A Variation of the Translation Attenuation Model Can Explain the Inducible Regulation of the pBC16 Tetracycline Resistance Gene in *Bacillus subtilis*

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Expression of the *tet* resistance gene from plasmid pBC16 is induced by the antibiotic tetracycline, and induction is independent of the native promoter for the gene. The nucleotide sequence at the 5’ end of the *tet* mRNA (the leader region) is predicted to assume a complex secondary structure that sequesters the ribosome binding site for the *tet* gene. A spontaneous, constitutively expressed *tet* gene variant contains a mutation predicted to provide the *tet* gene with a nonsequestered ribosome binding site. Lastly, comparable levels of *tet* mRNA can be demonstrated in tetracycline-induced and uninduced cells. These results are consistent with the idea that the pBC16 *tet* gene is regulated by translation attenuation, a model originally proposed to explain the inducible regulation of the *cat* and *erm* genes in gram-positive bacteria. As with inducible *cat* and *erm* genes, the pBC16 *tet* gene is preceded by a translated leader open reading frame consisting of a consensus ribosome binding site and an ATG initiation codon, followed by 19 sense codons and a stop codon. Mutations that block translation of *cat* and *erm* leaders prevent gene expression. In contrast, we show that mutations that block translation of the *tet* leader result in constitutive expression. We provide evidence that translation of the *tet* leader peptide coding region blocks *tet* expression by preventing the formation of a secondary-structure complex that would, in the absence of leader translation, expose the ribosome binding site. Tetracycline is proposed to induce *tet* by blocking or slowing leader translation. The results indicate that *tet* regulation is a variation of the translation attenuation model.

In gram-positive bacteria, inducible resistance to the ribosomally targeted antibiotics chloramphenicol and erythromycin is mediated by a form of regulation termed translation attenuation (10, 19, 33). Both example genes, *cat* and *erm*, specify proteins that function in the cytoplasm and confer resistance to the corresponding antibiotics. *cat* genes specify a protein that catalyzes the acetylation of chloramphenicol, and *erm* genes specify a protein that methylates specific residues in the 23S rRNA. The regulation of both genes is based on the nucleotide sequence of the mRNA 5’ to the coding region for the resistance protein, which we define as the leader region of the mRNA. The leader regions of the *cat* and *erm* mRNAs are predicted to fold into stable secondary structures that sequester the ribosome binding site (RBS) for the coding sequence that specifies the protein that confers antibiotic resistance (12, 20). Preceding and overlapping with the secondary-structure domain in the *cat* and *erm* mRNAs is a short open reading frame consisting of an RBS and a translation initiation codon, followed by a series of sense codons and a translation stop codon. The number of codons seen in leader open reading frames represented by the known *cat* and *erm* genes ranges from 7 to more than 25. It has been established that the *cat* and *erm* leader open reading frames are translated and that ribosome stalling during leader peptide translation forces the secondary structure into an alternative conformation that exposes the RBS for the coding sequence for the resistance protein. Thus, both *cat* and *erm* represent examples of a novel form of regulation that acts at the level of mRNA translation.

The genes (*tet*) specifying inducible resistance to tetracycline in gram-positive bacteria show a nucleotide sequence arrangement upstream of the resistance determinant that is reminiscent of that seen upstream of inducible *erm* genes, suggesting that *tet* regulation may follow the translation attenuation model. Indeed, on the basis of only the sequences of the inducible *tet* genes on plasmids pT181 and pTHT15 (16, 17), two groups proposed that the genes were regulated by translation attenuation. In contrast, a recent study of an inducible *tet* gene found in the chromosome of a strain of *Bacillus subtilis* led to a different interpretation (30). Induction was proposed to result from translation reinitiation, in which tetracycline causes a leader-associated ribosome to hop to the RBS and initiation codon of the coding sequence for the resistance protein. In the present study, we have analyzed the regulation of the inducible *tet* gene from plasmid pBC16 (6, 24). Our results indicate that this gene and probably related genes are regulated by a variation of the translation attenuation model. In this variation, translation of the leader open reading frame interferes with the formation and stability of a secondary-structure complex in the mRNA that exposes the RBS for the coding sequence of the resistance protein.

**MATERIALS AND METHODS**

Bacteria and plasmids. *B. subtilis* strain 1A40 (topC2 lys-3 metB10) from the *Bacillus* Genetic Stock Center, Ohio State University, was used unless otherwise noted. Plasmid pBC16 has been previously described and is a tetracycline resis-
tance plasmid that is related to pUB110 (6, 26, 29). The tet gene in pBC16 (24), including the leader region that extends 100 bp upstream from the coding region for the polypeptide responsible for tetracycline resistance, was amplified by PCR with two primers (5'-GC GGATCC AAAGTTAGGGAATTAA and 5'-CAAC TTTTGGGAAGTTTAGG) which place a BamHI site 5' to the tet gene. The fragment was cloned into pCR-BluntII-TOPO (Invitrogen). By site-directed mutagenesis, the first three codons of the tet resistance coding sequence, GTGGAATACA, were changed to ATGGATATC. To place a BamHI site 5' to the tet gene, the fragment was inserted in place of the wild-type GTG codons 2 and 3. The mutated tet-containing fragment was inserted between the BamHI and XbaI sites of pUB110. The resulting plasmid, pNT1, specified resistance to neomycin (10 \(\mu\)g/ml) and chloramphenicol at 3 \(\mu\)g/ml. After 2 h of incubation at 37°C, the cells were washed, harvested, lysed, and assayed for chloramphenicol acetyltransferase (CAT) activity at 25°C as previously described (11). Proteins were determined by the Bradford method (7). CAT specific activity is expressed as micromoles of chloramphenicol acetylated per minute per milligram of protein.

Site-directed mutagenesis. Site-directed mutagenesis was performed with the QuickChange site-directed mutagenesis kit (Stratagene) on plasmid DNA. Oligomers containing the desired mutation(s) were 25 to 35 nucleotides (nt) in length. Mutations were introduced into DNA fragments cloned into Escherichia coli plasmid vectors and sequenced, and the resulting fragment containing the desired mutation was recloned into pUB110. After reinsertion into pUB110, the region bearing the mutation(s) was again sequenced.

Computer-assisted mRNA folding. The mRNA corresponding to the leader sequence of the tet gene in pBC16 or pNC1 was folded by use of the Kinefold RNA-folding program (35, 36).

Northern blot assays. Cells were grown to mid-log phase in LB broth with or without tetracycline, and rifampin was added to 100 \(\mu\)g/ml. Aliquots (10 ml) were withdrawn immediately and at subsequent intervals. RNA was extracted according to the method of Mayford and Weisblum (20). RNA (10 \(\mu\)g) was electrophoresed on a 2% agarose–formaldehyde gel as previously described (2). The resolved RNA was transferred to GeneScreen nylon membrane and hybridized with the \(^{32}\)P-end-labeled primers noted in the legend to Fig. 4. The hybridization temperature was 42°C.

T1 endonuclease cleavage and primer extensions. RNA was isolated from cells (20), and 5 \(\mu\)g was suspended in 19 \(\mu\)l of T1 buffer (20 mM MgCl\(_2\), 100 mM NH\(_4\)Cl) and heated to 70°C for 5 min and quick cooled on ice. RNAse T1
(Fermentas) was diluted in water to 10 U/μl, and 1 μl was added to the RNA. After 10 min of incubation on ice, the reaction was terminated by addition of 60 μl (3 volumes) of cold stop solution (0.3 M sodium acetate, 10 mM EDTA, 0.5 mg/ml carrier RNA), immediately followed by addition of 2 μl of 10% sodium dodecyl sulfate to yield a final sodium dodecyl sulfate concentration of 0.24%. RNA was extracted with an equal volume of phenol-chloroform-isomyl alcohol and ethanol precipitated. Primer extensions were performed by addition of 0.8 pM end-labeled oligomer and Moloney murine leukemia virus reverse transcriptase (RT; Fermentas). Incubation was for 1 h at 37°C. Reaction mixtures were loaded on sequencing gels along with a sequencing ladder.

The concentration of T1 used was determined by testing serial dilutions of the enzyme on target mRNA. The enzyme concentration selected showed T1 nicking activity on the transcript and permitted RT to extend a primer to the 3′ end of the transcripts. A threefold dilution of this enzyme concentration eliminated detectable nicking of the RNA.

**RESULTS**

**Tetracycline induction results from activation of translation.** The construction of plasmid pNC1 (Fig. 1A) involved inserting the inducible tet gene from plasmid pBC16 (6) between the BamHI and XbaI sites of pUB110 and replacement of the tet coding sequence with the cat-86 coding sequence (see Materials and Methods). pNC1 specified tetracycline-inducible CAT activity in B. subtilis. Computer-assisted folding of the leader region of tet mRNA specified by pBC16 and of the leader region of the tet-cat fusion specified by pNC1 predicts a complex secondary structure that sequesters the RBS for the antibiotic resistance coding sequence (Fig. 1B), and we define the tet leader region as that portion of tet mRNA extending from the RBS for the leader peptide coding sequence (nt 1 to 6) to nt 110 (Fig. 1B).

B. subtilis cells carrying pNC1 grew on solid media at chloramphenicol levels not exceeding 3 μg/ml but failed to grow when the chloramphenicol level was 10 μg/ml. pNC1 containing cells capable of growth on 10 μg/ml chloramphenicol occurred spontaneously at a frequency of approximately 10^{-9}, and this phenotype cotransformed with plasmids isolated from the mutants. The mutant plasmids examined contained alterations of the tet leader region.

The constitutively expressed spontaneous mutant designated pNC1-F (Fig. 2A) resulted from duplication of the region of the leader that spans the tet RBS, providing the cat-86 reporter gene with a nonsequestered RBS. CAT activity specified by pNC1-F was constitutive and 100-fold greater than that observed with uninduced pNC1 (Fig. 2B). Since sequences upstream from the duplication were unaltered in the F mutant, the duplication itself was responsible for the high constitutive expression leading to the interpretation that it is the sequencing of the tet RBS which is the basis for the lack of expression of the wild-type gene in the absence of inducer.

If tet induction were due to a process that controlled only mRNA translation and not transcription, one would expect that tet-cat fusion mRNA would be present in cells grown without inducer. Figure 3 shows a Northern blot assay testing the levels of mRNA from pNC1-containing cells grown with and without inducer. Two different probes were used to assess mRNA levels, a 5′ probe for the tet leader (Fig. 3A) and a 3′ probe for cat (Fig. 3B). The level of tet-cat mRNA (the 1.8-kb band) was comparable in cells at mid-log phase regardless of whether or not the cells had been grown in tetracycline (compare Fig. 3A, uninduced and induced at time zero, and Fig. 3B, uninduced and induced at time zero). Rifampin (100 μg/ml) was added at mid-log phase (defined as time zero in Fig. 3), and samples taken 10 and 20 min later showed that a fraction of the mRNA from the induced cells had an extended half-life compared with mRNA from uninduced cells. Densitometer scanning of the 1.8-kb mRNA band demonstrated that when the 5′ probe was used, 15% of the time zero mRNA from induced cells was detected at 10 min and 13% was detected at 20 min. The values for the 3′ probe were 9% and 7% for the 10- and 20-min time points. In a separate experiment, virtually identical results were obtained. These data indicate that during induction two families of tet-cat mRNA were detected, a fast-decaying species and a minor slow-decaying species. Previous studies with erm and cat, and recently with a tet(L) gene (5, 9, 27, 32), have shown that induction increases the half-life of the corresponding mRNA, and this is believed to be due to protection afforded the mRNA by translating ribosomes or, alternatively, to leader-stalled ribosomes conferring stability on the
entire length of the transcripts. Since induction stabilized only a fraction (about 10%) of the tet-cat fusion transcripts, it follows that during induction only a fraction of the mRNA was translated or contained leader-stalled ribosomes. This interpretation is supported indirectly by the observation that the CAT activity specified by the induced CAT activity specified by the constitutive mutant was much greater than the CAT activity specified by the induced tet-cat fusion gene (Fig. 2B), arguing that induction activated the translation of a minority of the transcripts.

Testing the mRNA secondary structure predicted by computer-assisted folding. Computer-assisted folding of tet leader mRNA into the most thermodynamically stable state generated a structure with three stems (Fig. 1B). To test the validity of the computer prediction, T1 endonuclease cleavage studies were performed. T1 nuclease preferentially cuts single-stranded RNA 3' to G residues, and therefore T1 cleavage has been used to identify regions of RNA that are single stranded (34). We empirically determined the concentration of T1 that has been used to identify regions of RNA that are single stranded (34). We empirically determined the concentration of T1 that showed nicking activity and after nicking determined the locations of the nick sites as new sites of termination of reverse transcription. Typical results of T1 cleavage and reverse transcription of tet leader mRNA extracted from pBC16-containing cells grown without tetracycline are shown in Fig. 4. Figure 4A shows spontaneous and T1-caused primer extension stops across nt 17 to 78, and Fig. 4B shows the stops across nt 72 to 143. Spontaneous stops of reverse transcription are those seen when the RNA is not cleaved with T1 endonuclease and are thought to result from a change in RNA conformation that causes RT to occasionally drop off of the RNA, such as sites where corners are turned in the RNA and changes from the single-stranded state to duplexed states of RNA. The results from Fig. 4 are included in Fig. 1B, with spontaneous stops represented as arrows and the T1 cleavage sites as shaded G residues. The results of the T1 cleavage studies appear consistent with the “off” configuration of the RNA (Fig. 1B).

Is tet regulated by translation attenuation? Stasinopoulos et al. (30) found that an ochre mutation at leader codon 2 for the B. subtilis tetA(L) gene caused constitutive expression of the reporter gene. This observation appears contradictory to the translation attenuation model (e.g., see reference 1) and presumably led to their proposal of the novel ribosome restart model to explain tet regulation. We made ochre mutations at three positions in the pNC1 tet leader, at leader codons 1 and 2, and leader codon 2, and at leader codon 12. Ochre replacements of leader codon 2 and of leader codons 1 and 2 resulted in constitutive cat expression that was 20- to 50-fold higher than the basal expression of the wild-type gene (Fig. 5), and no induction by tetracycline was detected. By contrast, the ochre mutation at leader codon 12 resulted in normal tetracycline induction of cat-86 expression (Fig. 5), suggesting that induction is likely the result of an event such as ribosome stalling or slowing very early in the leader, certainly before leader codon 12.

An alternative approach to block leader translation involves eliminating the leader RBS. We changed the leader RBS from 5'AGGAGGA to 5'ACTATCA by site-directed mutagenesis. This rbs-1 mutation elevated basal cat expression 10-fold, and induction by tetracycline was not observed (Fig. 5). These data suggest that leader translation to an as-yet-unidentified point between leader codons 1 and 12 is essential to maintaining gene expression at a low basal level, i.e., the “off” state.

If leader translation favors the “off” configuration of the mRNA (Fig. 1B), a mutation that permits only inefficient initiation of leader translation would be expected to show an elevation of basal cat expression. It was previously demonstrated that substitution of CTG for an ATG initiation codon...
in *B. subtilis* caused a 70% reduction of translation efficiency, presumably reflecting reduced utilization of CTG for initiation (3). Replacement of the wild-type leader initiation codon ATG with CTG caused a threefold elevation in basal expression (Fig. 5). The CTG substitution allowed induction by tetracycline (Fig. 5); the maximum level of induced expression was comparable to that seen with wild-type pNC1 (Fig. 5).

To examine the role of the leader-encoded amino acids in
leader, taking care to avoid changes that would alter the predicted “off” secondary structure of the regulatory domain. While these two mutations altered the amino acid sequence between leader codons 3 and 11, induction of the resulting mutant was indistinguishable from induction of wild-type pNC1 (Fig. 6). Cys codons are seen twice in the mutant in which the leader initiation codon was changed from ATG to CTG. Light gray vertical bars represent uninduced cells, and dark bars represent induced cells.

The regulation, two frameshifting mutations were made in the leader, taking care to avoid changes that would alter the predicted “off” secondary structure of the regulatory domain. While these two mutations altered the amino acid sequence between leader codons 3 and 11, induction of the resulting mutant was indistinguishable from induction of wild-type pNC1 (Fig. 6). Cys codons are seen twice in the tet leader, and Cys is a commonly occurring codon in the leaders for other genes (Fig. 7). Replacing either Cys codon with a codon for an unrelated amino acid (tyrosine; UAU) did not, however, alter normal inducible expression (data not shown). Thus, it appears unlikely that the general amino acid sequence of the leader is critical to inducible expression.

A mechanism through which leader translation might block tet expression. Three naturally occurring constitutively expressed tet genes contain truncated leader open reading frames containing only 4 or 5 codons, which are much shorter than the 16 to 20 leader codons seen with inducible tet genes (Fig. 7). This observation is consistent with the idea that leader translation, at least to the position represented by leader codon 12 of the pBC16 tet leader, is critical to blocking downstream gene expression. Moreover, as first noted by Stasinopoulos et al. (30), the leader of the B. subtilis tetA(L) gene is optimized for efficient translation, and this is also true for the pBC16 tet leader; the leader RBS is a perfect complement with the 3’ end of 16S rRNA, and the spacing between the leader RBS and the ATG start codon is a consensus 7 nt. We therefore considered the possibility that leader translation favors the “off” conformation of the mRNA by reducing the stability of an alternative mRNA conformation, the “on” conformation, that provides the resistance determinant with a nonsequestered RBS. Hoshino et al. (16) and Khan and Novick (17) have proposed “on” conformations that do not account for the role of leader translation in maintaining the gene in the “off” state.

Leader mRNA will fold into various conformations as transcription processes along the DNA in a 5’-to-3’ direction. A transcript of the tet leader produced when the U nucleotide (nt 98) immediately 5’ to the tet RBS has just emerged from RNA polymerase will fold into the structure we define as the “on” conformation depicted in Fig. 8, with a ΔG of −15.4. This is the most thermodynamically stable conformation other than the “off” conformation (Fig. 1B), and the “off” conformation requires transcription through an additional 7 nt. Therefore, after the “on” conformation has formed (Fig. 8), continued transcription for an additional 7 nt places the “on” state in competition with the “off” conformation (Fig. 1B) and the “off” state is thermodynamically favored. Accordingly, when the additional 7 nt are added to mRNA in the “on” conformation, two outcomes seem possible. The first is reconfiguration to the “off” state. The second is entry of a ribosome at the tet RBS. If a ribosome occupies the tet RBS, that ribosome would reduce the probability (or rate) of reconfiguring the mRNA to the “off” state because nucleotides essential to forming the “off” state are occupied by the entering ribosome.

In the context of the proposed “on” conformation model, we examined a spontaneous mutant of pNC1, pNC1-X, selected for growth on 10 μg/ml chloramphenicol in the absence of inducer. pNC1-X contained a single base substitution (G→U) at nt 87 in the leader. This mutation is predicted to weaken the “off” conformation of the mRNA and strengthen the “on” state (pNC1-X in Fig. 9). pNC1-X expressed constitutive CAT levels 30-fold higher than basal CAT expressed by pNC1, and
pNC1-X appeared to retain a low level of CAT inducibility (Fig. 9). By site-directed mutagenesis, the G at nt 87 in wild-type pNC1 was changed to a C and to an A. The G → C mutation (in pNC1-C; Fig. 9) resulted in constitutive CAT expression, but the level of expression was about two-thirds of the CAT activity of pNC1-X (Fig. 9). This mutation weakened the “off” conformation but did not strengthen the “on” conformation. We suggest that the mutations in pNC1-X and pNC1-C (Fig. 9) both change the dynamics of refolding the “on” conformation to the “off” state by weakening the “off” conformation and, in the case of pNC1-X, additionally strengthening the “on” conformation.

The mutation that changed nt 87 from G to A (in pNC1-A) replaced a GU pair in the wild type with an AU pair in the mutant (Fig. 9). Our folding models predict that this mutation should have little or no effect on the stability of either the “off” conformation or the “on” conformation (Fig. 9), and this mutation resulted in normal tetracycline induction of CAT activity.

In our mRNA-folding models, nt 56 is unpaired in the “off” state (Fig. 1B) and in the “on” state is unpaired within a bulge in stem 2/H11032 (Fig. 8). We changed nt 56 from A to C by site-directed mutagenesis, yielding the mutation stm-1. The effect of this mutation is to add another GC pair in stem 2/H11032 of the “on” state (Fig. 8) with a concomitant decrease in the G of the “on” state from −15.4 to −20.2, which is a significant increase in the stability of stem 2’. This mutation conferred constitutive CAT expression at a specific activity of 3.5 and was not induced by tetracycline. Thus, a single nucleotide change that is predicted to stabilize the “on” state favored constitutive expression.

The altered stabilities of the “off” and “on” conformations seen with the pNC1-X mutant or with the stm-1 mutation likely reduce the rate of reconfiguration of the “on” to the “off” state. This would enhance the probability of ribosome loading at the tet RBS and hence constitutive expression. Constitutive expression at levels seen with the pNC1-F RBS duplication mutant (Fig. 2A and B) are not seen with pNC1-X and with the stm-1 mutation but at a reduced frequency compared with the wild type.

The model we suggest for tet regulation predicts that induction by tetracycline results only from translational activation of transcripts that are incompletely synthesized at the time of antibiotic addition and that completed leader transcripts, those transcripts present in cells before antibiotic addition, remain in the “off” conformation and are not induced by tetracycline. We therefore tested tetracycline induction of the tet-cat fusion
gene in pNC1 immediately after RNA synthesis was blocked by the addition of 100 µg/ml rifampin. When this type of experiment was applied to cat-86, we successfully obtained induction (12). In contrast, we were unable to induce the tet-cat fusion after rifampin addition (data not shown). While this is a negative finding, it is consistent with the proposal that induction of the translation of tet mRNA is only effective on transcripts which have not yet folded into the “off” conformation.

DISCUSSION

Studies of the inducible expression of cat and erm genes from gram-positive bacteria have established a form of gene regulation termed translation attenuation in which the cognate antibiotic induces translation of the mRNA for the resistance determinant. Inducers for both genes are antibiotics targeted to the 50S ribosomal subunit (14, 15). Tetracycline is a protein synthesis inhibitor targeted to the A site on the small ribosomal subunit (8). We were interested in determining if there were similarities between the regulation of a tetracycline resistance gene with the known regulation of cat and erm genes. At the outset, we were aware of the possibility that the expression level of tet genes might be reduced relative to the expression levels of most cat or erm genes. Both cat and erm specify proteins that function in the cytoplasm, whereas the preponderance of known tetracycline resistance genes, including that resident on pBC16, specify membrane-associated proteins that appear to function as antibiotic pumps (21, 31, 37). High-level

![Graphical representation of mRNA stability and induction](http://jb.asm.org/)

**FIG. 9.** Comparison of the effects of mutations at nt 87 on induction and predicted stabilities of the “off” and “on” states. pNC1-X was a spontaneous constitutively expressed mutant bearing a single mutation (G→U) at nt 87. pNC1-A and pNC1-C were made by site-directed mutagenesis and change nt 87 to A and C, respectively. Uninduced and induced CAT specific activities for wild-type (wt) pNC1 and the three mutant plasmids are shown at the top. Beneath the specific activities are shown the “off” and “on” conformations containing nt 87. Beneath each depiction of secondary structure is the calculated ΔG in kilocalories per mole. The stick diagrams to the left represent the upstream regions of leader mRNA and are shown only for the pNC1 depiction. Un, uninduced; Tet, tetracycline induced.
expression of a membrane-associated protein has the potential to alter membrane structure to the extent that cell growth might suffer. The anticipation that the expression level of the 

The promoter used to drive transcription of the tet-cat fusion in pNC1 was a promoter for the mob gene resident in pUB110 (data not shown). The native promoter for the tet gene in pBC16 was identified by primer extension mapping, and this promoter was cloned to the cat-86 gene in the promoter cloning vector pPL703 (22) and resulted in CAT levels with a specific activity of approximately 2. There was no indication of any influence of tetracycline on the function of the native promoter in pPL703.

Computer-assisted folding of the tet-cat mRNA specified by pNC1 (and the tet mRNA specified by pBC16) predicts that the tet RBS is sequestered in secondary structure. The constitutive mutant pNC1-F resulted from a duplication of the tet RBS such that the cat-86 coding sequence was provided with a nonsequestered RBS. Accordingly, we interpret the low basal expression in pNC1 results from the sequestration of the tet RBS. A second observation consistent with the translation attenuation model is that the level of tet-cat mRNA does not detectably differ between the uninduced and induced states. The observation that appears in disagreement with the translation attenuation model deals with the role of the leader open reading frame in regulation. In three instances, we observed that mutations that block leader translation also result in constitutive expression of the downstream reporter gene. To ensure that the effect of these mutations was not to alter the folding of the proposed “off” conformation, the mRNA corresponding to the leaders with either the rhs-1 mutation or the mutants designated TAA-1 and TAA-1,2 (Fig. 5) were computer folded. In the resulting structures, the tet RBS remained sequestered in secondary structure and the $\Delta G$ of each structure was between $\pm 22$ and $\pm 23$. This indicates that the leader sequence changes resulting in constitutive expression did not bring about a change in the structure of the mRNA such that the tet RBS was in a weakened secondary-structure complex.

Our data suggest that tet follows a variation of the translation attenuation model. In this variation, efficient leader translation causes ribosomes to queue on leader mRNA and interfere with base pair formation critical to stabilizing the “on” conformation. Translational activation of the resistance coding sequence results from blocking leader translation at an early location, either through the action of tetracycline or by mutation. In our model, when RNA polymerase transcribes to a site immediately preceding the tet RBS (i.e., when nt 98 emerges from RNA polymerase), there are two possible folding outcomes for the mRNA. If the upstream leader mRNA is under active translation, the “on” conformation is either not formed or is weak and the addition of 7 nt allows rapid formation of the “off” conformation. If, on the other hand, leader translation is blocked, then the “on” secondary structure forms and subsequent synthesis of an additional 7 nt exposes the tet RBS, allowing ribosome entry and tet translation. Once ribosomes enter at the tet RBS, the probability of the “on” conformation reconfiguring to the “off” state should decrease because entering ribosomes mask sequences essential to forming the “off” state.

The sequence of the pBC16 regulatory region, from the leader initiation codon through the RBS for the tet resistance determinant, shares 89% nucleotide sequence identity with the same region preceding the tetA(L) gene studied by Stasinopoulos et al. (30). The differences are 10 nt substitutions, with 3 of these substitutions falling within the duplexed portion of the RNA shown in Fig. 1B. Kinefold-assisted folding of the tetA(L) leader produced a structure, with a $\Delta G$ of $\pm 23$, very similar to the “off” state of the pBC16 tet leader. The high degree of conservation of this region suggests that both genes are regulated by a similar, perhaps identical, mechanism. However, our proposal for the regulatory mechanism differs fundamentally from the translation attenuation model suggested by Stasinopoulos et al. (30).

Stasinopoulos et al. (30) proposed that tetA(L) from B. subtilis is regulated at the levels of transcription and translation. In our study, there was no indication of a transcriptional component in pBC16 tet regulation, neither in the form of an antisense terminator nor at the promoter for the gene. The translational component of the regulation suggested by Stasinopoulos et al. (30) predicts that a ribosome stalled in the leader, or at the leader RBS, “jumps” from the leader site to an exposed tet RBS in response to tetracycline. However, their proposed conformation of the mRNA, in which the tet RBS is exposed, is extremely weak (30) and certainly disfavored on the basis of thermodynamic considerations. Several lines of evidence argue against this translation attenuation model, but we mention only two. The first is our finding that when leader translation is blocked, the reporter gene is expressed in the absence of inducer. This finding is the opposite of what is predicted by a reinitiation model. Secondly, implicit to a ribosome reinitiation model is the prediction that there should be a precise spatial relationship between a ribosome “takeoff site” in the leader and the downstream “landing site” for the ribosome. We therefore made an insertion of 6 nt (AUAAUU) between nt 98 and 99 which would, in theory, disrupt the putative spatial relationship and found that induction of this mutant was indistinguishable from induction of wild-type pNC1 (data not shown). This finding is therefore also inconsistent with a reinitiation model.

Our model for tet regulation proposes that induction requires coupling of translation with transcription, which is a feature not proposed for the regulation of cat and erm genes. Furthermore, our model also proposes that leader translation has an active role in maintaining the tet gene in an unexpressed state, which differs from the regulation mechanics proposed for cat and erm. Otherwise, the inducible regulation of the three antibiotic resistance genes appears to follow a common theme and is based on the ability of these three antibiotics to bind to ribosomes and inhibit translation.

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REFERENCES


