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Four Codons in the *cat-86* Leader Define a Chloramphenicol-Sensitive Ribosome Stall Sequence

ELIZABETH J. ROGERS, UN JIN KIM, NICHOLAS P. AMBULOS, JR., AND PAUL S. LOVETT*

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

Received 17 July 1989/Accepted 26 September 1989

Genes encoding chloramphenicol acetyltransferase in gram-positive bacteria are induced by chloramphenicol. Induction reflects an ability of the drug to stall a ribosome at a specific site in *cat* leader mRNA. Ribosome stalling at this site alters downstream RNA secondary structure, thereby unmasking the ribosome-binding site for the *cat* coding sequence. Here, we show that ribosome stalling in the *cat-86* leader is a function of leader codons 2 through 5 and that stalling requires these codons to be presented in the correct reading frame. Codons 2 through 5 specify Val-Lys-Thr-Asp. Insertion of a second copy of the stall sequence 5′ to the authentic stall sequence diminished *cat-86* induction fivefold. Thus, the stall sequence can function in ribosome stalling when the stall sequence is displaced from the downstream RNA secondary structure. We suggest that the stall sequence may function in *cat* induction at two levels. First, the tetrapeptide specified by the stall sequence likely plays an active role in the induction strategy, on the basis of previously reported genetic suppression studies (W. W. Mulbry, N. P. Ambulos, Jr., and P. S. Lovett, J. Bacteriol. 171:5322–5324, 1989). Second, we show that embedded within the stall sequence of *cat* leaders is a region which is complementary to a sequence internal in 16S rRNA of *Bacillus subtilis*. This complementarity may guide a ribosome to the proper position on leader mRNA or potentiate the stalling event, or both. The region of complementarity is absent from *Escherichia coli* 16S rRNA, and *cat* genes induce poorly, or not at all, in *E. coli*.

cat-86 is a plasmid gene that specifies chloramphenicol-inducible chloramphenicol acetyltransferase in *Bacillus subtilis* (1). Drug induction of *cat-86* expression results primarily from the activation of translation of *cat-86* mRNA rather than from activation of transcription of the gene (8). The primary event which causes induction of *cat-86* is chloramphenicol-mediated stalling of a ribosome at a specific mRNA site in a leader region located 5′ to the *cat-86* coding sequence (1). The stalled ribosome destabilizes an adjacent downstream secondary structure in *cat-86* mRNA that normally sequesters the *cat-86* ribosome-binding site (2). Thus, drug-mediated stalling of a ribosome that is translating the *cat-86* leader can result in the unmasking of the ribosome-binding site for the downstream *cat-86* coding sequence. This form of regulation is a variation of the attenuation regulatory model (16) and has been termed translational attenuation.

Induction of *cat-86* expression can also be achieved by stalling a drug-free ribosome at the correct site in the leader; stalling the ribosome is accomplished by starving host cells for specific leader-encoded amino acids (1, 7). It is inferred from this observation that chloramphenicol is needed solely to stall a ribosome at the correct leader site and that the drug is not necessary for an event subsequent to stalling.

cat-86 is induced by both chloramphenicol and the less well studied antibiotic amicetin but is not induced by several other antibiotics that are also known to block ribosome transit (9). Consequently, we suspected that the *cat-86* leader might contain a recognition sequence that allowed the stalling of a ribosome by chloramphenicol (and amicetin) at the location necessary to cause destabilization of the downstream RNA secondary structure. The present report describes experiments which demonstrate that a chloramphenicol-specific stall sequence exists in the *cat-86* leader and provides an initial characterization of the nature of the stall sequence.

* Corresponding author.

MATERIALS AND METHODS

Bacteria and plasmids. *Bacillus subtilis* BR151 (*trpC2 metB10 lys-3*) was used throughout. All plasmids were derivatives of pPL703 (Fig. 1). *cat-86* in pPL703 was activated by the P4 promoter (17). Bacterial growth and plasmid isolation and manipulations were as previously described (7).

Enzyme assays. Chloramphenicol acetyltransferase was assayed at 25°C by the colorimetric procedure of Shaw (22). Protein was determined by the method of Bradford (3).

Oligonucleotide mutagenesis. Site-directed mutagenesis was performed as previously described (7, 24, 25). DNA sequencing was by the dideoxy method using M13 vectors (21).

Induction of *cat-86*. Cells harboring *cat-86* were induced for 2 h with 2 μg of chloramphenicol per ml. Induction by amino acid starvation was as previously described (7).

RESULTS

Chloramphenicol stall sequence in the *cat-86* leader. Induction of *cat-86* results when a ribosome stalls in the leader such that its aminoacyl site occupies leader codon 6 (1). This precise positioning of ribosome stalling due to the action of chloramphenicol can be explained by proposing that a chloramphenicol-specific stall sequence exists in the leader. This stall sequence would cause the cessation of elongation of a translating ribosome in the presence of chloramphenicol. The existence of a chloramphenicol stall sequence in the leader was first indicated by the observation that insertion of a second copy of leader codon 5, designated 5A, between leader codons 5 and 6 prevented induction by chloramphenicol and that subsequent deletion of leader codon 6 restored induction (15). Thus, the inserted codon blocked induction

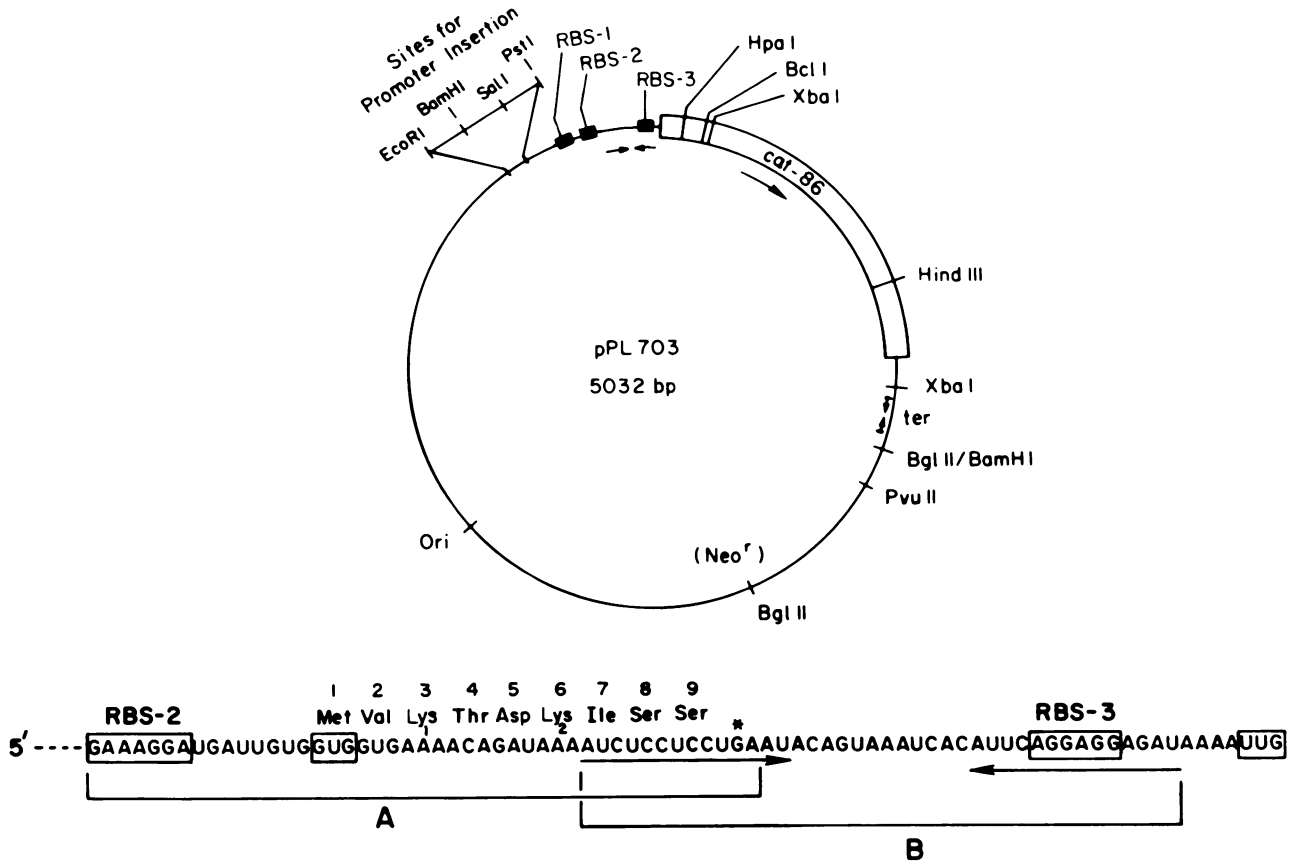

FIG. 1. Diagram of plasmid pPL703. (Top) pPL703 was constructed as a promoter-cloning plasmid by inserting a 1,250-base-pair *Pst*I-*Bgl*II DNA fragment from *Bacillus pumilus* between the *Eco*RI and *Bam*HI sites of the neomycin resistance (Neo^r) plasmid pUB110 by using a 21-base-pair *Eco*RI-*Pst*I linker containing internal *Bam*HI and *Sal*I sites. The 1,250-base-pair fragment contains *cat-86*. Promoter-containing fragments inserted into the linker activate transcription of *cat-86*. In this study, *cat-86* was activated by the P4 promoter (17). (Bottom) BAL 31 deletion studies and gene fusions have established that the minimal regulatory region essential to chloramphenicol inducibility consists of an 84-base-pair sequence located immediately 5′ to the *cat-86* coding sequence. Domains A and B constitute two functionally distinct regions of the regulatory sequence. Domain B specifies the RNA stem-loop that prevents translation of the *cat* coding sequence. Domain A contains a sequence that allows chloramphenicol to stall a ribosome which then destabilizes the RNA stem-loop. The boxed UUG codon is the translation start for *cat-86*. *, Translation stop codon UGA; RBS, ribosome-binding site.

by displacing an upstream stall sequence from the RNA stem-loop.

We suspected that the leader stall sequence should exist within the 28 nucleotides (nt) which are 5′ to leader codon 6. This is suggested because a ribosome on linear mRNA protects about 40 nt, and the aminoacyl site of a ribosome is probably located 12 nt upstream of the leading edge of a translating ribosome (11, 19). To identify this stall sequence, we took advantage of the observation that inserting the 5A codon between codons 5 and 6 of the leader prevented induction by displacing the stall sequence from the RNA stem-loop (15). Continued insertion of leader codons between codons 5 and 6 would be expected to eventually restore induction when the insertions reconstructed the stall sequence. We further reasoned that if the chloramphenicol stall sequence was determined entirely by nucleotides that are located 5′ to leader codon 6, the proposed reconstruction experiment would produce a leader with two complete stall sequences. Thus, a ribosome might stall at either of these sites in the presence of chloramphenicol, yet only stalling at the more downstream sequence, which is adjacent to the stem-loop, would lead to *cat-86* induction. Consequently, we performed this reconstruction experiment with a leader mutant of *cat-86* designated *cat-86* Thr (7). Leader codon 3 (designated 3*) of *cat-86* Thr is ACA (Thr) instead of AAA (Lys), which is present in the wild-type leader. *cat-86* Thr is inducible by chloramphenicol, but the extent of induction is less than half of that seen with the wild-type *cat-86* gene (Fig. 2). On the basis of our model for *cat-86* induction, it seemed likely that replacement of the Lys codon with a Thr codon might have weakened the stall sequence. Thus, use of *cat-86* Thr in the reconstruction experiment would be expected to produce a leader with two stall sequences, and the upstream stall sequence would be weaker than the downstream stall sequence.

The results of the reconstruction experiment with *cat-86* Thr demonstrate that insertion between leader codons 5 and 6 of codon 5A (Fig. 2, *cat-86* Thr + 5A) or codons 3A, 4A, and 5A (Fig. 2, Mut III) prevented induction, whereas insertion of codons 1A, 2A, 3A, 4A, and 5A permitted induction (Fig. 2, Mut IV). Subsequent deletion of the inserted 1A codon did not interfere with induction (Fig. 2, compare Mut V with Mut VI). Thus, we suspected that the stall sequence was within leader codons 2 through 5.

The inserted codons 1A through 5A restored induction but to a level lower than that observed with the wild-type *cat-86*

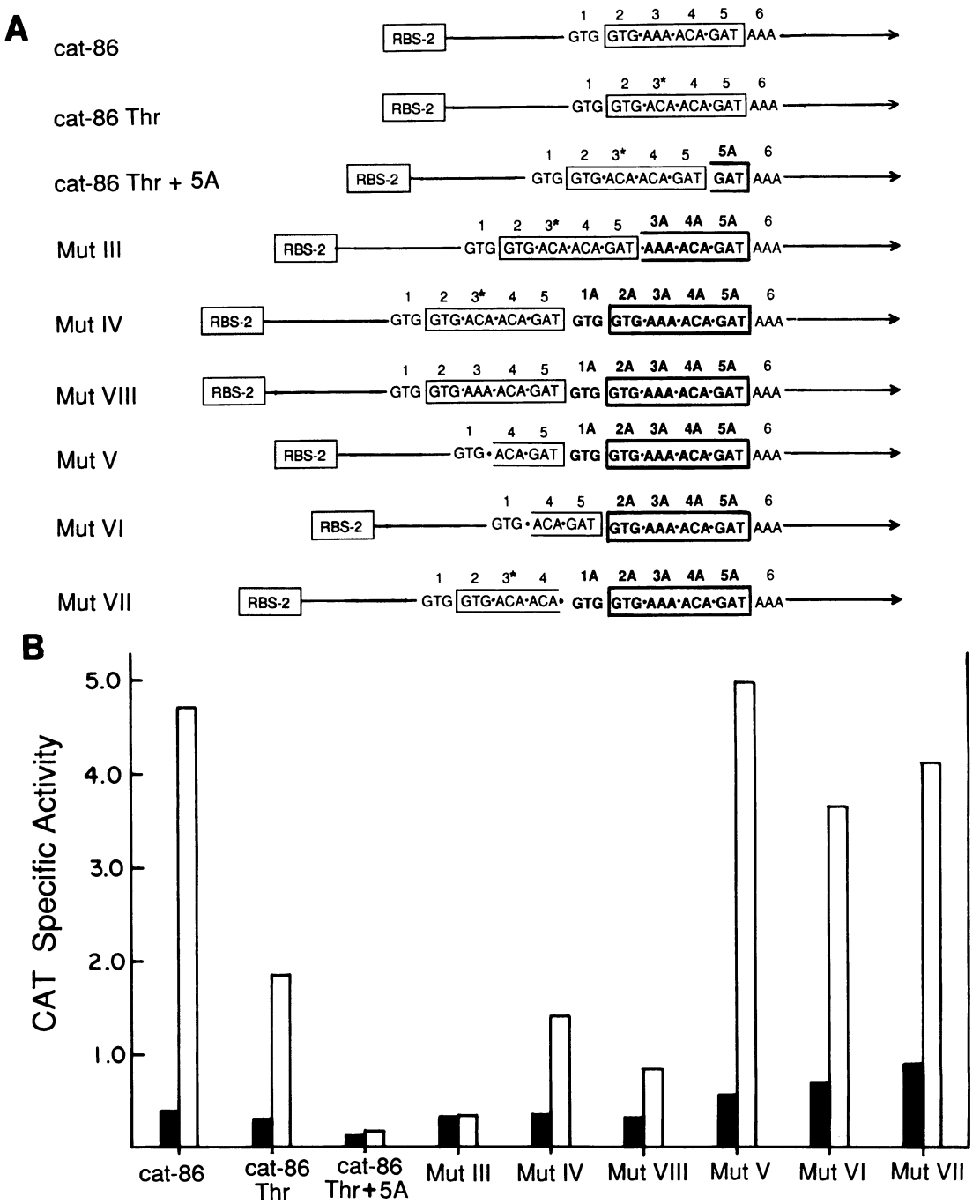

FIG. 2. Influence of the *cat-86* leader sequence on inducibility of *cat-86* by chloramphenicol. (A) Sequences of wild-type and mutant leaders; (B) induction levels of CAT after 2 h of incubation with (□) or without (■) 2 μg of chloramphenicol per ml.

gene (Fig. 2, compare *cat-86* with Mut IV). If this lower level of induction was the result of stalling competition because of the presence of the upstream stall sequence (e.g., codons 2 through 5), we would expect full induction to return upon removal of relevant portions of the upstream stall sequence. Full inducibility was restored to the reconstruction mutant when we deleted leader codons 2 and 3* (Fig. 2, compare Mut IV with Mut V). In a separate experiment, deletion of leader codon 5 also restored full inducibility (Fig. 2, Mut VII). Thus, the stall sequence is contained within leader codons 2 through 5.

Chloramphenicol induction of *cat-86* with tandem, wild-type leader stall sequences. To determine the inducibility of a version of *cat-86* in which the leader contained two wild-type stall sequences, codon 3* (Thr) in Mut IV (Fig. 2) was mutagenized to a Lys codon (AAA), which is the codon at position 3 in the wild-type leader (Fig. 1). The resulting leader (Fig. 2, Mut VIII) therefore contained two wild-type stall sequences, an upstream competing sequence and a downstream sequence that is in the correct position to allow *cat-86* induction. This mutant (Mut VIII) did not induce as well as did the version containing a weakened, upstream

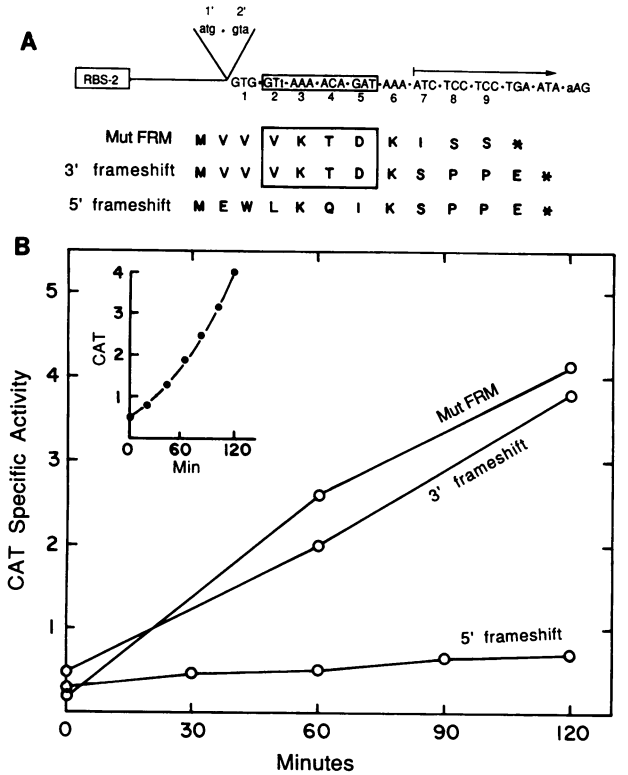

FIG. 3. Sequence of the leader of Mut FRM and chloramphenicol induction studies of Mut FRM and two frameshifted derivatives. (A) Mut FRM is a derivative of *cat-86*. The leader of Mut FRM contains nucleotide sequence changes from the wild-type leader, which are shown as small letters. The amino acid sequence of the leader of Mut FRM and the two frameshifted derivatives is also shown. (B) Chloramphenicol induction profiles of Mut FRM and a derivative with an A deleted from codon 6 (3′ frameshift) and one with the T deleted from codon 2′ (5′ frameshift). (Inset) Induction of the 5′ frameshift mutant by lysine starvation. RBS, Ribosome-binding site.

competing sequence (Fig. 2, Mut IV). Therefore, chloramphenicol-dependent ribosome stalling must have occurred at the upstream stall sequence, and the upstream stall sequence is displaced from the RNA stem-loop by six intervening codons. From this result, we infer that the RNA stem-loop structure in *cat-86* transcripts is not an essential component of the stall sequence.

Successful induction of *cat-86* requires that the stall sequence be presented in a correct reading frame. We wished to determine if the stall sequence was a function of the nucleotide sequence or codon sequence in the region of the leader spanning codons 2 through 5. Therefore, a mutant of *cat-86* was constructed in which the region encompassing leader codons 2 through 5 could be frameshifted by 1 nt while maintaining the original spatial relationship between the nucleotide sequence of leader codons 2 through 5 and the RNA stem-loop. To achieve this, we used a previously described mutant of *cat-86* in which leader codon 2 had been changed from GTG to GTT (15). This mutation did not alter the inducibility of *cat-86* but did prevent the formation of an unwanted translation termination codon in the −1 reading frame of the leader. We next added codons 1′ and 2′, ATG · GTA, to the 5′ end of the leader, and a single base change (C→A) was made in the 12-nt-pair region located between the inverted-repeat sequences (Fig. 3). The latter produced a TAA (ochre) codon in the −1 frame relative to the reading frame of the leader. This mutant construction, Mut FRM (Fig. 3), was then used in two experiments to determine if the original reading frame across the stall sequence was essential for drug induction. We initially introduced a control frameshift mutation by deleting an A from leader codon 6, which resulted in a −1 frameshift 3′ to the stall sequence. This mutation did not block the inducibility of *cat-86* (Fig. 3). To determine if a −1 frameshift mutation 5′ to the stall sequence altered induction, a T was deleted from leader codon 2′ of Mut FRM. This deletion prevented drug induction (Fig. 3). Thus, we infer that drug-mediated ribosome stalling that leads to *cat-86* induction can take place only when the stall sequence is presented in a correct reading frame.

The change of reading frame across the stall sequence blocked induction, and this blockage was presumed to be due only to an absence of chloramphenicol-mediated ribosome stalling in the leader. A testable alternative was also considered. Perhaps the sequence of the leader codons or of the leader amino acids is essential both for stalling and for a poststalling event which enables the ribosome to melt the RNA stem-loop. To resolve this question, the 5′ frameshift mutant was induced by starving host cells for lysine (7), because in this mutant a Lys codon (AAA) is present at the 5′ base of the RNA stem-loop (Fig. 3). Starving the host cells for lysine activated *cat-86* expression in the 5′ frameshift mutant (Fig. 3, inset). We therefore infer that the codon sequence (or amino acid sequence) of the leader is necessary for chloramphenicol-mediated stalling but not for a poststalling event.

DISCUSSION

Genetic studies with *cat-86* and pUB112 *cat* have established that induction requires a ribosome to be stalled with its aminoacyl site at leader codon 6 (1, 6). It has also been shown that stalling a ribosome at the correct *cat-86* leader site in the absence of chloramphenicol similarly results in induction of gene expression (1, 7). Thus, the primary, and probably exclusive, function of the inducing antibiotic chloramphenicol is to stall a ribosome in the leader. Net induction of chloramphenicol acetyltransferase protein by chloramphenicol could result if chloramphenicol nonspecifically stalled ribosomes at different sites in different *cat* leaders. Induction would then reflect the expression of that fraction of the total *cat* genes in a cell in which a ribosome was fortuitously stalled at the correct leader site. Alternatively, *cat* leaders could contain a specific sequence that causes a ribosome to become hypersensitive to stalling in the presence of chloramphenicol, i.e., a stall sequence. Our present results demonstrate that a specific chloramphenicol stall sequence exists in the leader and that this sequence is determined by leader codons 2 through 5.

The stall sequence blocks ribosome transit by a mechanism that remains to be determined. It seems clear, however, that the nature of specific leader amino acids is important to the stalling strategy. This conclusion is based partly on suppression studies of ochre mutations in the leaders of *cat-86* and the pUB112 *cat* gene. *sup-3* is a nonsense suppressor of *B. subtilis* which inserts lysine at ochre codons (10, 18). Replacement of *cat* leader codon 2, 3, 4, or 5 (the stall sequence) with the ochre codon (TAA) prevents induction in wild-type cells (1, 6). A *sup-3*-containing cell restores inducibility to leader mutants in which

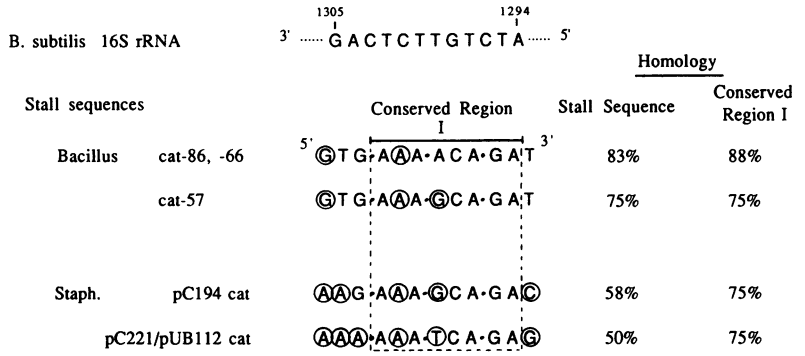

FIG. 4. Homology between stall sequences in *cat* leaders and a region of *B. subtilis* 16S rRNA. The sequence of *B. subtilis* 16S rRNA is from the work of Green et al. (12). The 5'-most nucleotide of 16S rRNA is assigned as nt 1. Stall sequences are from the leaders of six inducible *cat* genes whose leader sequences are summarized in reference 7. The *cat* genes were originally identified in *B. pumilus* (Bacillus) or *Staphylococcus* species (Staph.). The stall sequences for *cat-86* and *cat-66* are identical, as are the stall sequences for *cat* on plasmids pC221 and pUB112. Conserved region I was previously identified as a 8-base-pair region of near identity in all *cat* leaders (7). Circled bases are mismatches with the 16S rRNA sequence shown. Percent homology is calculated as the number of nucleotides which are complementary to the rRNA sequence divided by the number of nucleotides in the sequence. There are 12 nt in the stall sequence and 8 nt in conserved region I. The stall sequences are codons 2 through 5 of *cat* leaders. Conserved region I includes leader codons 3 and 4 and the first two positions of codon 5 (7).

the ochre codon replaces a Lys codon (leader codon 2 of the pUB112 *cat* and leader codon 3 of *cat-86* [16, 18]). *sup-3* does not restore inducibility to leader mutants in which the ochre codon replaces nonlysine codons (leader codons 4 or 5 [6, 18]). These data argue that the nature of the leader amino acids is a determinant of induction. Consistent with this interpretation is the overall similarity of amino acids 2 through 5 of the leaders of several inducible *cat* genes. Lys or Val is found as amino acid 2; amino acids 3 and 5 are Lys and an acidic amino acid, respectively; amino acid 3 is typically Thr or Ser, although Ala is found at this position in two of the six leaders examined. The consensus sequence is Lys-Lys-Thr/Ser-Asp (4, 7, 14, 23). Lastly, single nucleotide replacements in *cat* leader codons 3 through 5 which change the corresponding amino acid to an unlike amino acid diminish or abolish inducibility (6, 7, 15; U. J. Kim, unpublished data).

A second level of function may also exist embedded within the nucleotide sequence of the stall sequence. This was uncovered by considering mechanisms that might guide, or stabilize, the placement of a stalled ribosome on leader mRNA to effect destabilization of the RNA stem-loop (5). Of the 12 nt in the *cat-86* (and *cat-66*) stall sequence, 10 are complementary to an internal sequence in *B. subtilis* 16S rRNA (Fig. 4). This homology of 83% increases to 88% if only matches within an 8-nt subset of the stall sequence, conserved region I, are considered. Conserved region I was previously identified by Duvall et al. (7) and is conserved at seven of the eight positions in all *cat* leaders. Examination of the predicted stall sequences in other *cat* leaders for which sequence data are available reveals homology with 16S rRNA of 50 to 75%, if all 12 nt of the stall sequence are taken into account (Fig. 4). Since most of the matches are within conserved region I, the homology increases to 75% if only matches within conserved region I are considered. *Escherichia coli* 16S rRNA (20) lacks the region of homology found in *B. subtilis* 16S rRNA, which possibly accounts for the reduced or absent induction of *cat* genes in *E. coli* (13, 23; E. J. Rogers, unpublished observations). The role of the homology between the *cat* stall sequence and *B. subtilis* 16S rRNA remains to be fully explained. It is conceivable, however, that such an interaction may fine-tune ribosome positioning on leader mRNA or facilitate an event essential to ribosome stalling or both.

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