TRANSLATIONAL ATTENUATION: THE REGULATION OF BACTERIAL RESISTANCE TO THE MACROLIDE-LINCOSAMIDE-STREPTOGRAMIN B ANTIBIOTICS

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I. INTRODUCTION

Bacterial resistance to the macrolide-lincosamide-streptogramin B (MLS)* group of antibiotics appears to be regulated at the level of translation.[1] The regulatory system proposed for MLS resistance is strikingly similar to attenuation at the transcriptional level which controls many biosynthetic operons and has therefore been named "translational attenuation". This type of control and its attendant phenomena are the subject of this review. It is best viewed as an example, on the one hand, of the influence of RNA conformation on macromolecular synthesis and, on the other, as an instance of control at the level of translation. Transcriptional attentuation has been reviewed recently by Kolter and Yanofsky.[2] RNA folding as a controlling factor in protein synthesis is dramatically exemplified by the RNA phages.[3] Several additional examples of prokaryotic translation-level control can be cited.[4-6b]

The MLS antibiotics act by binding to the large ribosomal subunit and consequently inhibit protein synthesis.[7,8] The most common naturally occurring form of resistance to these antibiotics has been shown to be mediated by specific methylation of 23S rRNA resulting in a decreased ribosomal affinity for the MLS antibiotics.[9-11] MLS resistance of this type is extremely widespread among the bacteria, occurring in *Staphylococcus*,[12,13] Streptococcus,[14-17] Bacillus,[18,19] Bacterioides,[20] Clostridium,[21] Corynebacterium,[22] and in *Streptomyces* (including the organisms which produce MLS antibiotics).[23,24] Two types of MLS resistance have been observed. In the first, or constitutive type of resistance, the organism is resistant to all of the MLS antibiotics without prior exposure. In the second, or inducible type, resistance to all of the MLS agents requires prior or simultaneous exposure to one or another MLS antibiotic.[25-28] Most often in the *Staphylococcal* and *Bacillus* systems, only erythromycin (Em) and the closely related macrolide oleandomycin (Om) act as inducers. This induced resistance is phenotypic. Removal of the inducer results in the rapid loss of resistance to noninducing MLS antibiotics.[29] The induction of MLS resistance is the principal subject of this review.

* Abbreviations used: Cm, chloramphenicol; Em, erythromycin; EmA, erythromycin A; Fs, fusidic acid; Lm, linocomycin; MLS, macrolide-lincosamide-streptogramin B; Om, oleandomycin; r-protein, ribosomal protein; Tc, tetracycline; Ty, tylosin

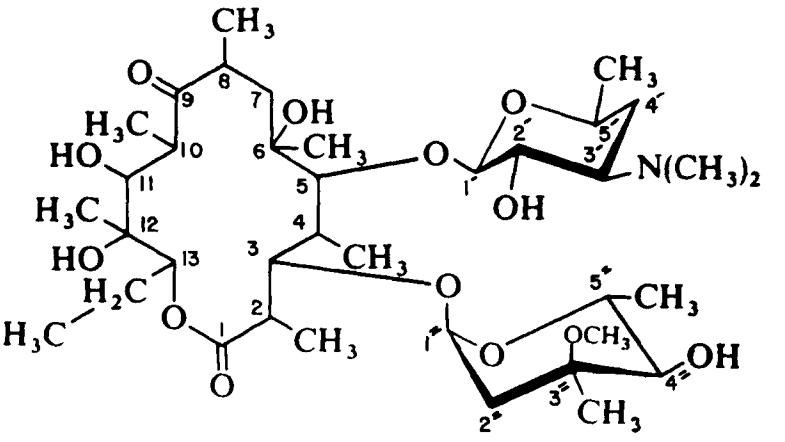

FIGURE 1. Structure of erythromycin A.

II. MLS STRUCTURE AND BINDING TO THE RIBOSOME

A discussion of the regulation of MLS resistance must properly begin with an account of the chemical structure and mode of action of this diverse group of antibiotics. The structure of erythromycin A (EmA), the prototypical macrolide, is shown in Figure 1. EmA consists of a macrocyclic 14-membered lactone ring (hence the term "macrolide") joined to a neutral sugar (cladinose) and a basic sugar (desosamine). A large number of macrolides have been described, which vary in both the size of the lactone ring and in its substituents, as well as in the nature of the sugar moieties. For instance, the carbomycin- and spiramycin-type macrolides have bulky disaccharides at the 5-position and the spiramycins contain an additional amino sugar at position 9.

All of the MLS antibiotics, of which literally hundreds have been described, bind to the large ribosomal unit of prokaryotes and inhibit protein synthesis.[7,8] A single binding site is present on each 50S ribosome and the dissociation constant for Em binding is about 10^{-8} to 10^{-7}.[30,31] Binding competition[32] and mutational cross-resistance studies suggest that the binding of these antibiotics involves overlapping ribosomal sites. The most extensive structure-function studies reported involve EmA.[33] These studies have demonstrated that EmA possesses a largely hydrophillic face on which the substituents on the 1, 6, 9, and 11 carbons are exposed. A second face is largely hydrophobic. On this surface the 3′ dimethylamino and the 2′ hydroxyl groups are exposed. Studies of the ability of various derivatives to compete with [^{14}C] EmA for binding to ribosomes have suggested that the 3′-dimethylamino, 2′-hydroxyl, 11 and 12-hydroxyls, 9-keto, and 3″-methoxy groups are important for ribosomal binding.[33,34] These substituents are exposed on both the hydrophillic and hydrophobic surfaces of EmA, and Mao and Putterman[34] have suggested that together with the 6-hydroxyl group they form hydrogen bonds with adjacent nucleotides arranged in a "semicircular shape" to surround the bound molecule of EmA. Studies involving chemical modification of 50S ribosomes led these authors to propose hydrogen bonding to nucleotides rather than to r-protein. However, since the binding free energy (~8 kcal) is too large to be explained by the formation of only 7 hydrogen bonds, Mao and Putterman[34] suggested that hydrophobic interactions provide additional stability. Perun,[33] based on similar studies involving Em derivatives, suggested that the 3′-dimethylamino and 2′-hydroxyl groups on the amino sugar initiate ribosomal attachment by hydrogen bonding, followed by the formation of additional hydrophillic or hydrophobic bonds. Any modification of EmA resulting in a decrease in binding to the ribosome causes a concomitant loss in antibacterial activity. There are some

derivatives which bind but are poor antibiotics.[35] These include 2′,4″-diacetyl EmA and 4″-acetyl erythromycin B. (Erythromycin B is the 12-deoxy derivative of EmA.)

III. RIBOSOMAL BINDING SITE FOR MLS ANTIBIOTICS

The binding of MLS antibiotics to the 50S ribosome can be decreased by the addition of chloramphenicol.[32,36] This competition suggests that MLS binding occurs at a ribosomal site that is near the peptidyl end of peptidyl t-RNA. Other lines of evidence support this conclusion. For instance, MLS antibiotics will not bind to polysomes, but removal of the nascent peptidyl-tRNA from polysomes using puromycin confers Em-binding capability.[37,38] It is likely that the peptidyl-tRNA sterically blocks access to the binding site since methymycin, a macrolide, and lincomycin (Lm) do inhibit the binding of [^{14}C] chloramphenicol to polysomes.[37] It was pointed out by Pestka[37] that these compounds are less bulky than other MLS antibiotics which do not inhibit chloramphenicol binding to polysomes. Methymycin, in fact, even exhibits a slight inhibition of transpeptidization on polyribosomes, suggesting again that it can gain access to a binding site in the presence of peptidyl tRNA.[39] The binding of Em to core particles of *E. coli* 50S ribosomes is restored by the addition of proteins L15 and L16.[40] In addition, mutational alterations of L4 and L22 can confer Em resistance.[41,42] These four proteins are among those considered to be at or near the peptidyl transferase center of the 50S ribosome.[43,44]

IV. MODE OF MLS ACTION

The detailed mode of action of MLS antibiotics is far from clear. The picture is confused by the fact that the various members of the MLS group affect protein synthesis differently. We will attempt to summarize some of the main features, concentraing on Em (and its close relative, Om). The MLS antibiotics can affect transpeptidization in in vitro model reactions.[45-53] They do not appear to inhibit the formation of the initiation complex.[38,48,49] In addition, Em inhibits neither the binding of aminoacyl-tRNA to the ribosome, nor the EF-G dependent hydrolysis of GTP.[48] Some interesting differences appear in the action of Em and Om compared to the other MLS antibiotics. Em and Om stabilize polyribosomes in vitro and in vivo, while all other macrolides tested cause their rapid disappearance.[54,56] It is likely therefore, that Em and Om, unlike the other agents, reduce the rate of ribosome movement on mRNA. In addition, Em and Om appear to block transpeptidization only when the peptidyl-tRNA donor is relatively large, whereas other macrolides, as well as lincosamides, can prevent transpeptidization when the donor is small.[47-49,57] In fact, Em actually stimulated the transfer of fmet, acetyl-phenylalanine, and acetyl-leucine to puromycin, while tylosin (Ty), spiramycin, and carbomycin inhibited these reactions.[45-47,49] Em inhibited polylysine and polyphenylalanine synthesis programmed by polyA and polyU, respectively, causing the accumulation of di- and trilysine and of di-, tri-, and tetraphenylalanine, while other macrolides such as Ty and niddamycin either caused accumulation of diphenylalanine or completely blocked peptide bond formation.[48,58] There is also an interesting substrate specificity apparent in the action of Em. Mao and Robishaw[49] have suggested that amino acids with hydrophillic and bulky side chains (e.g., *lys, pro*) are more sensitive to the action of Em than those with small or hydrophobic side chains (e.g., *leu, val*). Other reports support this generalization.[45,46,51] Thus, the length of the peptidyl-tRNA, as well as the hydrophillicity and the bulk of the amino acid side chains, contribute to Em sensitivity and probably determine at which residue the transpeptidization reaction will be blocked.

The precise step arrested by the various MLS agents is not known. Meninger and Otto[59] have suggested the Em and possibly all macrolides "stimulate the dissociation of peptidyl-tRNA from ribosomes, probably during translocation." In order to explain the unique ability

of Em (and Om) to prevent polysome breakdown as well as other properties of Em inhibition, they suggest that Em, unlike other macrolides, stimulates release of peptidyl-tRNA from the ribosomal A site, but does not stimulate release of deacylated tRNA from the P site. Freezing of deacylated tRNA on the P site, they propose, binds the ribosome to the RNA, preventing polysome breakdown. In fact, Em has been reported to inhibit the release of deacylated tRNA.[53,60] Evidence favoring stimulation of peptidyl-tRNA release by macrolides had also been reported earlier.[61,62]

Pestka[7] has suggested that the macrolides bind to a 50S ribosomal site near a binding site for peptidyl-tRNA and sterically block translocation without directly affecting peptidyl transferase activity. Larger macrolides and larger peptidyl-tRNA chains would favor his steric inhibition, as observed. This model is not mutually exclusive with Meninger and Otto's, since inhibition of translocation may be the cause of the Em-stimulated release of peptidyl-tRNA from the A site. Also consistent with the proposed inhibition of translocation are results reported by Cundliffe and McQuillen.[54] Em inhibited the puromycin reaction in vivo when added to *B. megaterium* protoplasts. This could have been due to a direct inhibition of the peptidyl transferase reaction. However, when the protoplasts were pretreated with chlortetracycline, which blockades the A site, thereby restricting peptidyl-tRNA to the P site, Em was no longer able to inhibit the subsequent reaction with puromycin. These results support the notion that the original inhibition of the puromycin reaction by addition of Em to the protoplasts was due to inhibition of translocation which had the effect of restricting nascent peptides to the A site. It also suggests that Em does not directly inhibit the peptidyl transferase center. Mao and Robishaw[49] have suggested that Em is an allosteric effector which induces a conformational change near the peptidyl-tRNA binding site, affecting the peptidyl transferase reaction rate. This model is not consistent with the above-mentioned data. On the other hand, models invoking conformational changes seem easier to reconcile with the fact that Em stimulates the puromycin reaction with certain N-blocked acetyl amino acids[45-47,49] than are steric-inhibition models.

Whatever the molecular details of MLS action, then, we can summarize the effects of these agents as follows. The MLS antibiotics inhibit transpeptidization by binding to the 50S ribosome. They most likely affect translocation, either by steric interference or by allosteric interaction with the ribosome. In particular, Em and the closely related macrolide OM, unlike the other MLS antibiotics, permit peptidyl chain growth to proceed before blocking further growth. Sensitivity to these two macrolides appears to depend on the hydrophillicity and bulk of the amino acid side chain. Em and Om are unique among the MLS antibiotics in not causing a rapid breakdown of polysomes. Finally, MLS antibiotics cannot bind to and affect chain growth on polysomes, suggesting that once formed, peptidyl-tRNA blocks access to the ribosomal MLS binding site. One such site is present on each 50S ribosome.

V. MLS RESISTANCE

In the laboratory, resistance to various MLS antibiotics can be selected as one-step mutations. In *E. coli* these mutations generally affect the large ribosomal subunit proteins L4 and L22,[41,42] although recently a probable 23S rRNA mutation leading to Em resistance in *E. coli* has been reported.[63] Similar mutational alterations in protein L17 of *B. subtilis* also result in Em resistance.[64] (L17 of *B. subtilis* corresponds to L22 of *E. coli*.[65]) These mutations are often correlated with a decreased ribosomal affinity for Em and usually exhibit cross-resistance only to closely related macrolides. Ribosomal protein alterations generally confer low level resistance (to less than 50 μg/mℓ Em).

Naturally occurring resistance to the MLS antibiotics is strikingly different. Organisms exhibiting MLS-type resistance are capable of growth in the presence of any of the MLS

antibiotics and are often resistant to very high concentrations (up to 5 to 6 mg/mℓ) of Em. MLS resistance is very widespread in nature.[12-24] In several cases the mechanism of MLS resistance has been examined and determined to be associated with a specific N^6, N^6-dimethylation of adenine in 23S rRNA.[9-11] Weisblum and co-workers[10] have demonstrated that in *S. aureus* this specific methylation occurs within a GAAAG sequence in 23S rRNA, and results in a decreased binding affinity of MLS antibiotics to the ribosome.[29] A ribosome reconstitution experiment has demonstrated that modification of the 23S rRNA is responsible for in vitro Em resistance.[11] The extent of in vivo labeling using [^{14}C]methionine has established that one to three dimethyl adenine residues are present in each molecule of 23S rRNA from MLS-resistant cells of *S. aureus*.[10] The location(s) of the methylated sequence within 23S rRNA has recently been determined.[141,142] In some *Streptomyces* spp., such as *S. viridochromogenes* and *S. lincolnensis*, resistance is associated with N^6-monomethylation of adenine.[24] In others, both di- and monomethylation occur (e.g., *S. hygroscopicus* and *S. fradiae*), and in still others N^6-dimethylation has been observed to occur exclusively.

It is interesting that other examples of antibiotic resistance associated with the presence or absence of rRNA methyl groups have been described. Thus, kasugamycin resistance is associated with the *absence* of methyl groups on two adjacent adenines near the 3′ end of 16S rRNA, which are normally present as N^6,N^6-dimethyladenine.[66] *Streptomyces azureus*, the organism which produces thiostrepton, constains extra pentose methyl groups (as 2′-o-methyladenosine) on 23S rRNA, and these are responsible for resistance to the antibiotic.[67,68] Methylases associated with kasugamycin sensitivity,[69] and with resistance to thiostrepton[70,72] have been isolated and partially characterized.

MLS-resistance methylases have been isolated from *B. subtilis*[72] and from *Streptomyces erythreus*,[73] the erythromycin-producing organism. The *Streptomyces* enzyme dimethylates approximately one adenine residue per 23S rRNA molecule in vitro. The enzyme isolated from *B. subtilis* is specified by the *ermC* gene of pE194, a 3.5 k.b. plasmid isolated from *S. aureus*[74] and introduced by transformation into *B. subtilis*.[75] The in vivo product of the *ermC* methylase is N^6,N^6-dimethyladenine.[75] This enzyme has a molecular weight of 29,000 and is an extremely basic protein. It utilizes S-adenosylmethionine as a methyl donor and is capable of methylating 50S ribosomes and 23S rRNA in vitro, but is inactive on 30S and 70S ribosomes and on 5S or 16S rRNA. The methylase cannot act on ribosomes or on 23S rRNA isolated from the homologous strain, grown under conditions which result in full in vivo methylation.[72,76] Thus, the in vitro and in vivo methylation sites are the same and the site specificity of methylation is determined by RNA-enzyme interactions in the absence of r-proteins. Em does not inhibit methylation even when present at concentrations sufficient to saturate the ribosomes used as substrate. It is likely, therefore, that methylation, which decreases the affinity of ribosomes for Em, does not occur within the Em binding site, but rather causes a long-range conformational alteration in 23S rRNA which decreases Em binding. It is worth noting that the N^6,N^6-dimethylation associated with kasugamycin sensitivity causes an alteration in the conformational stability of a hairpin loop near the 3′ end of 16S rRNA.[77,78] Since 70S ribosomes cannot be methylated in vitro, it is likely that the methylation site is blocked by the small ribosomal subunit. Interestingly, a class of *E. coli* mutants *(eryC)* which confer Em resistance, appear to affect the 30S subunit.[79] Thus, the MLS binding site may also occur near the interface of the two ribosomal subunits. Recently we have shown[76] that the *ermC* methylase (an *S. aureus* enzyme) cannot methylate in vivo methylated ribosomes from various other MLS-resistant organisms including *Streptococcus, B. licheniformis,* and *S. aureus* strains carrying MLS-resistance determinants other than *ermC*. It appears then that the methylation sites in these organisms are homologous.

The DNA sequences of three MLS determinants have been determined. *ermC* specifies a 29,000-dalton protein described above.[80,81] *ermD* is an MLS determinant cloned from the chromosome of *B. licheniformis* which specifies a 35,000-dalton polypeptide identified in

B. subtilis minicells.[18] The DNA sequence of *ermD* has recently been obtained.[82] Finally, the DNA sequence of the MLS determinant of pAM77, a plasmid isolated from *S. sanguis*, has also been determined and the inferred amino acid sequence has been published.[83] It is worth noting that no base sequence homology was detected between these three elements using stringent hybridization conditions.[18,84] The amino acid sequences of the three rRNA methylases, inferred from their DNA sequences, are compared in Figure 2. As pointed out by Horinouchi and Weisblum,[83] the *ermC* and pAM77 methylases are strikingly similar; half of the amino acids are identical. The authors state that this homology is noticeable on the nucleotide level as well. The three methylases have been compared pairwise using a dot matrix computer analysis.[85] The regions of similarity revealed by these comparisons have been used to align the *ermD* protein with the other two, and these comparisons are also shown in Figure 2. Table 1 summarizes some numerical results, comparing the proteins. In addition, the three methylases are quite basic and otherwise generally similar in their amino acid composition. The *ermC* and pAM77 proteins (from *S. aureus* and *Streptococcus sanguis*) are closely related, while the *ermD (B. licheniformis)* protein is more distantly related to the others. Certain regions (residues 46 to 60, 112 to 134, and 184 to 197 in *ermD*) seem to be particularly conserved.

VI. INDUCTION OF MLS RESISTANCE

It was observed more than 25 years ago that certain Em-resistance organisms exhibited resistance to other macrolides only after prior exposure to Em.[25] This was subsequently shown to be due to the induction of generalized MLS resistance by Em.[26,27] Using the *S. aureus* strain 1206, Weisblum and colleagues established that Em acts as an inducer of resistance to all other MLS agents tested, but that most of these other antibiotics do not display inducing activity.[28,29] Resistant cells began to appear within 20 min of exposure of this strain to 10^{-7} *M* Em, and resistance was lost after about two generations of growth in the absence of inducer. The appearance of resistant clones was inhibited by chloramphenicol and streptovaricin. It appeared from these early studies that Em, an inhibitor of protein synthesis, was capable of inducing resistance to MLS antibiotics, and that this induction required protein synthesis. Although many wild-type MLS resistant strains of *S. aureus* and *Streptococcus* exhibited inducible resistance, others expressed full MLS resistance in the absence of inducer. No investigation of these constitutive strains on the molecular (DNA) level has been reported.

The remarkable induction specificity noted above (only Em and Om are inducers) has been reported for several MLS determinants,[86,87a,87b] including one *(ermD)* which is located on the chromosome of *B. licheniformis*[18] and another which is located on a plasmid, pE194, originally isolated from *S. aureus*.[75] Some MLS-resistant organisms, on the other hand, have different induction specificities. In this regard, the *Streptomyces* are noteworthy.[24] In these organisms, MLS antibiotics other than Em were frequently found to be inducers. Often an antibiotic acted as an inducer of MLS resistance in the producing organism. Thus, Lm induced *S. lincolnensis*, the producer of Lm, and streptogramin-B induced *S. diastaticus*, a streptogramin-B producer. Several reports have appeared of mutationally altered induction specificities.[86,87] These mutants have not been studied further. It is not known whether they are altered in the MLS determinant, in the ribosome, or elsewhere.

In the *ermC* and *ermD* systems, induction of resistance has been shown to be accompanied by induction of synthesis of a single protein species identified in minicell extracts by polyacrylamide gel electrophoresis.[18,88] The *ermC* system has been the most extensively studied and is discussed first, followed by a description of the *ermD* system.

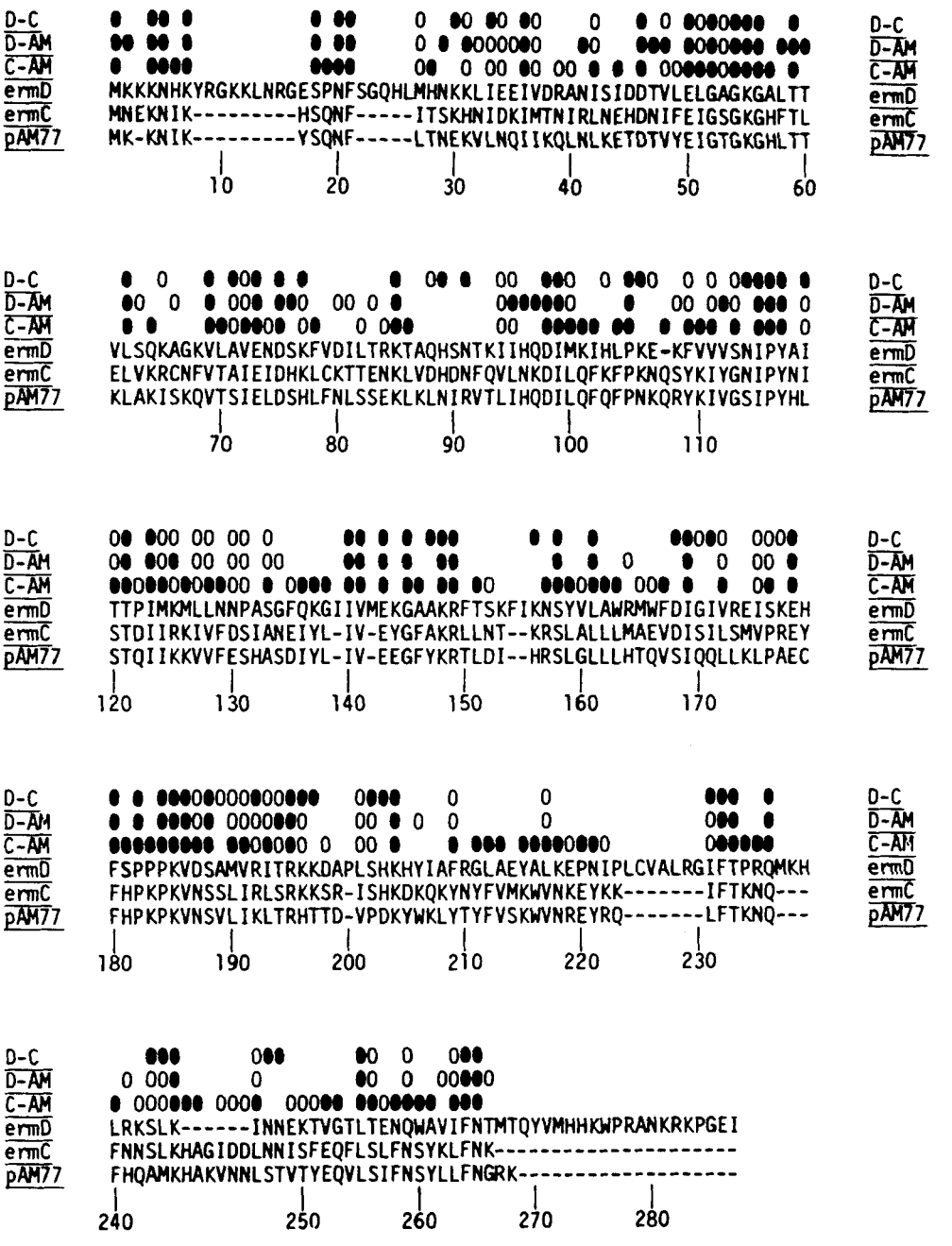

FIGURE 2. Comparison of amino acid sequences of the *ermC*,[80,81] *ermD*,[82] and pAM77[83] MLS-resistance methylases. (●) Identity. (○) Conservative replacements.[131] (The latter are defined in Table 1.)

VII. POSTTRANSCRIPTIONAL REGULATION OF THE *ermC* METHYLASE: mRNA STABILITY

A 29K Em-induced protein has been shown to be the product of the *ermC* determinant and to be identical to the rRNA methylase described above.[72,88] The purified methylase and the 29K protein comigrate on polyacrylamide gels. Like MLS resistance, both are induced

Table 1
COMPARISON OF *ermC*,[80,81] *ermD*,[82] AND pAM77[83] MLS-RESISTANCE METHYLASES

Proteins	Fraction of identical amino acids	Fraction of conservatively replaced[a] amino acids
ermD — ermC	0.31	0.18
ermD — pAM77	0.28	0.24
ermC — pAM77	0.50	0.19

[a] Conservative replacements are defined as follows: C; S,T,P,A,G; N,D,E,Q; H,R,K; M,I,L,V; F.Y,W.[131]

by Em and Om and not by Ty, Lm, or other MLS agents. None of the other four polypeptides known to be specified by pE194 is induced by Em. Regulatory mutants of pE194 which express MLS resistance constitutively express elevated levels of both the 29K polypeptide and of methylase activity. Finally, deletion mutants obtained by in vitro manipulation of *ermC* result in MLS sensitivity, altered electrophoretic mobility of the 29K protein, and in abolition of methylase activity.[89]

Recently we have fused the promoter and regulatory sequences of *ermC* to the *E. coli* β-galactosidase structural gene.[90] In *B. subtilis* strains carrying this construct, β-galactosidase activity is inducible by Em. The optimal rate of induction occurs using Em at 0.25 μ*M*. Increased β-galactosidase synthesis is detectable about 3 min after addition of inducer at 32°C. Higher Em concentrations induce poorly since the strain is sensitive to Em. However, when the wild-type *ermC* determinant is used, induction can be demonstrated using a wide range of Em concentration.[85,88] Plating on 8 m*M* Em yields colonies with a high plating efficiency, after about 3 days growth at 32°C. On the other hand, induction can also be demonstrated using extremely low Em concentrations. Since Ty is a noninducing macrolide, *B. subtilis* strains carrying pE194 fail to form colonies on 5 μg Ty per milliliter. In the presence of 10 n*M* Em, however, these strains slowly form colonies on Ty. With higher inducer concentrations, colonies also form on Ty-containing plates, but more rapidly. Any model of MLS regulation must explain these phenomena: slow induction with very high and very low Em concentrations, and fast induction with moderate concentrations.

We have demonstrated that the synthesis of *ermC* methylase is not regulated on the level of transcription.[1] Growing cultures of *B. subtilis* carrying pE194 were pulse-labeled with model of MLS regulation must explain these phenomena: slow induction with very high and very low Em concentrations, and fast induction with moderate concentrations.

We have demonstrated that the synthesis of *ermC* methylase is not regulated on the level of transcription.[1] Growing cultures of *B. subtilis* carrying pE194 wee pulse-labeled with [^{3}H]uridine with and without induction, RNA was isolated, and filter hybridization was used to assay the content of radioactive *ermC* transcript. Since the pulse (30 sec) was short compared to the half-life of *ermC* mRNA (see below), this experiment assayed the transcription rate of *ermC*. No difference was detected in the rate of transcription with or without induction. Further experiments,[1] carried out using *B. subtilis* minicells poisoned with rifampicin, demonstrated that the mRNA for the 29K methylase was markedly stabilized in the presence of Em. This effect was specific. Only the functional half-life (τ_F) for the 29K protein was affected by the presence of Em, and stabilization was not seen using Ty, a noninducing macrolide. Finally, we have demonstrated by Northern blot hybridization that Em physically stabilizes the 970 base *ermC* transcript in the presence of rifampicin.[91] This specific increase in both the functional and physical half-life of *ermc* mRNA suggests that regulation of *ermC* is posttranscriptional. The stabilization may represent the primary cause of increased methylase synthesis, or may be a secondary consequence of enhanced translation.

Our model, presented below, assumes that stimulation of translation is the primary effect. If this assumption is correct, the increase in methylase synthesis observed following induction would be a consequence of both increased translation and of mRNA stabilization.

Further proof of the posttranscriptional regulation of *-ermC* comes from experiments using a pE194 derivative in which the *ermC* promoter has been excised and replaced by a Hind III linker.[92] This construct also contains the chloramphenicol (Cm) resistance gene from plasmid pC194 and therefore exhibits a Cm^r EM^s phenotype. The regulatory region of this plasmid has been sequenced. Although it lacks the *ermC* promoter, the regulatory region (see below) and coding sequences of *ermC* remain intact. Hind III-digested chromosomal DNA from *B. licheniformis* was introduced by ligation into the Hind III site and this DNA used to transform an appropriate competent culture with selection for Cm and Em resistance. Colonies were obtained with a wide range of Em resistance levels and contained plasmids with inserts. These colonies exhibited inducible Em resistance. Thus, regardless of the promoter used for *ermC* transcription, the expression of em resistance can be inducible.

VIII. *Tyc* MUTANTS

Further information was obtained by the use of spontaneous plasmid mutants which express elevated levels of *ermC*-methylase in the absence of inducer. These we have called *tyc* mutants (for Ty constitutive) since they were isolated as strains capable of growth in the presence of Ty, a noninducing macrolide.[75] All such mutants that have been examined have been shown to be plasmid mutants which map near the promoter of *ermC*.[80,81,93,94] The *tyc* mutations are of two types: point mutations and duplications. The latter have been shown in several cases to consist of direct tandem duplications of a portion of *ermC* itself.[93] The structure of one of these *(tyc-16),* which consists of a 109 bp duplication, will be discussed in detail below. Another, *tyc-9,* carries a direct tandem duplication of 650 to 700 bp. Revertants of *tyc-9* carrying wild type-sized plasmids display the inducible Em-resistance phenotype.[80] Thus, the duplications are responsible for constitutivity. We have shown that these *tyc* strains, as well as strains carrying *tyc-1,* a point mutant, produce two to four times the wild-type basal level of methylase.[72,76] Northern blot hybridization experiments have revealed that *tyc-16,* a duplication mutant, and *tyc-1,* a point mutant, confer unusual stability on *ermC* mRNA in the absence of induction.[91] If *ermC* regulation is entirely posttranscriptional, we would expect *tyc* mutations, which alter normal regulation, to be located downstream from the transcriptional start site of *ermC,* so that the mutational change would affect the transcript. In the case of the duplication mutants *tyc-16* and *tyc-9*, this would result in transcripts which are 109 and 650 to 670 bases larger than the wild type. This expectation has been confirmed,[80] in contrast to typical constitutive mutants of transcriptionally regulated genes, which occur in *trans*-acting regulatory genes or in operator sequences and which therefore do not affect the structure of the target gene mRNA.

IX. INVOLVEMENT OF RIBOSOMES AS POSITIVE EFFECTORS OF *ermC* INDUCTION

Several lines of evidence demonstrate the involvement of ribosomes in *ermC* induction. When cultures carrying pE194 were fully induced by prior exposure to Em, no difference in the rate of methylase synthesis could be demonstrated in the presence or absence of inducer if the Em was removed and then added back to a portion of the culture.[1] This was interpreted as suggesting the operation of a methylation-mediated induction feedback loop involving Em binding to the ribosome. According to this model, binding to the ribosome is required for induction to occur. Mature ribosomes are then methylated, decreasing the ribosomal affinity for Em, and preventing further induction. To test this model, a chromosomal mutation

(ole-1) which confers Em resistance and alters *B. subtilis* ribosomal protein L17[95] was introduced into a minicell strain carrying pE194.[1] [^{14}C]Em binding to ribosomes in vitro demonstrated that this mutation reduced the ribosomal affinity for Em. In the *ole-1* minicells, methylase could not be induced. Thus, perturbation of the Em binding site on the 50S ribosome by alteration of L17 or by prior methylation prevented induction, providing strong support for the notion that inducer binding to the ribosome is needed for induction to occur.

Evidence consistent with this idea and with existence of the postulated induction feedback loop was obtained by studying the behavior of *ermC* structural mutants.[1,76] These were generated by deleting DNA from within the coding region near unique Hpa I[89] and Bcl I[96] sites. These deletions, of which about ten have been studied, yielded Em-sensitive phenotypes. In every case, an altered protein was present in minicell extracts and these proteins exhibited a dramatic "hyperinducible" phenotype. In the *ole-1* background, these altered proteins were noninducible. Finally, unlike the wild-type methylase, the mutant proteins remained inducible even when the minicell strain was preinduced. These results are entirely consistent with the postulated induction feedback loop and with the notion that Em must bind to ribosomes for induction to occur, since inactive protein cannot methylate 23S rRNA and the feedback loop is thus broken. However, another interpretation is also possible: feedback may be exerted by direct methylase interaction with the *ermC* transcript causing repression of synthesis, and the altered proteins may be inactive as repressors. None of our experiments exclude such autogenous regulation, although the *ole-1* and preinduction experiments establish that Em binding to the ribosome is required for induction, and therefore that methylation-mediated feedback probably does occur.

X. STRUCTURE OF *ermC*

The *ermC* gene has been sequenced in our laboratory and in that of Weisblum.[80,81] The correct DNA fragments for sequencing were identified by cloning experiments. Based on the sequence and on physiological and genetic data like those discussed above, both laboratories have proposed models for regulation.[80,81,93,94,97] A description of the *ermC* primary structure follows, after which the regulation model is presented.

A. Promoter

RNA polymerase binding studies and deletion analyses had established the direction of *ermC* transcription and the approximate location of the *ermC* promoter.[89] Interpretation of the sequence[80,81] suggested that the promoter was as shown in Figure 3, possessing reasonable homology with the promoter consensus sequences identified in *E. coli*.[98] S1 nuclease mapping confirmed the location of this promoter sequence.[80] More recently we have made *ermC* transcript in vitro in the presence of [γ-^{32}P]ATP and have enzymatically sequenced the 5′ terminus of this transcript, confirming that the start site for transcription is at one of the tandem A residues indicated in Figure 3.[99]

B. Coding Sequence

An open reading frame capable of encoding a 244-amino acid protein was identified from the DNA sequence.[80,81] The calculated molecular weight of this protein was 28,947, in excellent agreement with the measured molecular weight of purified methylase (29,000).[72,88] This coding sequence encompassed several restriction sites known to occur with the *ermC* gene and no other suitable open reading frame existed in the sequenced DNA, on either strand. The putative start codon for the methylase is AUG, which is preceded by a Shine-Dalgarno[132] sequence enhibiting the strong complementarity to the 3′ end of 16S rRNA now expected of ribosomal binding sites from gram-positive organisms (Figure 3).[100] The start codon begins 140 to 141 bases downstream from the transcriptional start site, defining a "leader" segment on the mRNA.

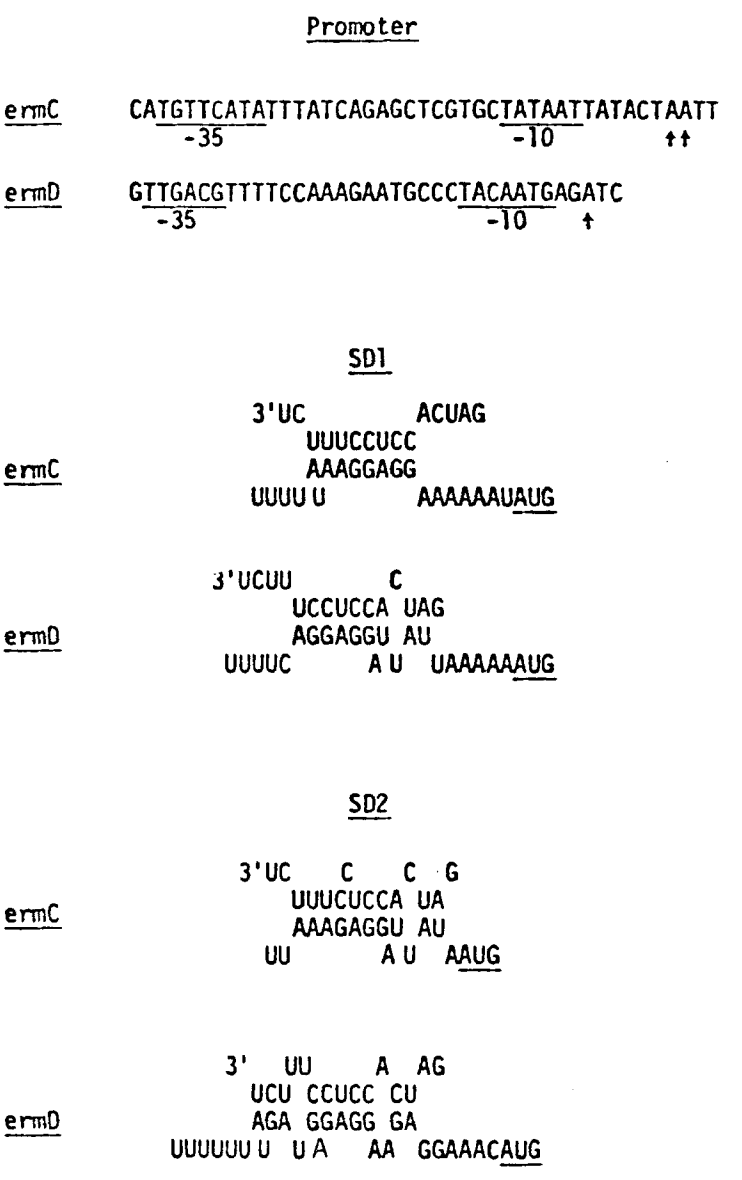

FIGURE 3. Promoter, SD1 and SD2 sequences of *ermC*[80,81] and *ermD*.[82,99]

C. Leader RNA

The primary structure of the leader RNA is reminiscent of the leader RNA sequences found in certain biosynthetic operons.[2] Immediately after the transcriptional start, a typical gram-positive Shine-Dalgarno[132] sequence is found (Figure 3), followed about 6 to 7 bases downstream by an open reading frame which starts with an AUG codon and terminates 19 amino acids later with a UAA. This putative peptide would be out of phase with the *ermC* coding sequence. In addition, several hairpin loop structures can be drawn, based on six complementary segments, which involve the leader and part of the coding sequence (Figure 4). The most stable structures (B, C) sequester the methylase initiation codon and part of the Shine-Dalgarno sequence (SD2) in base-paired structures. A less stable structure (A) does not sequester this ribosomal binding site. In none of these structures is the ribosomal binding site for the putative leader peptide (SD1 + AUG) involved in base pairing.

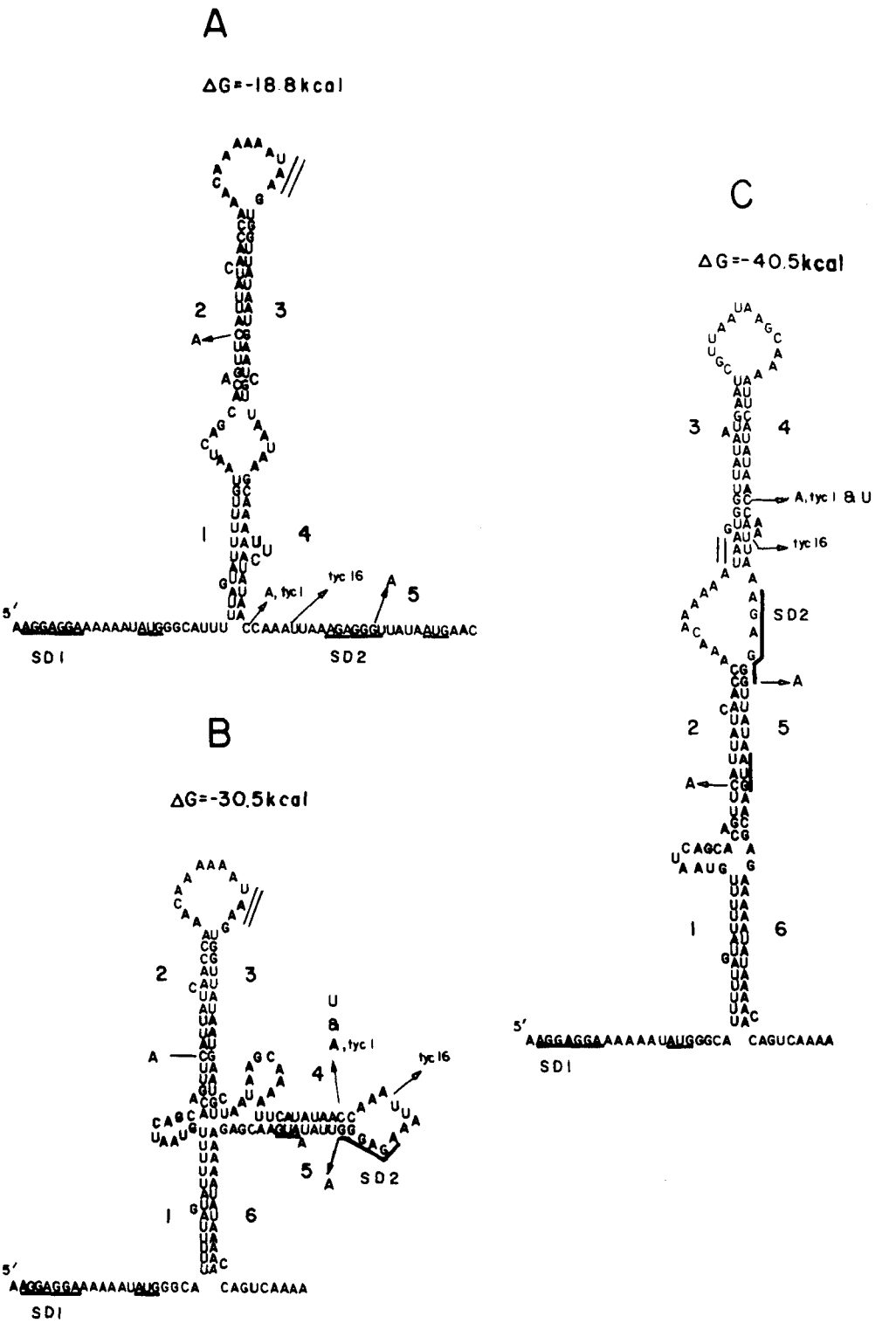

FIGURE 4. Proposed alternative structures for the regulatory region of *ermC* mRNA. The Shine-Dalgarno (SD)[132] sequences and their associated initiation codons are marked with single bars. The termination codon for the putative leader peptide is indicated by double bars. The approximate energies of the structures are given.[133,134] The locations of various point mutants and of *tyc-16*, a 109 base-direct tandem duplication, are given.[80,81,93,94]

XI. *ermC* INDUCTION MODEL

The induction model proposed by our group[80] and by Weisblum's[81] assumes that B and C are translationally inactive due to base pairing of SD2 and its associated AUG codon. It was proposed that the *ermC* transcript exists primarily in one of these inactive forms. In the presence of Em, ribosomes translating the leader peptide stall, freezing the transcript in an open configuration which permits translation to initiate at SD2 and results in increased synthesis of the methylase. The original models differed somewhat in that the structures proposed by Horinouchi and Weisblum[81] did not include segments 1 and 6. Our segments 2, 3, 4, and 5 were called 1, 2, 3, and 4 by those authors. Subsequently,[94] they recognized the two additional complementary segments, calling them A and A′. The present discussion will adhere to our original nomenclature. Horinouchi and Weisblum[81] also did not originally propose structures A or C, although they presented C in a later paper.[94]

We suggest that ribosomes translating the leader peptide stall in segment 2 when they have bound a molecule of Em (or Om). Stall at this position may be a consequence of the size and amino acid composition of the nascent peptide (see above). It is worth noting that the putative peptide (Figure 5) is rich in hydrophobic amino acids at the N terminus and rich in hydrophillic residues at the C terminus. Based on the properties of Em action noted above, it appears that the N- and C-terminal moieties of this peptide may be specialized to restrict Em-induced ribosome stall to the C-terminal portion, within segment 2. Thus, the distribution of amino acid residues in the peptide may serve a function analogous to the high concentration of regulatory amino acid residues in the leader peptides of attenuated biosynthetic operons.[2] Other MLS antibiotics, which fail to induce, would be expected to cause stall to occur earlier, perhaps preventing formation of the first (fMet-Gly) peptide bond. Our model is also consistent with the findings already noted, which suggest that Em causes a slowing down of ribosome movement on mRNA, and preserves polysomes, whereas the noninducing antibiotics promote rapid polysome breakdown.

Ribosome stall within structure C would directly free segment 5 which contains SD2 + AUG (Figure 6). Stall in segment 2 of structure B would have a similar effect. Following the direct freeing of segment 3, an isomerization would likely occur based on the calculated stabilities of the structures involved, leading to the same active structure postulated to result from ribosome stall in structure C. Once segment 5 has been freed, the second ribosomal binding site would be available for the loading of ribosomes, permitting methylase synthesis to proceed.

An apparent paradox in this system is that Em, an inhibitor of protein synthesis, is acting here as an inducer of protein synthesis. We must resolve this paradox by explaining why the ribosome which initiates methylase synthesis in the active (open) structure is not subsequently stalled in the presence of Em. We refer to two extreme situations discussed above: induction in the presence of very low or very high concentrations of Em. In the former case, most ribosomes will be Em free. In vitro the half-saturation concentration for Em-ribosome binding is about 1 μM.[1] At low inducing concentrations (<100 nM) Em-free ribosomes will be readily available to initiate methylase synthesis at SD2. Once the nascent peptidyl-tRNA exceeds a certain length, Em binding will not occur and a methylase molecule will be completed. Thus, at moderate and low Em concentrations, induction kinetics will reflect a compromise between the availability of ribosomes which carry a bound Em molecule (to allow stalling in segment 2) and those which are initially Em free (to permit methylase synthesis). Although at elevated intracellular Em concentrations ($\geqslant 1$ μM) nearly all non-methylated ribosomes will carry bound Em, induction still occurs. We have observed that the basal rate of methylase synthesis in the absence of inducer is about 5% of the fully induced level. It is therefore likely that some minor fraction of ribosomes will always be methylated, providing a pool of Em-free ribosomes for translation of methylase even in the

```
ermC    MetGlyIlePheSerIlePheValIleSerThrValHisTyrGlnProAsnLysLys
        1  2  3  4  5  6  7  8  9  10 11 12 13 14 15 16 17 18 19
```

FIGURE 5. Putative leader peptide sequences of *ermC*.[80,81]

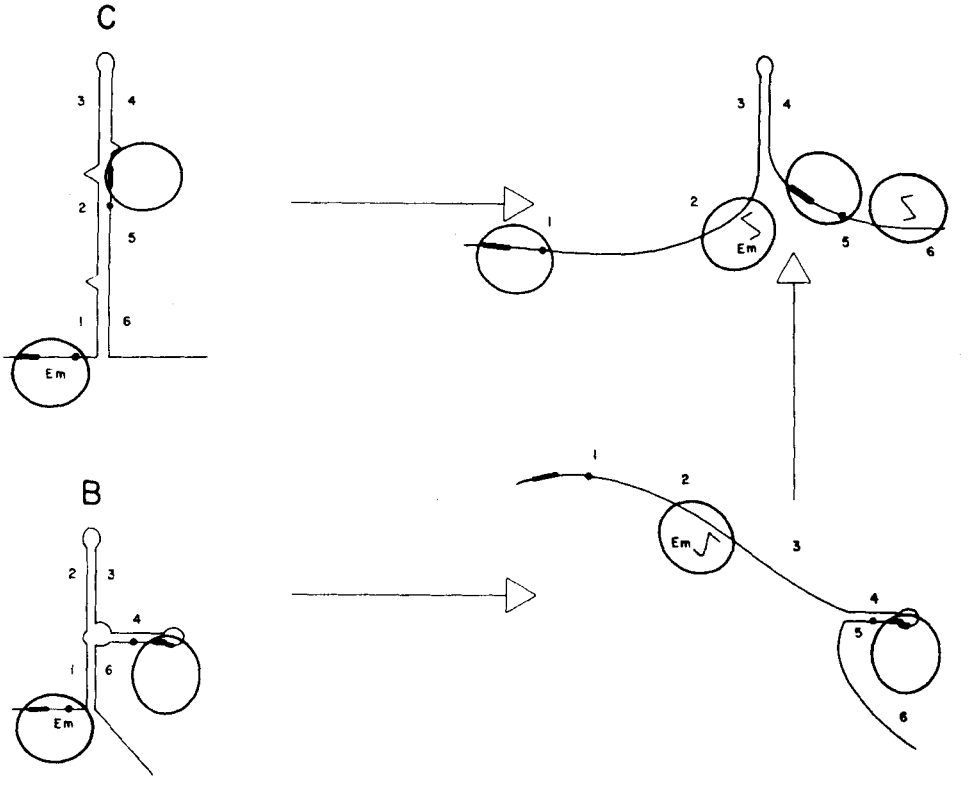

FIGURE 6. Model for induction of *ermC*. Ribosomes (elipses) are bound to SD1 and its associated AUG, and leader peptide translation begins. In the presence of Em, slowing of the ribosome occurs when the nascent peptide chain (zigzag line) reaches a critical length, and when certain sensitive amino acids are incorporated. This slowing of ribosome movement exposes segment 5 directly in structure C and following rearrangement in B. A ribosome, bound to SD2 (without interaction with the initiation condon), then rapidly commences methylase translation as the AUG codon is exposed.

presence of excess Em. Synthesis of methylase under these conditions would slowly but exponentially increase as the pool of methylated ribosomes was gradually augmented. This is what has been observed.[85]

It is worth noting that in the inactive structures B and C, SD2 is only partially base paired, while the associated initiation codon is sequestered. Jay et al.[101] have demonstrated that a synthetic deoxyribooligonucleotide containing a Shine-Dalgarno sequence but lacking an initiation codon can bind to ribosomes and facilitate binding of fmet-tRNA. Thus, oligonucleotide-dependent binding of fmet-tRNA does not require interaction with an initiation codon. This complex involves both 30S and 50S ribosomes and may be regarded as a preinitiation complex. If in the cell such a complex forms faster than it dissociates, SD2 may remain ribosome bound. When ribosome stalling occurs, the bound fmet-tRNA may rapidly interact with the newly exposed initiation codon permitting methylase synthesis to

proceed. Thus, we envisage a delicately poised mechanism in the absence of inducer, with *ermC* message turning over fairly rapidly ($\tau_F \simeq 6.0$ min at 32°C) but maintained in an inactive preinitiation complex.

XII. ANALYSIS OF REGULATORY MUTANTS

Support for the induction model derives from analysis of regulatory mutants. These fall into three categories: insertions (duplications), point mutants, and deletions. As noted above, about half of the spontaneously isolated *tyc* mutants are insertions, most of which are direct tandem duplications of *ermC* sequences. One of these *(tyc-16)* has been sequenced[93] and consists of a 109 base pair-tandem duplication of the sequence downstream from the point indicated in Figure 4. Since the duplication involves the downstream end of segment 4, all of segments 5 and 6, and a further sequence downstream and within *ermC,* we can envisage the two base-paired structures involving *tyc-16* RNA diagrammed in Figure 7. Structure II would be favored on both thermodynamic and kinetic grounds. Structure I would be entropically disfavored, since the 109 base-duplicated loop would be constrained. Structure II would also form first, since it involves pairing of promoter-proximal sequences and would therefore be kinetically trapped by the large activation energy required for II → I isomerization. Structure II is an "open" structure permitting constitutive methylase synthesis, as observed in *tyc-16* mutants. This notion (kinetic trapping of transcript structures) is a plausible and important one. It is plausible since the published rate constants for hairpin formation using $A_6C_6U_6$ are 2 to 3 × 10^4 S^{-1}, and the second order rate constants for double helix formation are in the 10^5 to 10^7 M^{-1}/S^{-1} range,[102-104] while the in vivo elongation rate of transcription is about 0.025 S^{-1} at 37°C.[105] Thus, incomplete transcripts should possess secondary structure, a notion in accord with current thinking about transcriptional termination and attenuation models. Direct evidence that secondary structure can form in nascent RNA chains and that conformational isomerization can occur during chain growth has been presented by Kramer and Mills[105a] using Qβ replicase. The kinetic trapping idea is important since it provides a rationale for the behavior of certain *tyc* mutants (such as *tyc-16*) and since it helps explain basal methylase synthesis (see below).

Several *tyc* mutants which result in single base changes have been sequenced (Figure 4).[80,81,94] *tyc-1* is a C → A transversion occurring in the downstream portion of segment 4. It raises the basal level of methylase synthesis about three- to fourfold, although synthesis can be further elevated somewhat by induction.[88] *tyc-1* would not destabilize pairing of segments 2 and 5, which is responsible for translational attenuation in C, the most stable structure. This strongly suggests that C, the equilibrium structure, is not solely responsible for attenuation of methylase synthesis. In structure B, *tyc-1* would markedly destabilize the initial segment 4 to 5 pairing (−11.7 to −2.8 Kal). What is more, the active structure A (Figure 4) would actually be stabilized by *tyc-1,* since the mutational change would permit additional base pairs to form. These relationships suggest the kinetic scheme shown in Figure 8. We suggest that structure A, involving segments 1 to 4, will form first. This is an open structure which permits methylase synthesis to proceed, accounting for at least some of the observed uninduced (basal) synthesis. When segment 5 is transcribed, it will pair with 4, and segment 1 will then be free to pair with newly transcribed segment 6, forming structure B. We may thus regard segments 1 and 6 as "stabilizers" which favor this A → B conversion. Since *tyc-1* allows additional base pairs to form in A and destabilizes 4 to 5 pairing in B, this isomerization would occur less rapidly and to a lesser extent, and a higher basal synthesis would occur, as observed. It is possible that translation of the leader peptide is strongly coupled to transcription, since SD1 is always exposed in structures A, B, and C. Such translation might tend to unpair segments 1 and 4, lowering the activation energy for A → B isomerization. Continued translation would then favor the formation of C, the equilibrium

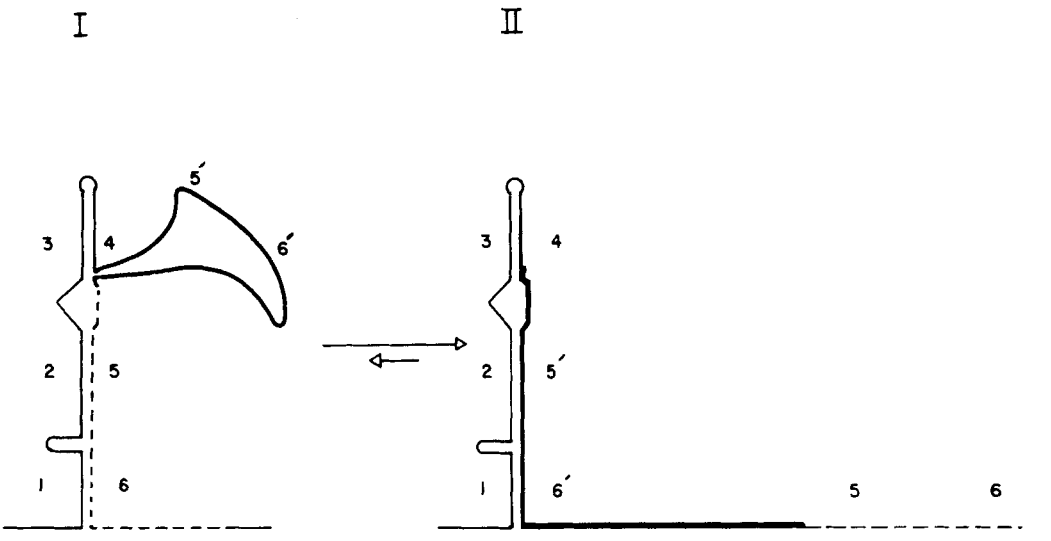

FIGURE 7. Alternative structures for the *tyc-16* mRNA. *tyc-16* contains a 109 base direct tandem duplication.[93] The duplicated copies are shown by heavy and by dashed lines.

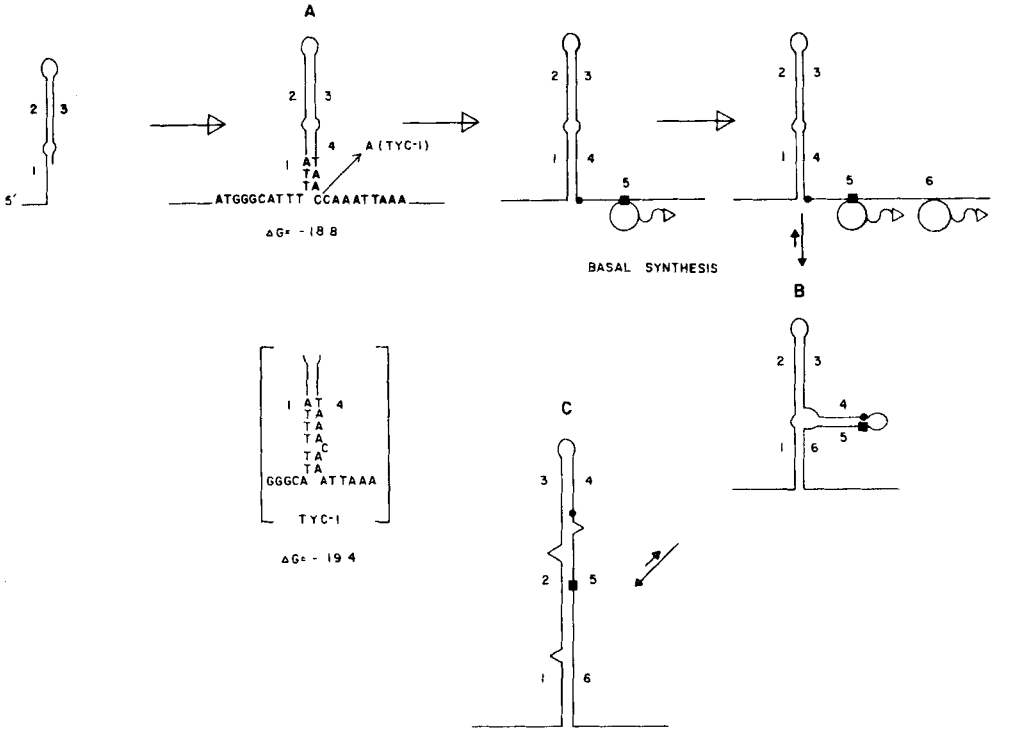

FIGURE 8. Kinetic trapping. We suggest that structures form during transcription in the order A, B, C (see Figure 4). In metastable structure A, SD2 is unsequestered, permitting basal (uninduced) methylase synthesis. Circles denote ribosomes and wavy lines denote nascent methylase. the *tyc-1* mutant replacement (C → A) permits extra base pairs to form in A, but destabilizes sequestering of SD2 in B. The SD sequences are shown by solid squares, and the location of the *tyc-1* mutation by solid circles.

structure, after transcription of segment 6 is complete. Thus, we propose the transcription coupled conversion A → B → C, possibly also coupled to translation. The properties of *tyc-1* and of *tyc-16* are consistent with this kinetic model. Horinouchi and Weisblum[94] have described four additional constitutive point mutants (Figure 4). One is identical to *tyc-1*. Another is a C → T transition at the same position as *tyc-1*. This can be rationalized similarly to *tyc-1*, although it would not result in formation of additional base pairs in A. A third mutation is a G → A transition in segment 5 which affects the same base pair in structure B as does *tyc-1*. This mutant would act similarly on structure B as the previous one, but would also destabilize base pairing of segments 5 and 2 in C, and is particularly interesting since it occurs within the base-paired portion of SD2. A final point mutation isolated and sequenced by Horinouchi and Weisblum is a C → A transversion which occurs within segment 2. This change would directly destabilize the base pairing involving the methylase initiation codon in C. In structure B, this mutation would destabilize 2 to 3 pairing favoring more rapid isomerization to structure C. Thus, the net effect would be increased methylase synthesis due to freeing of the initiation codon. It would be interesting to determine the relative uninduced synthesis of these last three *tyc* mutants, but quantitative data are not available. In conclusion, it appears that the available point and insertion mutations can be rationalized in terms of the regulation model, with the added consideration of kinetic effects.

Additional evidence favoring the model derives from deletion analysis. Horinouchi and Weisblum[94] have isolated a constitutive mutant which deletes 62 bp including the leader peptide coding sequence, the leader peptide termination codon (UAA), and the single bases immediately preceding the leader peptide initiation codon and immediately following the termination codon. This remarkable constitutive mutation thus removes segments 1 and 2, allowing 3 and 4 to pair and exposing the second ribosomal binding site. This mutant permits the conclusion that the leader peptide is not required for MLS resistance or for expression of *ermC*.

Further deletion mutations were constructed and analyzed in our laboratory.[93] The rationale for this study was as follows. Examination of structures B and C suggests that deletion of successive overlapping segments of the regulatory region proceeding from some upstream point would generate a series of mutants with various attenuation phenotypes. A mutant with a deletion terminating before SD1 should have the normal wild-type phenotype, i.e., inducible and Em resistant (I^+R). Any mutant with a deletion extending past SD1 will be I^-, since the model proposes that a ribosome must load at SD1 in order to stall in segment 2. Since the inactive structures are more stable than the active ones, deletions terminating just past SD1 or extending into segment 1 should permit the synthesis only of low amounts of methylase, corresponding perhaps to the wild-type basal level synthesis. These mutants might exhibit a low (partial) resistance (PR) to Em and should therefore have a new phenotype (I^-PR). A mutant with a deletion extending through segment 2 would be fully resistant and I^-, since S2 + AUG would be exposed. A deletion which extended through segment 3 would again produce a I^-PR phenotype, since segments 4 and 5 would pair when 4 was not preempted by 3 (Figure 4). Deletions extending through segment 4 would produce a I^-R phenotype and those extending into segment 5 would result in Em sensitivity (S). A series of deletions were generated using the exonuclease Ba131, all of which removed the *ermC* promoter and were shown to have fused *ermC* to an upstream promoter. These deletants were shown to fall into the expected four phenotypic classes: I^+R (wild type), S, I^-R, and a new phenotype, I^-PR. The latter was characterized by the formation of tiny colonies on EM and the production of low levels of methylase, uninducibly. The phenotypes of these deletants were confirmed by polyacrylamide electrophoresis of labeled minicell extracts. The Em-sensitive deletants were shown by restriction site mapping to have extended into the *ermC* coding sequence. Twelve deletants were sequenced and their downstream termination points are diagrammed in Figure 9. Δ14 leaves the SD1 sequence intact and exhibits

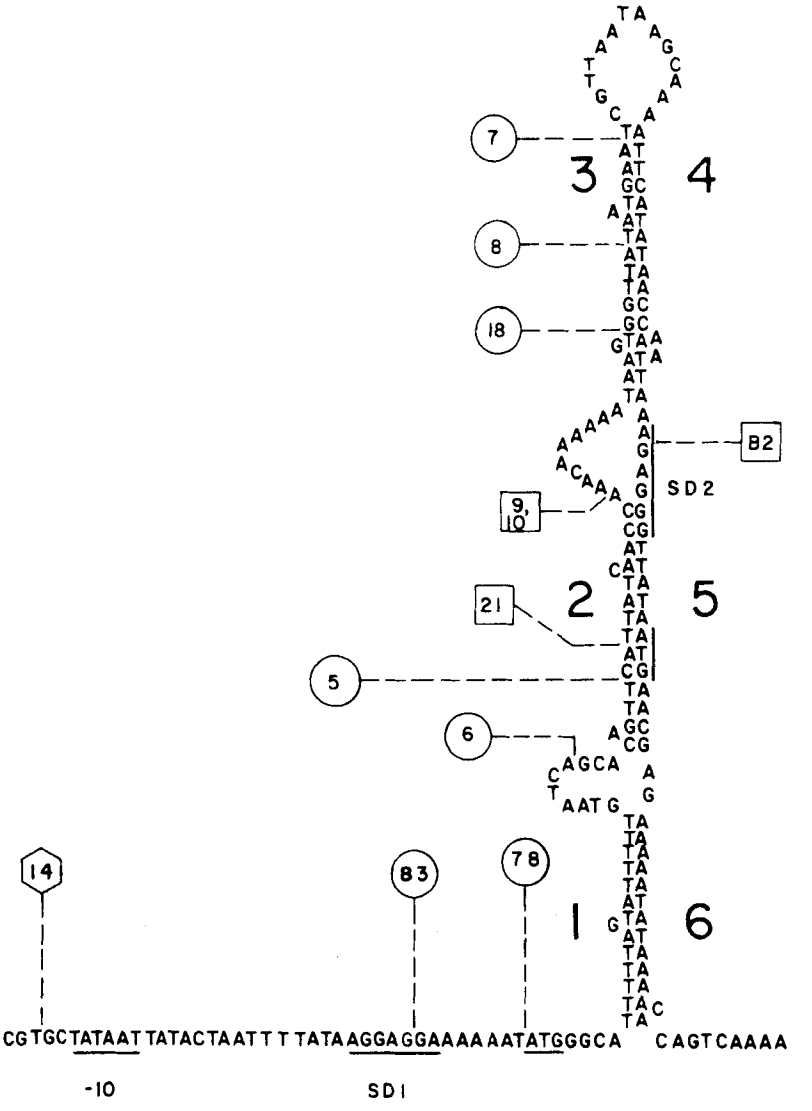

FIGURE 9. Termini of deletions.[93] The locations of the downstream deletion termini, on inactive structure C, are given. In each case the sequence to the left of the flag is replaced by an upstream sequence. The shapes of the flags indicate the phenotypes of the mutants: R,I$^+$ (hexagon); PR,I$^-$ (circle); R,I$^-$ (square). The sequence is written as DNA since it includes the region upstream from the wild type transcriptional start site. In each deletant, transcription initiates at an upstream promoter, to which the *ermC* sequences were fused. We have shown, using hybridization-blotting, that this upstream promoter is probably identical in each deletant.[91,93]

a wild-type (I$^+$PR) phenotype. Thus, the nontranscriptional regulation of *ermC* is confirmed, since the *ermC* promoter is deleted in this mutant. Δ83 and Δ78 remove all or most of SD1, resulting in low level constitutivity (I$^-$PR). These two mutations do not damage the predicted secondary structure, strongly suggesting that induction requires translation of the leader peptide. Δ5 and Δ6 extend further, removing segment 1 (Δ6) and part of segment 2 (Δ5). These also result in a I$^-$PR phenotype. Δ5 ends just before the portion of segment 2 which is predicted to pair with the initiation codon of SD2 + AUG, thus leaving this codon base paired. Δ21, on the other hand, removes two additional bases leaving this AUG codon

largely unpaired and resulting in a dramatic increase in methylase synthesis (I^-R). This strongly suggests that a critical factor in translational attenuation is sequestration of the initiation codon. Δ9 and Δ10 terminate identically between segments 2 and 3 (although their upstream termination points are different). These result in high level constitutivity (I^-R), since segment 2 is left unpaired. Δ18, Δ8, and Δ7 remove successively larger portions of segment 3 and result in I^-PR phenotypes. These destabilize 3 + 4 pairing (structure C) or 2 + 3 pairing (structure B), presumably permitting 4 and 5 to pair and resulting in low level synthesis. Finally, ΔB2 extends through segment 4 removing all sequences capable of pairing with SD2 + AUG and generating an I^-R phenotype. The phenotypic consequences of this series of mutations are in striking accord with predictions from the translational attenuation model.

Comparison of the amount of methylase produced by the I^+R and I^-PR deletion mutants in the absence of induction permits some insight into the factor affecting basal (uninduced) synthesis. Since *ermC* transcription in these mutants does not utilize the normal *ermC* promoter, we cannot compare their production of methylase to the wild-type basal level; however, they can be compared to one another. Among the PR mutants, those which have lost SD1 but possess segments 1 to 6 (Δ78 and Δ83) exhibit higher methylase synthesis than those which are lacking segment 1 (Δ5 and Δ6). This is to be expected from the kinetic trapping model presented above, since in the absence of segment 1, the postulated kinetically trapped intermediate (structure A) would not form (see Figure 8). Interestingly, the mutant carrying Δ14, which does not affect SD1, produces a higher basal level than do Δ83 and Δ78, which lack SD1. These three deletions do not affect segment 1. This effect is likely due to "translational activation", since ribosomes may transiently open structures B and C while translating the leader peptide. For this to occur, SD1 must be intact. This idea is reminiscent of the explanation offered for the polar effect of RNA bacteriophage coat protein amber mutations on replicase synthesis.[106] Thus, the behavior of these mutants is consistent with the idea that both kinetic trapping and translational activation contribute to uninduced synthesis. These considerations are germane since as noted above, regulation of basal synthesis probably determines the ability of the cell to be induced for the *ermC*-methylase by high Em concentrations.

It was pointed out above that although induction is accompanied by an increase in *ermC* mRNA stability, we have assumed that this stabilization is not the primary cause of increased methylase synthesis. This assumption must be kept in mind since a plausible model can be envisaged in which ribosome stalling causes isomerization of mRNA from an unstable to a stable conformer. All of the available data are consistent with such a model, although the deletion analysis just presented is strongly suggestive of a direct effect of mRNA conformation on translational activity. Of course, it is possible that both are true, that isomerization activates translation and simultaneously results in an extended mRNA half-life.

XIII. *ermC* INDUCTION AND THE OVERLAPPING PROTEINS

In addition to the 29K methylase and the postulated leader peptide, three other polypeptides (32K, 23K, and 18K) are specified by *ermC*.[89] These proteins are not induced by Em. In fact, the synthesis of the 32K protein appears to decrease as the 29K protein is induced.[107] Peptide mapping and mutational analysis have revealed that the coding sequences for these three polypeptides overlap one another and that of the 29K methylase and that all four proteins are translated in the same reading frame.[89] The ability of Em to induce the synthesis of only one of these proteins suggests strongly that they are not degradation products and are not related as products and precursors. No function for the 32K, 23K, and 18K proteins has been suggested. Based on the DNA sequencing data, on analysis of mutants, and on the mechanism of MLS regulation discussed above, we can propose a model for the regulation

and synthesis of these overlapping proteins. The deletion mutants already discussed, which lack SD1, do not produce the 32K protein but do produce the 23K and probably the 18K polypeptides.[93,108] A mutant with a short deletion which removes a Hpa I site near the methylase C terminus does not produce any of the four overlapping proteins.[89] We propose that these proteins terminate identically, but initiate at four different points. Examination of the *ermC* sequence suggests the scheme shown in Figure 10. In minicell extracts, the 32K and 23K proteins are made in low yield and the 29K (methylase) and 18K proteins in higher yield (without induction). The proposed SD sequence for the 23K protein is "weaker" than that for the 18K protein, in accordance with these observations. The postulated SD sequence for the 32K protein (SD1), however, shows exclient complementarity to the 3′ terminus of 16S rRNA. If SD1 were used to initiate the 32K protein, then the first 19 amino acids of this protein would be identical to the *ermC* leader peptide. Amino acids 18 and 19 are *lys* and are encoded by tandem AAA codons. We suggest that this A_6 tract causes ribosome slippage during translation, causing a −1 frameshift. This would allow readthrough past the normal UAA termination codon of the leader peptide and would permit translation to proceed uninterrupted until the UAA termination codon for the 29K methylase is reached. The resulting protein would thus contain the 19 amino acid leader at its N terminus, all of the methylase sequence at its C terminus, and an additional 21-amino acid segment between. The predicted molecular weight of this protein would be about 33K, in good agreement with the observed value. Since the *ermC* induction model requires ribosomal stalling within the leader peptide coding segment, we would expect synthesis of the 32K protein to decrease concomitantly with induction, in agreement with our observations.[107] This model for protein overlap is susceptible to direct verification by protein sequencing. If proven, it will strengthen the induction model, especially since it would provide direct confirmation that SD1 + AUG is active as an in vivo site of initiation of protein synthesis. The synthesis of a newly identified phage f2 protein of unknown function has also been ascribed to a naturally occurring frameshift.[135] Near the point at which this frameshift must occur is an A_4 sequence. The arrangement postulated for the coding sequences of the 29K, 23K, and 18K proteins is similar to that of the C and Nu3 gene products of phage λ,[136] the A and A* proteins of ΦX174,[137] and the two *cheA* polypeptides of *E. coli*.[138] In all of these systems the proteins initiate differently, but terminate at the same points.

XIV. REGULATION OF THE *ermD* AND pAM77 MLS METHYLASES

We have recently completed the DNA sequence of *ermD*, a chromosomal determinant cloned from the *B. licheniformis* chromosome.[18,82] High resolution S1-nuclease mapping has identified the transcriptional start site of this gene (Figure 3). An open reading frame preceded by an SD sequence (Figure 3) was readily identified as the coding sequence for the *ermD*-inducible protein. This product, identified as a 35,000-dalton protein in *B. subtilis* minicells, is induced by exposure to Em and Om, but not by Ty, clindamycin, or Lm.[18] The positions of the transcriptional and translational start sites define an *ermD* leader region of 358 bases, considerably larger than that of *ermC*. Within this leader are several short open reading frames, at least one of which is preceded by a likely SD sequence (Figure 3).[82] This leader sequence can be folded into an extremely elaborate structure, involving several complementary segments. The secondary structure computer program of Zuker and Stiegler[139] predicts an extensively folded structure involving the first 430 bases of the *ermD* transcript, with a predicted energy of −135.6 Kcal. Deletion mutants of *ermD* have been isolated after in vitro treatment with the exonuclease *Ba131*. Several of these exhibit constitutive expression of *ermD* methylase and are deleted for a portion of the *ermD* leader.[82] Thus, the *ermD* transcript, like that of *ermC*, has a leader region which serves a regulatory function and which possesses extensive potential secondary structure. It remains to be seen whether *ermD* regulation is mediated by translational attenuation.

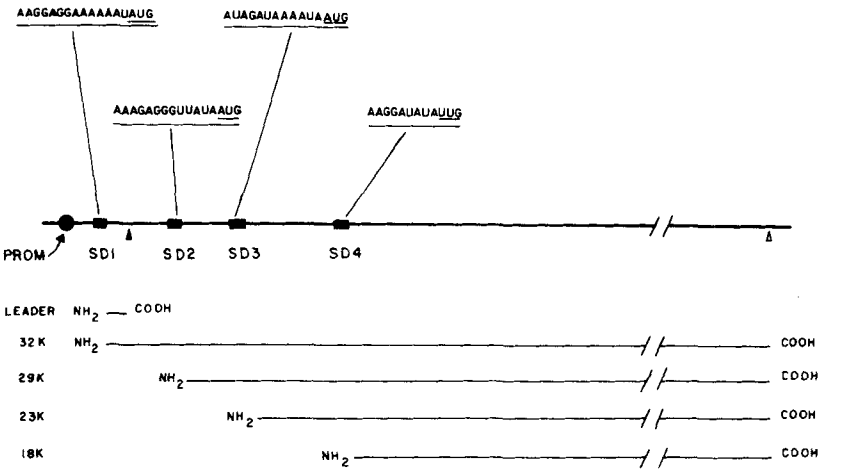

FIGURE 10. Model for *ermC* protein overlap. It is proposed that four of the five polypeptides encoded by *ermC* are coterminal at their COOH termini (△), but that their translation initiates at different points as shown (SD1-4). This would require that the 32K protein shift reading frame at the normal termination point for the leader peptide (▲).

Horinouchi et al.[140] have reported the sequence of the MLS-resistance determinant carried by pAM77, a *Streptococcus sanguis* plasmid. The resistance conferred by pAM77 is induced by a wide range of MLS antibiotics, including Em, clindamycin, and streptogramin B. The transcriptional and translational start sites of this gene were deduced from the DNA sequence and together define a 155-base 5′ leader which contains an open reading frame capable of encoding a 36-amino acid peptide. The leader region also contains a series of overlapping complementary sequences. One potential hairpin loop would sequester the methylase ribosomal binding site (SD2). The authors propose a model in which antibiotic-induced stall of a ribosome during translation of the peptide would release an RNA segment which would then preempt the 5′ arm of the hairpin loop which sequesters SD2, thus making the latter available for initiation of methylase synthesis. Horinouchi et al.[140] suggest that the longer leader peptide in pAM77, compared to that in pE194 (36 vs. 19 amino acid residues), is responsible for the ability of the pAM 77 determinant to respond to a broader range of MLS inducers, by providing "twice as many codons as potential sites for ribosomal stall." Whatever the detailed mechanism, the striking similarity in gross organization of the pAM77 MLS determinant to that of *ermC* and *ermD* suggests that this gene is also regulated by translational attenuation.

XV. *tet* GENE OF pT181

pT181 is an *S. aureus* plasmid which confers inducible resistance to tetracycline (Tc).[109,110] Although the resistance mechanism has not been elucidated for this determinant *(tet)*, it appears likely that it depends on decreased net uptake of antibiotic as in the case of Tc resistance in *E. coli*[111,112] and in *Bacillus.*[113] In *E. coli*, Tc resistance is under the control of a *trans*-acting negative control element.[114-116] The complete DNA sequence of pT181 has been determined by Khan and Novick.[117] The *tet* gene encodes a 35,000-dalton polypeptide, required for resistance, with a predicted abundance of hydrophobic amino acids, consistent with its presumed membrane site. Khan and Novick[117] have proposed, based on their DNA sequencing data, that induction of the *tet* product occurs by a translational attenuation mechanism. The likely promoter for this determinant was deduced from the DNA sequence.

Downstream from this, and preceding the start site for the probable resistance protein, is a leader sequence of about 160 bases which includes a potential open frame for a 16-amino acid peptide. Both of these frames appear to be preceded by reasonable SD sequences. The leader peptide would initiate with an AUG codon and the resistance protein with UUG, known to be an initiation codon in gram-positive organisms.[100] Most strikingly, this leader region can be folded into the stem-loop structures shown in Figure 11. Khan and Novick[117] suggest that Tc will stall a ribosome within segment II, at the base of inactive structure A. This would destabilize structure A, permitting isomerization to active structure B. Tc acts by inhibiting the binding of aminoacyl-tRNA to the ribosomal A site, and probably stalls inhibited ribosomes on the mRNA.[8] This proposed mechanism seems quite plausible and deserves further investigation.

XVI. OTHER SYSTEMS

Several other inducible antibiotic resistance genes are obvious candidates for translation level control similar to that exhibited by *ermC*. These involve antibiotics which affect protein synthesis and which interact with ribosomes: chloramphenicol (Cm) and fusidic acid (Fs).

Cm inhibits the ribosomal peptidyl transferase activity.[8] Fs appears to sequester GDP and EF-G near the ribosomal A site, thus preventing proper interaction of aminoacyl-tRNA with that site.[8] Plasmid-mediated Cm resistance is due to acetylation of Cm by an enzyme activity [chloramphenicol acetyl transferase (CAT)] specified by various resistance determinants.[118] The Cm resistance determinant of pC194 has been sequenced.[119] Based on the sequence and on molecular cloning experiments, it was suggested that CAT induction involves autogenous repression by the CAT protein.[119] It is not known whether CAT synthesis is regulated on a transcriptional or translational level, but a stem-loop structure can be drawn, involving the CAT SD sequence.[119] However, little experimental evidence exists, and the question of CAT regulation remains open.

The mechanism of inducible Fs resistance is unknown, and since absolutely no evidence is available concerning the mechanism of regulation of Fs resistance, it remains an attractive candidate for a translational attenuation-type control mechanism.

Regulation by translational attenuation is formally analogous to transcriptional attenuation, except that control is exerted on the level of translation. Aside from this difference, both types of systems involve alternative conformational states of mRNA leader structures and coupling of these folded states to the level of cellular protein synthesis by using ribosomes as regulatory effectors.[2] Another interesting attenuation type control is the regulation of *ampC*, a chromosomal β-lactamase gene of *E. coli*.[120,121] In this system, expression is growth-rate dependent. Regulation is thought to occur via transcriptional attenuation. An SD sequence in a leader portion of the *ampC* transcript is followed by the sequence AUGUAA. This sequence is immediately followed by a potential stem-loop structure which appears to function as a transcriptional terminator. It was suggested[121] that a ribosome can form an initiation complex at the AUGUAA sequence and pause before release, preventing the termination structure from forming and permitting transcription to proceed. Thus, ribosomes would act as positive effectors of *ampC* expression. Since ribosome content is proportional to growth rate, *ampC* expression would be growth-rate dependent.

In addition to *ermC*, several other prokaryotic translation level control systems have been studied. The *E. coli* RNA phages use translational regulation.[3] This regulation in certain cases involves RNA secondary structure as a control factor: in the regulation of replicase synthesis and probably in the prevention of false starts from potential internal initiators. In both of these cases, base pairing of initiator sequences prevents initiation of translation as proposed in the cases of *ermC*, *ermD*, the pAM77 MLS gene, and *tet*. Regulation of replicase seems to involve opening of an inactive base-paired structure when ribosomes traverse the

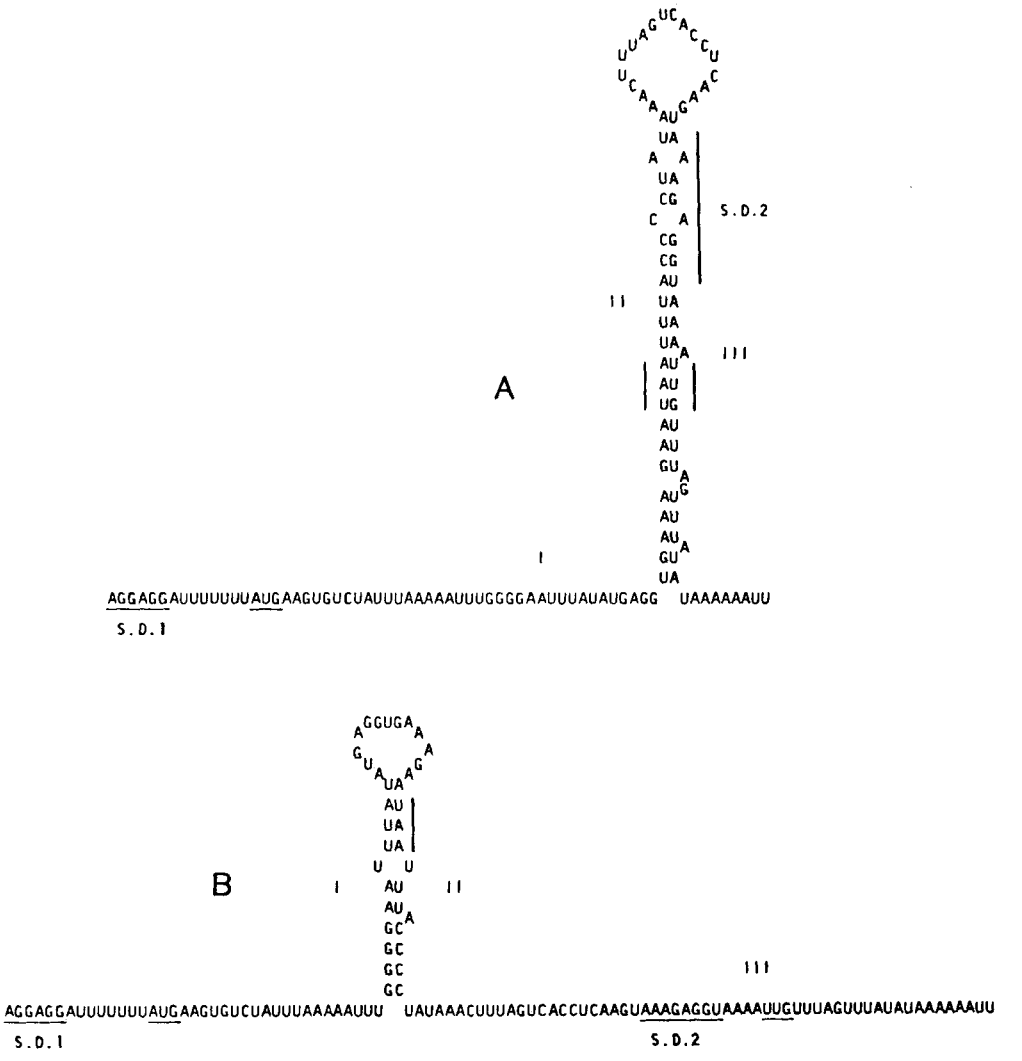

FIGURE 11. Proposed inactive (A) and active (B) secondary structures of the pT181 *tet* mRNA. Khan and Novick[117] have proposed that a ribosome stalled by the action of Tc in segment II causes isomerization to the active structure, permitting translation of the *tet* product to initiate near SD2.

coat protein gene; coat protein nonsense mutants are polar on replicase. A different base-paired structure involving the replicase initiator appears to be a binding target for coat protein, which thereby acts as a repressor of replicase synthesis. Thus, sequestration of the initiation codon and SD sequence of replicase again prevents translation. Several other cases of negative autogenous translational regulation involving protein binding to mRNA have been described: the *regA* gene and gene 32 systems of phage T4,[4,5] and several ribosomal protein operons.[6,122-126] It is worth remembering that a role for *ermC* methylase as an autogenous repressor has not been ruled out.

A particularly intriguing aspect of *ermC*, *regA*, and r-protein translational regulation is the enhanced stability of mRNA in all three of these systems when the transcripts are in the active state.[123,127-129] It seems likely that specific control of mRNA stability is an important component of regulation in these systems, and the mechanism of this control needs further investigation.

XVII. EVOLUTION OF ATTENUATION CONTROL

Why is the expression of some genes regulated translationally? Why are attenuation mechanisms used in preference to *trans*-acting regulatory elements, whether transcriptional or translational? Why are some systems subject to negative regulation and some to positive? Two kinds of answers to these questions may be envisaged. We may consider that a particular type of control operates efficiently for a particular system and has evolved for this reason. An interesting example of this approach is provided by Savageau's analysis of regulation.[130] Alternatively, it may be that control systems which respond to environmental changes evolve to utilize available mechanisms for sensing these perturbations. Thus, selection of a particular control system may reflect use of the most probable evolutionary pathway. Viewed in this way, MLS induction has evolved to utilize ribosomal interactions with Em and with mRNA, since these interactions were available, and since it was more probable that an Em-sensing device would evolve by building upon them than that an entirely new mechanism would evolve. Utilization of these interactions, then, determined the evolutionary development of a translation-level attenuation mechanism. Similarly, the translation-level repression control of gene 32 protein and of r-proteins evolved to utilize the preexisting (or better, independently existing) ability of gene-32 protein and of r-protein to bind RNA and at least in the case of r-protein, to recognize specific RNA sequences or structures. In general, attenuation mechanisms may have thus evolved to respond to perturbations which can affect ribosome performance, e.g., low intracellular concentration of aminoacyl-tRNA, increased Em, and probably increased Tc concentrations. Translational repression mechanisms utilize end products which can independently bind to RNA: r-protein, gene-32 protein, and probably the *regA* product. Transcription level controls may have evolved as intrinsically more economical mechanisms when suitable sensing devices were not available. The two ways of regarding the evolution of control mechanisms described above are not of course, mutually incompatible.

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