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Regulation of the inducible chloramphenicol acetyltransferase gene of the *Staphylococcus aureus* plasmid pUB112

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Analyses of deletion mutants of the gene for chloramphenicol (Cm) acetyltransferase (CAT) carried by the staphylococcal plasmid pUB112 revealed a regulatory region, which is indispensable for Cm-inducible *cat* gene expression, located 70 bp in front of the CAT-coding sequence. This region consists of a possible ribosome binding site followed by an open reading frame coding for a peptide of nine amino acids and overlaps partially with an inverted repeat capable of forming a stem-loop structure. Deletion of the ribosome binding site and of parts of the open reading frame abolishes inducibility and results in a low-level *cat* gene expression, if the inverted repeat remains intact. Deletion of the 5′ part of the possible stem leads to high-level constitutive CAT synthesis. The inverted repeat, therefore, exhibits negative control on *cat* gene expression whereas the preceding ribosome binding site is needed to enhance CAT synthesis in the presence of an inducer. These results suggest that translation of a leader peptide is a prerequisite for Cm-induced *cat* gene expression and that ribosome stalling on *cat* leader mRNA caused by Cm opens the stem-loop structure thereby releasing its negative effect on CAT synthesis.

Key words: Gram-positive chloramphenicol resistance/inducible gene expression

Introduction

Genes which confer chloramphenicol (Cm) resistance by the enzyme chloramphenicol acetyltransferase (CAT) are widespread within different bacterial species (Shaw, 1983). Among them are the *cat* genes of Gram-positive bacteria which are inducible by sub-inhibitory concentrations of the drug and of related antibiotics (Shaw, 1983). Four of those genes have been sequenced and their regulation has been studied in some detail. Two of them are coded on the small *Staphylococcus aureus* plasmids pC194 (Horinouchi and Weisblum, 1982) and pC221 (Shaw *et al.*, 1985) and the others, *cat*-86 and *cat*-66, were isolated from the chromosome of *Bacillus pumilus* (Harwood *et al.*, 1983; Duvall *et al.*, 1984). These studies on *cat* gene regulation indicate that neither the natural *cat* promoter nor the functional enzyme are necessary for induction. The regulation presumably occurs at the post-transcriptional level (Duvall *et al.*, 1983; Byeon and Weisblum, 1984). An inverted repeat structure preceding the CAT-coding region plays a key role in Cm-inducible regulation (Ambulos *et al.*, 1984). RNA transcribed from this inverted repeat could form a stable stem-loop in which the ribosome binding site of the *cat* gene is sequestered. This RNA cannot be translated because the *cat* Shine-Dalgarno sequence is not available for base-pairing with 16S rRNA. Accordingly, induction is accomplished by opening this stem-loop or hindering its formation. It has been proposed that this conformational change in mRNA structure is mediated by ribosomes modified by the inducing antibiotic (Duvall *et al.*, 1985). Recent results obtained for the *B. pumilus cat*-86 gene suggested transcriptional termination behind the stem-loop as an additional regulatory mechanism (Lovett, 1985). It seems that this *cat* gene differs from that of pC194 in so far as here no regulation of transcription could be detected (Byeon and Weisblum, 1984). In spite of all these data the mechanism by which Cm-modified ribosomes could open the stem-loop structure remains unclear, and no sequences on *cat* mRNA have been identified so far, which could be involved in ribosome-mediated *cat* gene induction.

The *cat* gene investigated in this study is encoded on pUB112, a 4-kb plasmid also from *S. aureus*, which replicates in *Bacillus subtilis* and confers Cm-resistance to this host (Brückner *et al.*, 1984). To examine the mechanism by which Cm is able to stimulate CAT synthesis in Gram-positive bacteria, we created several deletion mutants of the regulatory region of the pUB112 *cat* gene by means of the enzyme *Bal*31. Characterization of these mutants revealed a control region indispensable for induction. This region consists of a potential ribosome binding site and a small open reading frame encoding a polypeptide of nine amino acids. It is immediately followed by an inverted-repeat structure, which is also involved in *cat* gene regulation.

Results

Nucleotide sequence of the pUB112 cat gene and transcription initiation

The *cat* gene of pUB112 has been identified on a *Taq*I-*Mbo*I fragment located between positions 2.1 and 3.0 kb on the pUB112 circular map and it is inducible in *B. subtilis* (Brückner *et al.*, 1984). This fragment should, therefore, carry all information necessary for regulated *cat* gene expression. Determination of the nucleotide sequence (Figure 1) yielded an exact fragment length of 901 bp. Comparison with the nucleotide sequence of a *Taq*I-*Mob*I fragment of another *S. aureus* plasmid, pC221 (Shaw *et al.*, 1985), which also contains a *cat* gene, revealed that these two fragments are nearly identical. There are only 17 bp exchanges and one additional G:C base pair on the pUB112 fragment at position 896. 7 bp are different within the CAT-coding region and only four of these substitutions cause alterations in the amino acid sequence. None of the conserved amino acids found in four other CAT variants is affected by these changes. The amino acids Asp and Met, present in both staphylococcal CAT variants which have been sequenced until now, are replaced by members of the same group, Ser and Ile (residues 689 and 810, Figure 1), respectively. The two other replacements occur at positions (465 and 832) where no amino acid conservation among all other CAT variants has been detected (Shaw *et al.*, 1985). It is apparent that the plasmids pUB112 and pC221 share an almost identical type C *cat* gene (Fitton and Shaw, 1979) although they belong to different incompatibility groups (Jordanescu *et al.*, 1978).

The start of the *cat* mRNA has been deduced from the results

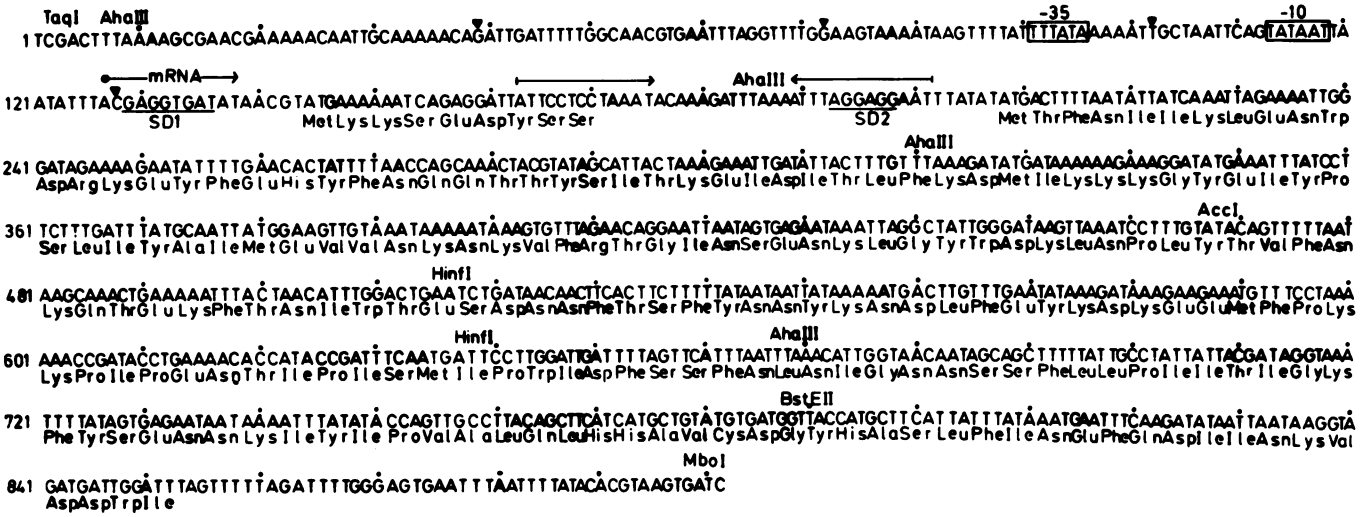

Fig. 1. Nucelotide sequence of the *TaqI-MboI cat* fragment of pUB112 displayed as the non-coding DNA strand. The putative RNA polymerase recognition sites are boxed. Two possible Shine-Dalgarno sequences (SD) are underlined. Two open reading frames following these ribosome binding sites represent a small leader peptide and the CAT-coding region. The amino acid sequences have been deduced from the nucleotide sequence. The opposing horizontal arrows indicate the inverted repeat structure. The vertical arrows point to the ends of *Bal*31 deletions which did not affect the inducibility of the *cat* gene. The deletion derivatives were designated as follows: pCR125 (residue 39); pCR54 (72); pCR2 (102); pCR1886 (128).

of *in vitro* transcription with *B. subtilis* RNA polymerase $E\sigma^{55}$. Using the isolated *Aha*III fragment (residue 8 – 195, Figure 1) as template, which had been subcloned by the addition of a 10-mer *Hind*III linker, a 68-base long run-off transcript could be detected. Dinucleotide priming of the *in vitro* system yielded stimulation with UpU, UpA and ApC and run-off transcripts of 70, 69 and 68 bases, respectively. The DNA sequence around the start site of the *cat* transcript is, therefore, TTAC. The synthesis of the 68-base long run-off RNA at nucleotide concentrations (5 μM) below that required for initiation of RNA synthesis could only be stimulated by the addition of high concentrations (250 μM) of ATP. Thus, *cat* mRNA is initiated *in vitro* at the A at position 127 (Figure 1), two bases upstream of the first Shine-Dalgarno sequence. This is in agreement with the results published by Shaw *et al.* (1985) on the pC221 *cat* gene and confirms once more the near identity of the two genes.

Characterization of cat gene deletion mutants

Previous work had suggested that the *TaqI* site of the *TaqI-MboI cat* fragment is near the beginning of the *cat* gene (Brückner *et al.*, 1984). To narrow the location of the gene and to isolate regulatory mutants, the 1400-bp *TaqI cat* fragment from pUB112 was digested with *Bal*31 enzyme to produce deletions beginning at both *TaqI* sites of the fragment. After joining *Hind*III linkers to the *Bal*31-generated ends, the *Mbo*I site on the *TaqI* fragment which is located near the end of the *cat* gene (Figure 1) was used to clone *Hind*III-*Mbo*I fragments into the polylinker region of the shuttle vector pRB273 (Brückner *et al.*, 1984). *Escherichia coli* K12 was then transformed, and selected Ap-resistant colonies were replica plated on agar plates containing 10 μg Cm/ml. Cm-resistant (CR) and Cm-sensitive (CS) clones were identified in this way and several of them were further analysed. Plasmids, which contained *cat* fragments of different sizes, were introduced into *B. subtilis*, and CAT activity of the resulting transformants was measured with and without induction by Cm or fluorthiamphenicol (Ftm), a fluorinated derivative of thiamphenicol, which is able to induce the *cat* gene but is not a substrate for CAT and is, therefore, not inactivated by acetylation (Syriopolou *et al.*, 1981). By these determinations we identified plasmids which showed altered *cat* gene expression as compared with the plasmid containing the entire *TaqI-MboI* fragment (pRB273-CR5). Finally, the end points of several deletions were determined by DNA sequencing. The CAT activities of representative clones are summarized in Table I and the end points of the corresponding deletions are shown in Figures 1 and 2.

Table I. Specific CAT activities in crude extracts of *B. subtilis* containing *cat* gene deletion derivatives

	A			B		
	CAT activity (nmol Cm acetylated/min/mg protein)			CAT activity, transcribed from *veg*II promoter		
Plasmid	Basal	Induced by Cm	Induced by Ftm	Basal	Induced by Cm	Induced by Ftm
pCR5	30	125	269	–	–	–
pCR125	31	128	–	–	–	–
pCR54	31	115	–	–	–	–
pCR2	6	25	–	–	–	–
pCR1886	<1	6	10	306	975	2109
pCR198	<1	1.5	1	82	104	98
pCR1809	<1	1.1	<1	–	–	–
pCS1837	<1	<1	<1	85	95	89
pCS1827	<1	<1	<1	–	–	–
pCS1825	<1	<1	<1	–	–	–
pCS1808	<1	<1	<1	91	88	92
pCS1816	<1	<1	<1	219	227	223
pCR1807	54	60	53	–	–	–
pCR94	64	56	63	2750	2554	2846

From the non-induced basal activities (Table I, panel A) we see that two clones, pCR125 and pCR54, exhibited the same *cat* gene expression as the entire *TaqI-MboI* fragment, indicating that here no essential functions had been deleted. (Because all plasmids described in this section are derivatives of pRB273, we designated them for convenience only pCR and pCS, respectively.) Plasmid pCR2, on the contrary, showed a 5-fold decrease in expression, and CAT synthesis mediated by pCR1886 had dropped below detectable levels. These reductions were probably due to the deletion of the −35 *cat* promoter region on pCR2 and to the

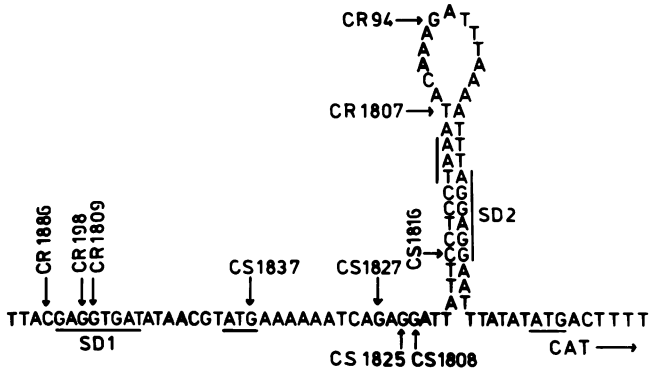

Fig. 2. Stem-loop configuration of the anti-sense DNA strand of the region preceding the pUB112 *cat* gene, and end points of regulatory deletion mutants. The sequence from position 125 to 216 (Figure 1) is shown. Potential Shine-Dalgarno sequences and start and stop codons are underlined. End points of deletions are indicated by arrows. The end point of the shortest fully inducible clone (pCR1886) is also indicated. The relative stability (ΔG) of the stem-loop structure is -19.8 kcal/mol.

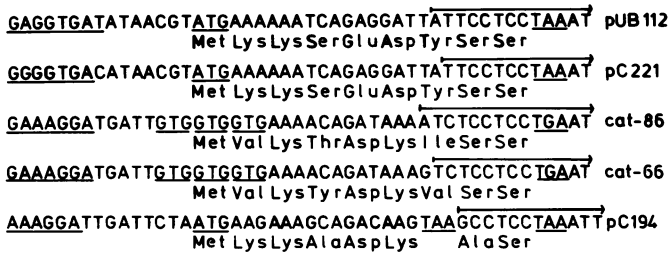

Fig. 3. Comparison of regulatory sequences preceding *cat* genes of Gram-positive origin. The sequences have been aligned by placing the stop codon of the putative leader peptides at the same position. The 3′ end of these sequences is defined by the 5′ part of the inverted repeat structures which are indicated by an arrow. The 5′ ends reflect the last bases which are complementary to *B. subtilis* 16S rRNA. Possible Shine-Dalgarno sequences, start and stop codons of interest are underlined.

loss of the whole promoter region on pCR1886 (Figure 1). Residual CAT synthesis of pCR1886 and of subsequent deletion derivatives probably depended on transcriptional readthrough from promoters of the vector. Surprisingly, CAT synthesis is markedly enhanced on pCR1807 and pCR94, which contained the shortest *cat* fragments investigated, and even higher than on the plasmid with the undeleted *Taq*I-*Mbo*I fragment.

Because we were interested in the mechanism of induction, we determined next whether CAT synthesis could be induced in these deletion derivatives. As shown in Table I, panel A, after exposure of the appropriate *B. subtilis* cultures to Cm or Ftm at sub-inhibitory concentrations, six clones had retained inducibility (pCR125, pCR54, pCR2, pCR1886, pCR198 and pCR1809). Although pCR198 and pCR1809 differ from pCR1886 by only 3 and 4 bp, respectively, inducibility of both is considerably reduced. Derivatives carrying fragments with larger deletions than pCR1809 could not be induced any more either by Cm or by Ftm (pCS1837−pCR94). The end points of the deletions of pCR198 and pCR1809 are, therefore, located within a region which is important for inducibility of *cat* gene expression. In this region a possible ribosome binding site can be seen, which is shortened by 2 and 3 bp on pCR198 and pCR1809, respectively. In all other deletion derivatives which were found to be non-inducible, this potential ribosome binding site is missing. Interestingly, this site is followed by an open reading frame for a peptide of nine amino acids.

Another regulatory element can be identified by comparing the basal CAT synthesis of the two clones with the shortest fragments, pCR1807 and pCR94, with pCR1886 or pCS1837. Although the natural *cat* promoter is missing on all these plasmids, pCR1807 and pCR94 showed a substantial increase in *cat* gene expression. To test the possibility that a promoter had been generated at the novel joint of the deletion on pCR1807 and pCR94, we carried out *in vitro* transcriptions with *B. subtilis* $E\sigma^{55}$ and *E. coli* RNA polymerase using isolated DNA fragments of the respective plasmids as templates. Under conditions where we should have been able to detect weak promoter activities we did not observe any initiation of transcription near the deletion junctions. It is, therefore, very unlikely that the increase of CAT activities on pCR94 and pCR1807 is due to the generation of a promoter at the deletion joint in front of the *cat* gene. As depicted in Figure 2, these two clones are deprived of the 5′ part of the inverted complementary repeat structure, which obviously exerts negative control on CAT synthesis. Ambulos *et al.* (1984) have already isolated similar deletions of the pC194 *cat* gene, which have been shown to have lost inducibility by Cm. The negative effect on CAT synthesis of the inverted repeat, however, could not be demonstrated by these data.

As found by others (Duvall *et al.*, 1983), our deletion analyses confirm that the natural *cat* promoter does not participate in *cat* gene induction.

CAT synthesis under the control of a heterologous promoter

Because CAT synthesis mediated by deletion derivatives without the *cat* promoter was mostly below a detectable level, we wanted to confirm our data on *cat* gene regulation with higher and, therefore, more measurable CAT activities. For this purpose, we put representative deletion mutants under the control of the heterologous *veg*II promoter which had been isolated from the chromosome of *B. subtilis* (Moran *et al.*, 1982) and which is part of the *veg* promoter system directing transcription during vegetative growth of *B. subtilis*. The *veg*II promoter is contained on an *Eco*RI-*Hind*III fragment of ~100 bp; the RNA start site is located within the *Hind*III recognition sequence (S.F.J.LeGrice, personal communication). This promoter fragment carries, therefore, no translation initiation signals which might interfere with *cat* gene regulation. After conversion of its *Eco*RI site to a *Hind*III site, the *veg*II-promoter fragment was cloned into the unique *Hind*III site of the respective plasmids. As summarized in Table I, panel B, results analogous to the former ones were obtained. pCR1886-*veg* could be induced by Cm ~3-fold and by Ftm ~7-fold. This is in good agreement with the induction level observed for the complete *cat* gene on pCR5 (4-fold and 8- to 9-fold, respectively) indicating that pCR1886 had retained all sequences necessary for regulated *cat* gene expression. There was again a substantial decrease in inducibility for pCR198-*veg*, and CAT was synthesized constitutively from all other plasmids which carried larger deletions. As the comparison of pCS1808-*veg* and pCR94-*veg* reveals, disruption of the possible stem-loop structure enhances the basal level of CAT synthesis ~30-fold. All these data are consistent with our former conclusions obtained from deletion derivatives missing a promoter immediately in front of the *cat* gene. However, two differences in CAT activities became apparent which had not been detected before with low-level CAT measurements. The basal *cat* gene expression of pCR1886-*veg* is 3- to 4-fold enhanced compared with pCR198-*veg*, pCS1837-*veg* or pCS1808-*veg*. The 2-fold increase in CAT synthesis of pCS1816-*veg* over pCS1808-*veg* is probably due to a destabilization of the putative stem-loop structure. In this deletion mutant the stem would be shortened by 4 bp thus relaxing the negative effect of this structure to some extent.

Interestingly, the *cat* Shine-Dalgarno sequence remains base paired within this shortened stem.

Discussion

We have identified a regulatory sequence for the *cat* gene of the *S. aureus* plasmid pUB112 which is indispensable for Cm-induced CAT synthesis. This sequence consists of a potential Shine-Dalgarno sequence (SD1), followed by an open reading frame encoding a polypeptide of nine amino acids, and is located ~70 bp upstream from the CAT-coding region. Partial deletion of SD1 markedly reduces the inducibility of Cm resistance in *B. subtilis*, and its total deletion leads to constitutive *cat* gene expression. RNA transcribed from this region can form 8 bp with the 3′ end of *B. subtilis* 16S rRNA. The calculated free energy (Tinoco *et al.*, 1973) for this mRNA-rRNA interaction is −17.8 kcal/mol. Such a strong Shine-Dalgarno complementarity was previously found to be required for initiation of protein synthesis by ribosomes of Gram-positive bacteria (McLaughlin *et al.*, 1981; Band and Henner, 1984). For mRNAs transcribed from plasmids pCR198 and pCR1809 this complementarity is reduced by 2 and 3 bp thus diminishing the free energy of their binding to 16S rRNA to −15.4 and −11.4 kcal/mol, respectively. In parallel, the inducibility of CAT synthesis falls considerably. For further quantitative considerations on *cat* gene expression we have to compare plasmids containing either the *veg*II or the *cat* promoter, because the basal CAT level directed by promoterless deletion derivatives could not be measured. With pCR198-*veg* CAT activity is only slightly stimulated by Cm or Ftm, whereas expression of the *cat* gene on pCR5 and pCR1886-*veg*, which both contain the complete SD1 sequence, can be induced 3- to 4-fold by Cm and 7- to 9-fold by Ftm. On pCS1837-*veg*, the entire SD1 sequence is missing and cells carrying this derivative cannot be induced any more. As expected, deletions extending beyond the end point of the pCS1837 deletion also lead to constitutive CAT synthesis. Thus, Cm-inducibility of *cat* gene expression depends on SD1 and we can, therefore, conclude that binding of ribsomes to this Shine-Dalgarno sequence is a prerequisite for Cm-mediated *cat* gene regulation.

In addition to SD1 and the following open reading frame, an inverted repeat structure in front of the CAT-coding region plays an important role in *cat* gene regulation. Plasmids which have lost one half of the inverted repeat exhibited an increased basal CAT synthesis as compared with clones carrying the entire structure. For example, pCR94-*veg*, which is deprived of the 5′ part of the inverted repeat, showed a 30-fold higher CAT activity than pCS1808-*veg*. Obviously, CAT synthesis is under negative control by this inverted repeat. The negative control is probably achieved by sequestering the Shine-Dalgarno sequence of the *cat* gene (SD2) within the stem. Translation of the *cat* mRNA would then be reduced because ribosomes could not bind to the *cat* ribosome binding site. Alternatively, the stem-loop structure could act as a Cm-regulated transcriptional terminator. We cannot distinguish between these possibilities by our experiments but it was suggested by others that the main regulatory event occurs at the post-transcriptional level (Duvall *et al.*, 1983; Byeon and Weisblum, 1984). Regardless of whether *cat* gene expression is regulated transcriptionally or post-transcriptionally or even at both levels (Lovett, 1985), the central problem of *cat* gene induction seems to be the mechanism by which a conformational change in *cat* mRNA structure preventing the formation of the stem-loop can be achieved by the antibiotic.

As already mentioned, SD1 is followed by a small open reading frame for a peptide of nine amino acids whose start codon is 7 bp apart from SD1. This region, therefore, fulfils all requirements needed for initiation of protein synthesis in Gram-positive bacteria. Under the assumption that this peptide is synthesized, we would propose the following mechanism for Cm-induced alteration of *cat* mRNA secondary structure.

In the absence of the inducing antibiotic, *cat* mRNA can form a stable stem-loop structure which exhibits negative control on CAT synthesis. In this mRNA conformation SD1 is accessible for ribosomes and the small leader peptide can be synthesized. Binding of Cm to the 50S subunits of ribosomes would freeze them on *cat* leader mRNA. Ribosome stalling at the 3′ end of the coding sequence for the leader peptide, which is located within the stem, would then prevent the inverted repeat sequences from base pairing, keeping it open and, thereby, releasing the negative effect on *cat* gene expression. Subsequently, CAT synthesis could be achieved by Cm-free ribosomes. This mechanism would be consistent with the findings that Cm slows ribosome movements on mRNA and preserves polysomes (Gale *et al.*, 1981). Furthermore, it has been shown for the *cat*-86 gene of *B. pumilus* that CAT induction is mediated by ribosomes (Duvall *et al.*, 1985).

However, one question concerning this mechanism remains open: would not ribosomes open up the stem-loop structure while translating the leader peptide? Indeed, we believe that this is the case to some extent. The basal CAT activities of clones which synthesize the leader peptide (pCR1886-*veg*) and of clones which have lost this ability (pCS1837-*veg*, pCS1808-*veg*) differ ~4-fold (Table I). This increase in basal *cat* gene expression of pCR1886-*veg* over pCS1837-*veg* and pCS1808-*veg* could be a hint that ribosomes translating the leader peptide partially destabilize the stem-loop thus releasing its negative effect to some extent. We believe that this would be an attractive explanation for the differences in basal CAT activities observed with the clones mentioned above. In spite of this possible effect of ribosomes in the absence of the inducing antibiotic, only stalling of them at a specific site on *cat* leader mRNA would lead to complete disruption of the stem and to fully induced CAT synthesis.

Two possibilities are conceivable to explain how the stem-loop structure could act negatively on CAT synthesis. If *cat* gene expression is controlled at the transcriptional level, CAT induction would be a consequence of enhanced transcriptional readthrough analogous to the control mechanism found in several amino acid biosynthetic operons (Kolter and Yanofsky, 1982). Suggesting post-transcriptional regulation, CAT induction would be very similar to the erythromycin (Em)-induced expression of the Em-resistance gene, *erm*C, also originating from *S. aureus* (Dubnau, 1984). In this system, stalling of Em-blocked ribosomes translating a leader peptide causes an alteration of mRNA secondary structure, which allows translation of *erm*C mRNA. On *erm*C mRNA several alternative secondary structures can be formed whereas on *cat* mRNA only one stem-loop structure is apparent. Apart from this difference, similarities between both systems are striking. The structural genes, whose ribosome binding sites are sequestered within a stem, are preceded by small leader peptides; both genes confer resistance to antibiotics, which bind to the 50S ribosomal subunit, affect transpeptidization, and are able to stabilize ribsomes on mRNA. We would therefore suggest that the pUB122 *cat* gene is regulated by a translational attenuation mechanism analogous to that described for *erm*C induction.

If our model is of general significance for *cat* gene induction in Gram-positive bacteria we would expect also to find similar regulatory sequences in front of other *cat* genes. Until now, the control regions of five *cat* genes of Gram-positive origin have

been sequenced, and their regulatory sequences are presented in Figure 3. The inverted repeat is present in the 5′ non-coding region of all of them. It varies somewhat in size (12 – 14 bp) and base composition, but the *cat* Shine-Dalgarno sequence is always sequestered in the stem. A sequence complementary to *B. subtilis* 16S rRNA at essentially the same position as SD1 on pUB112 is also present in front of the other *cat* genes. The calculated free energy for these mRNA-rRNA interactions varies from −18.6 kcal/mol for pC221 to −14 kcal/mol for both *B. pumilis* genes, and −11.8 kcal/mol for pC194. Although the Shine-Dalgarno complementarity is not the only parameter which defines the strength of a translation initiation site (Gold *et al.*, 1981), we can conclude that three of these sequences could function as a ribosomal binding site; only the mRNA-rRNA complementarity of pC194 seems to be rather weak. The potential Shine-Dalgarno sequences of pC221, *cat*-86 and *cat*-66, are followed by a coding sequence for a leader peptide of the same length, assuming that for both *B. pumilus* genes the central of three consecutive GTG triplets functions as an initiation codon. The last two amino acids, two serines in all peptides, are encoded within a sequence of the inverted repeat structure which is complementary to the *cat* Shine-Dalgarno sequence. Therefore, these three *cat* genes could be regulated in the same manner as described for pUB112.

The only exception among the listed *cat* genes appears to be that of pC194 where the potential leader peptide sequence is interrupted by a stop codon just before the inverted repeat. Furthermore, neither the possible Shine-Dalgarno sequence nor the first part of the coding region of the leader peptide is transcribed (Byeon and Weisblum, 1984). These authors presented a hypothesis for pC194 *cat* gene induction, according to which a sequence of the 23S rRNA complementary to the 5′ part of the stem should bind to nascent *cat* mRNA thereby exposing the *cat* Shine-Dalgarno sequence for interaction with ribosomes. We can exclude this mechanism for pUB112 *cat* gene induction because our SD1 deletions with the intact inverted repeat, which all contained this sequence complementary to 23S rRNA, should not have lost inducibility.

Although we have not proven that a leader peptide is really synthesized we do not believe that binding of ribosomes to SD1 without subsequent translation can explain the regulatory effect of Cm on *cat* gene expression. We therefore propose that stalling of Cm-modified ribosomes translating the leader peptide within the stem results in opening the stem-loop structure thereby allowing CAT synthesis. In further studies the existence of the leader peptide will be investigated and the influence of the stem-loop structure on transcription and translation will be studied to evaluate all aspects of *cat* gene regulation.

Materials and methods

Bacterial strains and plasmids

The construction and characterization of the shuttle vector pRB273 has been described previously (Brückner *et al.*, 1984). This plasmid was maintained within *B. subtilis* BD170 (*trp*C2, *thr*5) and within *E. coli* K12 71/18 [Δ(*lac-pro*), F′(*lac*I, *lac*Z), Δ15, *pro*, *sup*E]. Plasmid pUB112 was obtained from R.Novick, NY, in *S. aureus* strain RN2438.

Plasmid DNA manipulations and transformation

Plasmid DNA isolation, restriction and ligation reactions and transformation of *E. coli* have been described (Brückner *et al.*, 1984) and followed standard procedures (Maniatis *et al.*, 1982). Plasmids were constructed in *E. coli*, and after characterization transformed to competent *B. subtilis* cells (Contente and Dubnau, 1979). *Bal*31 digestion was carried out under conditions recommended by the supplier (Boehringer, Mannheim, GmbH).

DNA sequencing

For DNA sequencing the dideoxy chain termination method (Sanger *et al.*, 1977) with covalently closed plasmid DNA as template was used. A 16-mer primer complementary to sequences adjacent to the *Eco*RI site of pBR322 (Sutcliffe, 1979) was purchased from New England Biolabs. This primer allowed clockwise sequencing of fragments inserted into the *Eco*RI, *Cla*I or *Hind*III site of pBR322. The 5′ non-coding strand of the *cat* gene of pUB112 has been determined by sequencing the *Taq*I-*Mbo*I fragment. Subsequently a primer of 16 bases complementary to position 272 – 287 (Figure 1) of the non-coding strand inside the *cat* gene was used to obtain the sequence of the coding strand and the end points of the *Bal*31 deletions cloned into pRB273. Furthermore, *Aha*III fragments (Figure 1) provided with *Hind*III linkers were cloned into pBR322. Sequencing these fragments in either orientation yielded the DNA sequence of both strands of the *Taq*I-*Mbo*I fragment up to position 666. In our *cat* derivatives of pRB273 we determined the sequence of the coding strand using the *Eco*RI primer of pBR322.

CAT activity determinations

CAT activity was measured by the spectrophotometric method (Shaw, 1975) in sonicated extracts of *B. subtilis* (Goldfarb *et al.*, 1982). For our CAT determinations we used 10 ml of a *B. subtilis* culture grown in TSB medium (BBL) containing 5 μg kanamycin/ml to the mid-logarithmic phase (OD_{550nm} = 1.5 – 1.8). The cells were broken in a volume of 1 ml, and 5 – 50 μl of this crude extract or of appropriate dilutions were assayed. Each extract was tested three times and two to three extracts were prepared for each clone. Protein concentrations were determined according to the Coomassie blue assay (Bradford, 1976). From a portion of each culture, plasmid preparations and subsequent restriction analyses were carried out to ensure that no segregational or structural instability had occurred. Specific CAT activities are expressed in nmol Cm acetylated per min per mg protein (Table I).

Induction of the cat gene

Because acetylated Cm is not active as an inducer of the *cat* gene the induction rate depends on the CAT activity present prior to stimulation by Cm. Higher basal CAT activities lead to rapid acetylation of the inducer and, therefore, to reduced induction rates. To obtain sufficient induction, cultures with different basal CAT activities have to be induced by different Cm concentrations. Therefore, we used 0.1 μg Cm/ml for deletion derivatives which were deprived of a functional promoter and exhibited minimal basal CAT activities. Cultures of *B. subtilis* containing plasmids with the natural *cat* or *veg*II promoter were induced with 1 μg Cm/ml. Stimulation of CAT synthesis by Cm is always followed by a rapid inactivation of the inducer. In order to minimize this feedback regulation we exposed the cultures only a short time (45 min) to Cm.

These difficulties can be avoided by using a fluorinated derivative of Cm, Ftm, which cannot serve as a substrate for CAT. With Ftm, *B. subtilis* cultures can be induced independently of the basal CAT level, and no inactivation of Ftm by increasing amounts of CAT can occur. The induction rate obtained by Ftm under our conditions (0.1 μg/ml, 2 – 2.5 h) is 2- to 3-fold higher than with Cm. As an exception, CAT synthesis on pCR198 seemed to be better inducible by Cm. We have no explanation for this result, but it might reflect a slightly different mode of action between Cm and Ftm. To differentiate whether a deletion derivative is inducible or not, we performed induction with both Cm and Ftm.

In vitro transcription

In vitro transcription with purified *B. subtilis* $E\sigma^{55}$ RNA polymerase was carried out as described previously (Brückner *et al.*, 1984). Dinucleotide primed transcription (Downey *et al.*, 1971) was carried out with 250 μm ApU, UpU, UpA, ApC, CpG, GpA, ApG or GpG, respectively, and 5 μM nucleoside triphosphates.

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