and chloramphenicol is elevated when leader 1 is present in the mRNA. All experiments reported here were performed with pPL703 in which leader 1 was intact.

RESULTS

Evidence for a stall site in the cat-86 leader. Sequences essential to the inducible expression of cat-86 reside within an 84-bp regulatory region that immediately precedes the cat-86 coding sequence (1a-3, 16). This regulatory region can be divided into two functional domains (Fig. 1). Domain B spans a pair of inverted repeats which dictate a stem-loop structure in the corresponding mRNA. This stem-loop structure sequesters the cat-86 ribosome-binding site RBS-3. Thus, the RNA stem-loop structure is predicted to block translation of cat-86 mRNA. Domain A, which is 5' to and overlaps with domain B, contains a ribosome-binding site, RBS-2, and an open reading frame of 9 codons. Activation of the translation of cat-86 mRNA has been shown to require
the stalling of a ribosome in domain A of the mRNA, such that the aminocayl site of the stalled ribosome occupies leader codon 6 (1, 8). Ribosome stalling that placed the aminocayl site at an earlier leader codon, codon 3, 4, or 5, failed to activate cat-86 expression (1, 8).

We hypothesize that sequences exist in the cat-86 leader region that permit the antibiotic inducers to stall a translating ribosome such that its aminocayl site occupies leader codon 6. Furthermore, it is likely that such a stall site(s) is located at a precise distance 5' to leader codon 6. If this hypothesis was correct, a mutation which increases the distance between the putative stall site and leader codon 6 would block drug-mediated induction of cat-86. We therefore inserted an extra aspartic acid codon (GAT) between leader codons 5 and 6 (GAT - AAA), generating a mutant version of the gene, designated cat-86 + 5A, containing leader codons 5, 5A, and 6 (GAT - GAT - AAA) (Fig. 2). When this mutant plasmid was tested for induction, the insertion mutation prevented cat-86 induction by either amicetin or chloramphenicol (Fig. 2).

Two explanations for the effect of the 5A insertion mutation can be suggested. As noted above the mutation may displace the putative stall site from leader codon 6. Hence, drug-mediated ribosome stalling would take place in the cat-86 + 5A mutant, but the stalled ribosome would have its A site occupied by leader codon 5A, not by codon 6. The consequence is that the stalled ribosome would not be in the correct position in the leader to cause destabilization of the RNA stem-loop structure. An alternative explanation is that the inserted 5A codon itself disrupts the stall site. For example, perhaps leader codons 4 (Thr), 5 (Asp), and 6 (Lys) must be adjacent to permit stalling to occur and insertion of the extra Asp codon disrupts this sequence. If the insertion mutation merely displaces the stall site and does not interrupt it, deletion of codon 6 (AAA) from the cat-86 + 5A mutant would be expected to restore inducibility. We generated this mutant, designated cat-86 + 5AΔ6 (Fig. 2). cat-86 + 5AΔ6 was found to be fully inducible with chloramphenicol and amicetin. Thus, we favor the view that the insertion mutation causes displacement of the proposed stall site but does not disrupt the site.

Is RBS-2 the stall site? RBS-2 is the ribosome-binding site for the regulatory leader (2) (Fig. 1). RBS-2, GAAAGGA, exhibits a calculated ΔG of binding to 16S rRNA of −14 (26) and is therefore probably a moderately strong site of ribosome attachment (19, 20). However, the sequence of RBS-2, as well as the sequences for ribosome-binding sites for other cat leaders (5, 11, 12, 24), is atypical when compared with the ribosome-binding site for the cat coding sequence, AGGAGGA (Fig. 1). To determine whether the specific sequence of RBS-2 was essential for drug induction of cat-86, we changed RBS-2 from GAAAGGA to AGGAGGA. This mutation is predicted to increase the ΔG of binding to 16S rRNA to −16. This mutant version of cat-86, designated cat-86 RBS-2-, was inducible by both amicetin and chloramphenicol (Table 1). Thus, RBS-2 itself is probably not the sequence that is recognized as a stall site by ribosomes that are complexed with an inducing antibiotic.

To confirm that the function of RBS-2 as a site of initiation of translation was indeed important to cat-86 expression, we also changed the sequence from GAAAGGA to GAAATTA. This change is predicted to eliminate RBS-2 as a site of primary ribosome attachment. This mutant version, designated cat-86 RBS-2-−, was not induced by amicetin or chloramphenicol. This result is consistent with the results of a previous study (1), in which it was shown that a ribosome must be capable of translating the first 5 codons of the regulatory leader for cat-86 to be inducibly expressed.

**Mutation that selectively blocks amicetin induction of cat-86.** In an effort to identify a leader site that permits an inducible antibiotic to stall ribosomal transit and thereby activate cat-86, we considered the following. The leaders for six inducible cat genes contain an 8-nucleotide sequence that is highly conserved (8) (Fig. 3). This region, designated conserved region I, spans leader codons 3 and 4 and includes the first two positions of leader codon 5. A second conserved sequence of 10 nucleotides, conserved region II, occurs in the left inverted repeat (8). Based on the occurrence of the two conserved regions in all known cat leaders and the conservation of the distance between conserved regions I and II (eight nucleotides), Duvall et al. (8) speculated that conserved region I might represent a sequence that is involved in the stalling of ribosomes by drugs and that conserved region II might contain the sequence which is masked by a stalled ribosome. Selected missense mutations introduced into conserved region I failed to completely block drug-mediated induction of cat-86, although certain of these mutations reduced the extent of induction by both chloramphenicol and amicetin (E. J. Duvall, unpublished data). One explanation for this observation is that a putative chloramphenicol stall site in cat leaders may actually be an ideal

| Gene          | Sp act of the following inducer: |            |
|---------------|----------------------------------|------------|
|               | None | Chloramphenicol (2 μg/ml) | Amicetin (0.5 μg/ml) |
| cat-86        | 0.48 | 4.67                  | 3.57        |
| cat-86 RBS-2↑ | 0.47 | 2.78                  | 2.80        |
| cat-86 RBS-2↓ | 0.05 | 0.08                  | 0.07        |

* Each gene in pPL703 was activated by the P4 promoter fragment. Plasmids were maintained in BR151, and inductions were for 2 h at 37°C. Values are CAT specific activities (micromoles per minute per milligram of protein).
sequence for stalling and that minor changes in the sequence diminish, but do not abolish, its function.

Previously published data (1) support the view that the proposed leader stall sites for both chloramphenicol and amicetin may be close to each other. Moreover, since the product of cat-86, CAT, fails to inactivate amicetin, the ability of amicetin to induce cat-86 expression possibly reflects the fortuitous occurrence of an amicetin stall site in the leader. We therefore compared the leader sequence surrounding conserved region I in cat genes which could be induced by amicetin with the same sequences found in cat genes which could not be induced by amicetin. In the leaders for amicetin-inducible cat genes, the codon immediately 5' to conserved region I (leader codon 2) was GTG, a valine codon. In leaders for cat genes which were not amicetin inducible, leader codon 2 was either AAA or AAG, which are lysine codons. Accordingly, we changed the cat-86 leader codon 2 from GTG to AAA. This mutant, designated cat-86 L2-AAA, retained full inducibility by chloramphenicol but was not detectably induced by amicetin (Fig. 4).

The leaders for amicetin-inducible cat genes such as cat-86, cat-57, and cat-66 are predicted to use GTG as the initiator codon (Fig. 3). Leaders for cat genes which are not inducible with amicetin contain ATG as the initiator codon. We therefore modified cat-86 L2-AAA so that the presumed initiator GTG became an ATG codon. We designated this doubly mutated gene cat-86 L1-ATG L2-AAA. This second mutation caused an increase in the level of cat-86 expression in both the induced and uninduced states; this increase in expression may have been due to an increase in the efficiency of translation of the leader resulting from the use of ATG as the initiator codon. However, the conversion of GTG to ATG did not detectably influence the extent of induction by chloramphenicol.

**DISCUSSION**

A ribosome that translates the cat-86 regulatory leader induces gene expression when the ribosome is stalled at a position which places the ribosomal A site at leader codon 6 (1). The inducers chloramphenicol and amicetin may only be involved in causing the stalling event, since the stalling of drug-free ribosomes at the correct location in the leader also induces cat-86 (8). The site which permits stalling to occur in the presence of an inducer is perhaps a nucleotide sequence in the leader, an amino acid sequence in the corresponding leader peptide, or both. The stall site, or at least a major component of that site, must be located 5' to leader codon 6. This is suggested because the ribosomal A site, which is stalled at leader codon 6 during induction, likely represents the leading portion of a ribosome, and a ribosome has been reported to span 35 to 40 nucleotides in mRNA (21). Moreover, leaders for all known inducible cat genes contain a highly conserved 8-nucleotide sequence (conserved region I) located 2 nucleotides 5' to leader codon 6 (8).

In the present study we showed that insertion of an extra codon immediately 5' to codon 6 blocks induction and that the subsequent deletion of codon 6 restores induction. This result is consistent with our belief that the distance between the putative stall site and codon 6 must be exact for the induction mechanism to result in destabilization of the RNA stem-loop structure. RBS-2 is the ribosome-binding site for the cat-86 leader. Three nucleotide changes in RBS-2 which increase its strength of binding with 16S rRNA did not alter inducibility by either amicetin or chloramphenicol. Thus, we suspected that the drug stall site(s) is likely represented by a sequence between RBS-2 and codon 6. As a first approach toward identifying a possible drug stall site in this region, we took advantage of the observation that cat genes which originated in B. pumilus (cat-86, cat-66, cat-57) are amicetin inducible, whereas cat genes from S. aureus (those on plasmids pC194, pC221, and pUB112) are not (10). By changing a single GTG codon immediately 5' to conserved region I in the cat-86 leader to AAA, a codon found at this position in cat genes which are not inducible by amicetin, we eliminated amicetin from serving as an inducer of cat-86 expression. Thus, the nature of leader codon 2 appears to play a role in amicetin induction, although we do not yet know whether this is due to the amino acid specified by the codon or the sequence of the codon.

The change at leader codon 2 which prevented amicetin from inducing cat-86 had no detectable effect on induction by chloramphenicol. It is therefore evident that the aspects

![Conserved Region](image-url)
of cat-86 induction which are common to both inducing agents are unaffected by the mutation. The single feature of the proposed induction mechanism which differs for the two drugs is the nature of the leader stall site. Thus, it is conceivable that leader codon 2, or the corresponding amino acid valine, may constitute a portion of the site that permits amicetin to stall a ribosome. The mechanism that leads to the induction of cat-86 expression appears to share fundamental similarities with the attenuation model which has been proposed (13) to explain a major component of the regulation that controls expression of amino acid biosynthetic operons in Escherichia coli. An additional antibiotic-inducible gene, erm, which is regulated by erythromycin is also controlled by a mechanism that seems fundamentally similar to classical attenuation (7, 28). One of several differences between the drug-inducible systems and classical attenuation is the event which causes ribosomal stalling. In the case of classical attenuation, codons for the amino acid(s) which is the end product of the regulated biosynthetic operon are richly represented at the leader site where stalling of a ribosome can modulate operon expression (13). Stalling is regulated by the availability of the end product amino acid. In the case of cat-86 and ermC, stalling appears to be controlled by the binding of an antibiotic to a ribosome that is translating the regulatory leader (1, 7, 8, 28). We suppose that stalling occurs when the drug-ribosome complex encounters a specific leader sequence. In both systems, the stalling of a ribosome in the regulatory leader is proposed to alter the mRNA secondary structure, which regulates downstream gene expression at the level of either transcription (13) or translation (7, 9, 28).

ACKNOWLEDGMENT

This investigation was supported by Public Health Service grant AI-21350 from the National Institutes of Health.

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