Chloramphenicol induction of cat-86 requires ribosome stalling at a specific site in the leader

(antibiotic resistance/attenuation/Bacillus subtilis/plasmids/posttranscriptional regulation)

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ABSTRACT The plasmid gene cat-86 specifies chloramphenicol-inducible chloramphenicol acetyltransferase in Bacillus subtilis. Induction by the antibiotic is primarily due to activation of the translation of cat-86-encoded mRNA. It has been suggested that the inducer stalls ribosomes at a discrete location in the leader region of cat-86 mRNA, which causes the destabilization of a downstream RNA secondary structure that normally sequenses the cat-86 ribosome binding site. It is the destabilization of this RNA secondary structure that permits translation of the cat-86 coding sequence. In the present report, we show that ribosomes that were stalled in the cat-86 leader by starvation of host cells for the amino acid specified by leader codon 6 induced gene expression to a level above that detected when cells were starved for the amino acids specified by leader codons 7 and 8. Starvation for amino acids specified by leader codons 3, 4, or 5 failed to activate cat-86 expression. These results indicate that the stalled ribosome that is most active in cat-86 induction has its aminoacyl site occupied by leader codon 6. To determine if chloramphenicol also stalled ribosomes in the cat-86 regulatory leader such that the aminoacyl site was occupied by codon 6, we separately changed leader codons 3, 4, 5, and 6 to the translation termination (ochre) codon TAA. Each of the mutated genes was tested for its ability to be induced by chloramphenicol. The results show that replacement of leader codons 3, 4, or 5 by the ochre codon blocked induction, whereas replacement of leader codon 6 by the ochre codon permitted induction. Collectively, these observations lead to the conclusion that cat-86 induction requires ribosome stalling in leader mRNA, and they identify leader codon 6 as the codon most likely to be occupied by the aminoacyl site of a stalled ribosome that is active in the induction.

Duvall et al. (8) recently demonstrated that starvation of Bacillus subtilis for the amino acid specified by codon 6 of the cat-86-encoded regulatory leader induced gene expression. Amino acid starvation is predicted to stall ribosomes such that the aminoacyl site is occupied by the codon for the deprived amino acid. Consequently, amino acid starvation experiments can identify the location in the cat-86 leader of the aminoacyl site of a stalled ribosome that is active in the induction of gene expression. In the present report we precisely define, by amino acid starvation experiments, the codons in leader mRNA that can lead to induction of cat-86 when occupied by the aminoacyl site of a stalled ribosome. An antibiotic inducer of cat-86, chloramphenicol, is shown to stall a ribosome in the regulatory leader such that the ribosomal aminoacyl site also occupies codon 6.

MATERIALS AND METHODS

Plasmids and Bacterial Strains. Plasmid pPL703-Spac and mutant derivatives described in the text were used throughout. pPL703 (5032 base pairs (bp)) is a promoter-cloning plasmid (Fig. 1; ref. 9) in which the promoterless cat-86 gene was activated by inserting the Spac promoter fragment (10) at a site approximately 144 bp 5' to the cat-86 gene. Strains of B. subtilis used are listed in Table 1.

Enzyme Assays. Chloramphenicol acetyltransferase (CAT) was assayed by the colorimetric procedure (14), and protein was measured by the method of Bradford (15). CAT specific activity is expressed as micromoles of chloramphenicol acetylated per min per mg of protein at 25°C.

Amino Acid Starvation Experiments. Amino acid-requiring mutants of B. subtilis containing pPL703-Spac or mutant plasmids were grown at 37°C to logarithmic phase in minimal medium containing amino acid supplements (8). Cells were washed twice with cold, unsupplemented minimal medium and were resuspended in prewarmed minimal medium containing all amino acids except for one that was required for growth. The culture was then shaken at 37°C, and samples were removed for CAT assays at intervals over a 2-hr period. In some cases, samples were removed for CAT assays only at the time of resuspension into the second growth medium (0 time) and after the 2-hr incubation. All starvation assays were performed at least three times. Data presented are from individual assays but are typical of the replicates. Mock-starvation was performed by including the required amino acid in the second growth medium (8), and this was performed only when starvation for a particular amino acid induced cat-86 expression. Mock-starvation always failed to induce cat-86. Lysine is specified by two codons in the leader of cat-86, designated Lys-1 (codon 3) and Lys-2 (codon 6). Starvation for lysine to achieve ribosome stalling at Lys-1 was performed by the use of cat-86-Tyr, in which a tyrosine codon, TAT, replaced Lys-2 (8). Stalling at Lys-2 was

Two classes of inducible antibiotic-resistance genes in Gram-positive bacteria are regulated by mechanisms that seem to be variations of the attenuation regulatory model (1). Production of the cat and erm gene products is induced by chloramphenicol and erythromycin, respectively, and the gene products confer resistance to the antibiotic inducers (2-5). In both examples, the coding sequence for the drug-resistance determinant and the coding sequence for an upstream regulatory leader peptide are separated by a sequence in DNA that dictates a secondary structure in mRNA. This RNA secondary structure (as demonstrated for cat-86 and ermC; refs. 6 and 7) blocks translation of the drug-resistance determinant by sequestering the ribosome binding site (RBS). It has been suggested that ribosomes translating the regulatory leaders are stalled by the inducing antibiotics at a discrete location, which leads to destabilization of the downstream RNA secondary structure and translation of the drug-resistance determinant.

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Abbreviations: RBS, ribosome binding site; CAT, chloramphenicol acetyltransferase.
performed by use of cat-86-Thr in which a threonine codon, ACA, replaced Lys-1 (8). The rationale behind these experiments is as follows: Starvation of B. subtilis for an amino acid stalls transit of ribosomes at all codons for the deprived amino acid. Stalling at specific codons in the cat-86 leader destabilizes the RNA stem–loop, which permits initiation of translation of the cat-86 coding sequence (8). Translation of the cat-86 coding sequence requires all 20 amino acids, and this translation can occur in cells starved for 1 amino acid (8). We therefore feel that the appearance of the deprived amino acid in the cell must result from the turnover of preexisting proteins. This general protein turnover probably is triggered by starvation for a single amino acid and is likely to result in the availability of all 20 amino acids at low levels. We presume that amino acid starvation, which destabilizes the RNA stem–loop (e.g., lysine deprivation), allows ribosomes to bind to RBS-3, the RBS for cat-86 (Fig. 1), and to begin translation of cat. These ribosomes are predicted to stall in cat at a codon for the deprived amino acid. When protein turnover makes available the deprived amino acid, albeit at a low level, a fraction of the total stalled ribosomes in the cell resumes translation. If the ribosome that is stalled in the leader resumes translation, then the stem–loop probably reforms, sequestering the cat RBS. However, ribosomes that have previously initiated translation of cat-86 have the opportunity to continue translation. Since amino acids released by protein turnover are in limited quantity, cells are maintained in a partially starved state. Thus, stalling at codons for the deprived amino acid and translation of that same codon elsewhere in mRNA are events that continue to occur throughout the duration of the experimental period.

**Induction of cat-86 by Antibiotics.** Cells harboring cat-86, on pPL703-Spac or mutant derivatives, were grown to logarithmic phase in antibiotic medium 3 (Difco; penassay broth), and chloramphenicol (2 μg/ml) or amicetin (0.5 μg/ml) was added. Incubation was continued for 2 h, and cells were harvested for CAT assays. Amicetin is an alter-
native inducer of cat-86 (16); all inductions with amicetin gave results comparable to those resulting from induction by chloramphenicol.

Site-Directed Mutagenesis. Methods used to introduce mutations into the cat-86 leader 2 region have been described (8, 17). Sequencing of DNA was by the dideoxy method (18). The mutations inserted into the cat-86 regulatory leader are predicted not to influence the stability of the RNA secondary structure present in leader mRNA based on the rules of Tinoco et al. (19).

RESULTS

Correlation Between Codon Positions in the cat-86 Regulatory Leader and Amino Acid Starvation That Leads to cat-86 Expression. The minimum region essential to inducible expression of cat-86 consists of an 84-bp sequence 5' to the cat-86 coding sequence (20, 21). This regulatory region contains two functional domains (Fig. 1). Domain B spans a pair of inverted-repeat sequences, which dictate a stem–loop structure in cat-86 mRNA. The RBS for the cat-86 coding sequence, RBS-3, is sequestered within this RNA secondary structure. Hence, domain B blocks translation of cat-86 mRNA, and we have previously shown that chloramphenicol induction of cat-86 is due to activation of the translation of cat-86 mRNA (7). Domain A is 5' to, and overlaps with, domain B. Domain A contains an RBS, RBS-2, and an open-reading frame of nine codons, which we previously designated leader 2. In the present study, leader 2 is simply referred to as the regulatory leader since leader 1, which is located upstream from leader 2, is not essential to chloramphenicol induction, but the presence of leader 1 appears to stabilize cat-86 mRNA (ref. 21; N.P.A., unpublished results). In a previous study (8), it was shown that ribosome stalling in the cat-86 regulatory leader (Fig. 1) can be achieved by starving cells for amino acids specified by leader codons. A ribosome stalled at the leader codon located immediately 5' to the inverted repeats (codon 6) activated cat-86 expression. By contrast, stalling a ribosome at an early leader codon (codon 3 or 4) failed to activate the gene (8). We believe that ribosome stalling at codon 6 in domain A destabilizes the cat-86 RNA stem–loop in domain B. To perform a controlled set of starvations for amino acids specified by codons in the vicinity of codon 6 (Lys-2), we constructed by congression a set of nearly isogenic derivatives of BR151 having requirements for serine (BR151-Ser), isoleucine (BR151-Ile), and threonine (BR151-Thr). Starvation for aspartic acid was performed in strain 1A295 because the aspB66 allele, which confers an aspartic acid requirement, could not be introduced into BR151 by congression. Data from these experiments show that starvation for amino acids specified by the four carboxyl-terminal codons of the leader activated cat-86 expression (Fig. 2). Starvation for amino acids specified by codons in the amino-terminal portion of the leader (lysine, threonine, or aspartic acid; Fig. 2) and starvation for non-leader amino acids (tryptophan, tyrosine, and leucine; data not shown) failed to activate cat-86.

The aspartic acid codon (codon 5) is adjacent to the Lys-2 codon (codon 6), and ribosome stalling at Lys-2 activated cat-86, whereas stalling at the aspartic acid codon did not. Since starvation for aspartic acid was performed in a strain not isogenic with BR151, it was possible that factors unrelated to stalling might have influenced the results. We therefore converted the aspartic acid codon to a codon for tyrosine, TAT, by site-directed mutagenesis. Insertion of this mutant gene into a tyrosine-requiring mutant of B. subtilis, strain 1A206, with subsequent starvation for tyrosine failed to induce cat-86. We have previously shown that converting Lys-2 to TAT permits tyrosine starvation in strain 1A206 to activate the gene (8). Thus, the data demonstrate that stalling a ribosome such that the aminoacyl site is placed at leader codon 6 induces cat-86, whereas stalling that places the aminoacyl site at codon 5 fails to induce gene expression.

Location of the Aminoacyl Site of a Ribosome Stalled in the cat-86 Leader by Chloramphenicol. Chloramphenicol is believed to stall ribosomes at a site in the regulatory leader, which leads to destabilization of the RNA stem–loop. There is presently no direct evidence for the identity or the location of the leader sequence that permits stalling to take place. However, it is possible to determine the leader codon that occupies the aminoacyl site of a chloramphenicol-stalled ribosome. For example, we suspect that chloramphenicol can induce cat-86 only when a ribosome has translated to a location in the leader that brings the potential leader stall sequence to a correct position on the ribosome. Thus, replacing early leader codons with the translation termination (ochre) codon TAA should prevent a ribosome from translating to the leader stall sequence and, therefore, will block chloramphenicol induction of cat-86. Hence, the most amino-terminal location of an inserted ochre codon that permits chloramphenicol induction of cat-86 should mark the leader codon that occupies the aminoacyl site of a drug-stalled ribosome.

Leader codons 3–6 were individually changed to the ochre codon; the mutant genes are designated cat-86-Ter-3, -Ter-4, -Ter-5, and -Ter-6. Each of the four mutant genes in pPL703 was transcriptionally activated by inserting the Spac promoter into BR151 by congression. Data from these experiments show that starvation for amino acids specified by the four carboxyl-terminal codons of the leader activated cat-86 expression (Fig. 2). Starvation for amino acids specified by codons in the amino-terminal portion of the leader (lysine, threonine, or aspartic acid; Fig. 2) and starvation for non-leader amino acids (tryptophan, tyrosine, and leucine; data not shown) failed to activate cat-86.

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![Figure 2](image-url)
and was transformed into *B. subtilis* strain PY22. The uninduced and chloramphenicol-induced levels of expression of the mutant genes were then compared with that of wild-type cat-86 (Fig. 3). When the ochre codon replaced leader codons 3, 4, or 5, inducibility of the mutant genes was blocked regardless of whether the inducer was chloramphenicol (Fig. 3) or the alternative inducer amicetin (data not shown). In addition, the uninduced level of cat-86 expression in PY22 harboring cat-86-Ter-3, -Ter-4, or -Ter-5 was about 4-fold lower than the uninduced level of the wild-type cat-86 gene in the same strain (Fig. 3). The wild-type cat-86 gene and the mutant genes cat-86-Ter-3, -Ter-4, and -Ter-5 were also assayed for expression in PY122, a host that is largely isogenic with PY22 except that PY122 contains the sup-44 mutant allele, which is a suppressor of the ochre codon (11). In PY122 the induced and uninduced CAT levels were comparable to the levels observed in PY22 (data not shown). Similar results were obtained when these mutant genes were tested for inducibility in another *B. subtilis* strain 1A459, which carries the sup-3 mutant allele (Table 1). Thus, the noninducibility phenotype of cat-86-Ter-3, -Ter-4, or -Ter-5 was not detectably suppressed by either sup-44 or sup-3.

**cat-86-Ter-6** contains the ochre codon as a replacement for leader codon 6. In wild-type host bacteria (either PY22 or BR151), the uninduced level of expression of cat-86-Ter-6 was about 5-fold higher than the uninduced level of expression of wild-type cat-86 (Fig. 3). Chloramphenicol induction of PY22 cells (or BR151 cells) carrying the mutant gene increased expression to a level comparable to that seen when wild-type cat-86 was induced in the same strains. The high level of uninduced expression of cat-86-Ter-6 (Fig. 3) probably reflects a transient stalling of ribosomes at the termination codon. It is known, for example, that when a ribosome encounters an ochre codon, release from mRNA requires the action of a protein release factor (22). Thus, the time spent by a drug-free ribosome at the ochre codon in the cat-86-Ter-6 leader may facilitate destabilization of the RNA stem–loop. The data obtained by the amino acid starvation experiments (Fig. 2) and by analysis of the inducibility of the cat-86 Ter mutants (Fig. 3) demonstrate that a stalled ribosome that is active in destabilization of the RNA stem–loop has its aminoacyl site occupied by leader codon 6.

**DISCUSSION**

The present study reveals two important features of the mechanism that permits induction of cat-86 expression. First, induction requires the stalling of a ribosome in the leader region of cat-86 mRNA. Second, a ribosome that is active in cat-86 induction is one that is stalled in the leader region at a location that places the aminoacyl site at a codon 3' to leader codon 5. We believe that leader codon 6 is the preferred location for the aminoacyl site of a stalled ribosome that is active in induction, based on several observations. The highest level of cat-86 expression obtained in the amino acid starvation experiments was achieved when cells were starved for the amino acid specified by leader codon 6; induction was not detected during starvation for amino acids encoded by leader codons 3, 4, or 5, and only weak induction was achieved by starving for amino acids specified by codons 7 and 8. Furthermore, the inducibility of cat-86 by chloramphenicol (or amicetin) was retained when leader codon 6 was changed to the ochre codon, whereas inducibility was abolished when the ochre codon replaced leader codon 5.

Our experimental results appear to provide a partial explanation of the details that lead to the activation of the translation of cat-86 mRNA by ribosome stalling. We believe induction occurs when a stalled ribosome masks sequences in the left inverted repeat that contribute to the formation or stability of the RNA stem–loop. When the aminoacyl site of a stalled ribosome occupies codon 5, induction of cat-86 cannot be detected. Thus, a ribosome at this position must not be able to mask a sufficient number of nucleotides in the left inverted repeat to destabilize the stem–loop conformation. Our data also suggest that when the aminoacyl site of a ribosome is stalled at codon 7, induction is much weaker than when the aminoacyl site is stalled at codon 6. This suggests the possibility that while stalling at codon 7 may mask the left inverted repeat a ribosome at this position may also interfere with the entry of a second ribosome at RBS-3 (see Fig. 1). Since efficient induction is obtained when the aminoacyl site is stalled at codon 6, perhaps a ribosome at this location masks the left inverted repeat sequences but does not interfere with the access of another ribosome to RBS-3.

Our genetic data become more meaningful when viewed in the context of the physical size of a ribosome. A *B. subtilis* ribosome is reported to span about 40 nucleotides of linear mRNA (23). The distance between leader codon 6 and RBS-3 is 30 nucleotides. This distance would seem adequate for a ribosome stalled at codon 6 to permit entry of a second ribosome at RBS-3, if it is assumed that the aminoacyl site represents the leading edge of the stalled ribosome. However, if the physical presence of a stalled ribosome extends 10 nucleotides 3' to the aminoacyl site, this places the stalled ribosome in close proximity to the ribosome entering at RBS-3. Under these constraints, moving the stalled aminoacyl site from codon 6 to codon 7 could influence the ability of a ribosome to enter at RBS-3.

A question that is not addressed in the current study relates to the nature of the signal in the leader region that causes ribosomes to stall in the presence of chloramphenicol. It is this signal that presumably causes a ribosome to stall such that its aminoacyl site occupies codon 6. The inability of two

**Fig. 3.** Chloramphenicol induction of versions of cat-86 in which the ochre codon replaced leader codon 3, 4, 5, or 6. Mutant derivatives of cat-86 were constructed in which TAA replaced the Lys-1 codon (cat-86-Ter-3), the Thr codon (cat-86-Ter-4), the Asp codon (cat-86-Ter-5), and the Lys-2 codon (cat-86-Ter-6). Wild-type cat-86 and each mutant plasmid were introduced into PY22 and each was induced for 2 hr with chloramphenicol (2 μg/ml) or amicetin (0.5 μg/ml). Induction by both drugs gave comparable results, and only the chloramphenicol inductions are shown. Uninduced CAT activity is represented by solid bars; chloramphenicol induced CAT activity is indicated by open bars. Chloramphenicol induction of wild-type (wt) cat-86 in PY22 is shown at the left.
mutant alleles, sup-44 and sup-3, to suppress the noninducibility phenotype due to ochre codon replacements at leader codons 3, 4, or 5 is similar to a result obtained with the PUB112 cat leader (24-26). These data may indicate that the amino acid sequence of the leader is an important signal for stalling. However, further study of the question is clearly necessary since the nature of the amino acid inserted at ochre codons due to the product of sup-44 or sup-3 has not been identified.

A remarkable aspect of the regulation of inducible cat and erm genes is the similarity with the attenuation mechanism that is involved in the regulation of amino acid biosynthetic operons (1). Both systems activate gene expression when a ribosome stalls in a leader by sensing an environment signal, either the absence of an amino acid or the presence of a specific antibiotic. This stalling results in changes in mRNA conformation that lead to either transcription of the regulated genes in the case of amino acid biosynthetic operons (1) or activation of the translation of mRNA as in the case of cat-86 and ermC.

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