

Translational Coupling in *Bacillus subtilis* of a Heterologous *Bacillus subtilis*-*Escherichia coli* Gene Fusion

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Translational coupling was demonstrated in a gene fusion in which the promoter and the N-terminal region of the *Bacillus subtilis* subtilisin (*aprA*) gene were fused to a promoterless Tn9-derived chloramphenicol acetyltransferase (CAT; EC 2.3.1.28) gene. Expression of this gene fusion results in the production of a native-sized CAT product, whereas the Tn9-derived CAT gene is usually not translated from its own ribosome binding site in *B. subtilis* (D. S. Goldfarb, R. L. Rodriguez, and R. H. Doi, Proc. Natl. Acad. Sci. USA 79:5886-5890, 1982). A 178-base-pair deletion, which removed part of the signal peptide and the propeptide of the *aprA* gene and created a translational stop codon 230 base pairs upstream of the CAT gene ribosome binding site, reduced expression of the CAT gene. A *Bam*HI 10-mer linker insertion into this deletion site, which restored the reading frame and simultaneously removed the translation stop codon, restored CAT gene expression. The data indicate that expression of the CAT gene was dependent on translation of the truncated *aprA* gene into the ribosome binding site of the CAT gene.

Previous studies on the insertional activation of the Tn9-derived promoterless chloramphenicol acetyltransferase (CAT; EC 2.3.1.28) gene in *Bacillus subtilis* showed that the expression of the CAT gene in plasmid pGR71 (5) was dependent on the presence of a *B. subtilis* promoter in the inserted fragment, the utilization of a *B. subtilis* ribosome binding site for initiation of translation, and a correct in-phase fusion which resulted in an open reading frame between the inserted *B. subtilis* sequence and the CAT gene. The protein product that resulted from expression of such a hybrid gene in *B. subtilis* consisted of an N-terminal polypeptide from *B. subtilis* fused to the Tn9-derived CAT (6). The fusion products were catalytically active and resulted in chloramphenicol-resistant clones. These results also indicated that the Tn9-derived ribosome binding site of the CAT gene could not be used efficiently by the *B. subtilis* translation system to produce a chloramphenicol resistance (Cm^r) phenotype and that native-sized CAT was not produced (6).

In the course of these studies, we observed one case in which a DNA insert activated the expression of the promoterless CAT gene during the stationary phase of growth but not during the exponential phase (7). Furthermore, we found that only native-sized CAT was synthesized (27), in contrast to our previous studies in which only fusion products were observed (6). This indicated that the gram-negative ribosome binding site of the CAT gene in this clone was being used by the *B. subtilis* translation system.

A transcriptional analysis of this DNA insert (called the S fragment [7]) revealed that the promoters were typical sigma-37 and sigma-32 promoters (11). Sequence studies revealed that the S fragment encoded the N-terminal half of the *B. subtilis* subtilisin (*aprA*) gene containing its promoter region, ribosome binding site, signal sequence, propeptide, and N-terminal part (49 amino acids) of the mature subtilisin (28).

We investigated the nature of the fusion between the S fragment and the CAT gene in plasmid pGR71 to determine the mechanism which allowed the efficient utilization of the gram-negative ribosome binding site of the CAT gene in *B.*

subtilis. The sequence studies revealed that the *aprA* gene was not fused with the CAT gene in a translation phase which could result in an active, fusion CAT product. In fact, the out-of-phase fusion resulted in translation of the fusion mRNA from the *aprA* ribosome binding site up to and terminating within the CAT ribosome binding site. Our results indicate that this fortuitous translation termination allowed translation initiation from this gram-negative ribosome binding site which was very weakly utilized by the *B. subtilis* translation system. This result is similar, but not identical, to the translation coupling phenomenon (17) which has been observed in other native and synthetic operons.

MATERIALS AND METHODS

Bacterial strains and media. *B. subtilis* DB102 (*hisH nprR2 nprE18*) and *B. subtilis* DB104 (*hisH nprR2 nprE18 ΔaprA3*) (9) were used in this study. *B. subtilis* DB104 and *B. subtilis* DB102 cells containing recombinant plasmids were streaked to grow overnight on tryptose blood agar base (Difco Laboratories, Detroit, Mich.) plates with 5 μg of kanamycin (Sigma Chemical Co., St. Louis, Mo.) per ml, suspended in 2XSG medium (13) containing 5 μg of kanamycin per ml to dilute components made during the late stationary phase, and allowed to grow with kanamycin to the times indicated below.

Transformation of *B. subtilis*. Transformation was carried out by the procedure of Spizizen (23) as modified by Kawamura et al. (10).

Transformants were tested for the presence of the correct plasmids by a rapid miniscreen method (see below).

***B. subtilis* plasmid preparation for miniscreening.** Cells were grown at 30°C overnight on tryptose blood agar base plates containing 0.5% glucose and 5 μg of kanamycin per ml. The fresh colonies were suspended in 2 ml of L broth containing the antibiotic, and cells were grown for 3 h at 37°C. A 1.5-ml portion of each culture was centrifuged for 1 min in a Microfuge (Brinkman Instruments, Inc., Westbury, N.Y.); the pellets were washed once with SET buffer (20% sucrose, 50 mM EDTA, 50 mM Tris hydrochloride [pH 7.6]) and suspended by vigorous vortexing in 100 μl of SL buffer (SET buffer plus lysozyme at 2 mg/ml). To this suspension was added 4 μl of RNase solution (10 mg of pancreatic

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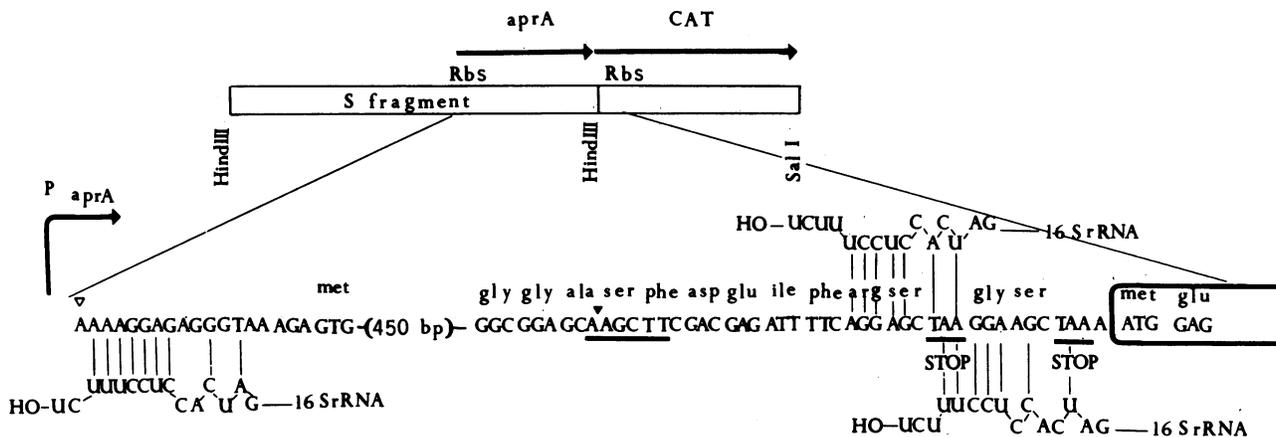


FIG. 1. The ribosome binding sites of the gene fusion containing the N terminus of *B. subtilis* *aprA* gene and the promoterless Tn9-derived CAT gene. Note the 450 bp between the ribosome binding sites of the *aprA* and the CAT genes. The open reading frame of the *aprA* gene ends with two translation termination codons TAA (UAA) in the ribosome binding site of the CAT gene. The 3' terminus of the 16S rRNA of *B. subtilis* is shown to complement the mRNA. Symbols: ▽, the first base to be transcribed upstream of the GTG (Met); ▼, the *Hind*III site between the *aprA* and CAT genes. There were two possible ribosome binding sites for the CAT gene.

RNase A per ml in 0.1 M sodium acetate–0.3 mM EDTA [pH 4.8], heated to 80°C for 10 min), and the mixture was incubated for 10 min at 37°C.

After this treatment, 200 μ l of freshly prepared lysis solution (1% sodium dodecyl sulfate, 0.2 N NaOH) was added and mixed gently by inverting the Eppendorf tubes several times; the tubes were then placed on ice for 5 min. To these tubes 143 μ l of cold 1.5 M potassium acetate (pH 4.8) was added, and the tubes were inverted again several times and placed on ice for at least 20 min. The tubes were then centrifuged at 4°C for 10 min, and the supernatants were carefully removed, mixed with an equal volume of phenol-chloroform (1:1), and centrifuged for 2 min in a Microfuge. The upper phase was then mixed with an equal volume of isopropanol, and the mixture was allowed to stand for 5 min at room temperature. The tubes were centrifuged at room temperature for 5 min, and the supernatants were decanted. The pellets were washed two times with 1 ml of 75% ethanol by centrifugation for 3 min. The DNA pellets were dried at reduced pressure and suspended in 10 μ l of distilled water. The yield of pGR71 derivatives from *B. subtilis* with this method was on the order of several micrograms of DNA.

Polyacrylamide gel electrophoresis. Polyacrylamide gels were used to study and analyze the electrophoretic mobility of CAT made by *B. subtilis* DB104 cells containing various recombinant plasmids as well as that of purified native CAT from *Escherichia coli* HB101 containing pBR328 plasmid (12). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis molecular weight standards (Bio-Rad Laboratories, Richmond, Calif.) were used as markers. Half of the gel was used for transblotting proteins as described below, and the other half was stained with Coomassie blue.

CAT assays. CAT activity was determined spectrophotometrically as described by Shaw (21). In all assays, the change in A_{412} was determined with 10 to 20 μ l of cell extract prepared as described by Goldfarb et al. (6).

Protein concentration assay. Protein concentration was determined by the method of Lowry et al. (15) with bovine serum albumin as the standard protein.

Immunological (Western) blot analysis of CAT. Aminoethoxyphenol paper was prepared by the method of Seed (20) and stored under nitrogen at 4°C in the dark. The procedure for transfer of the protein from the sodium dodecyl sulfate-

polyacrylamide gel to the paper has been described by Towbin et al. (25) and modified by Christmann and Dahmus (3). After transfer, the blot was treated sequentially by a modification of a method described by Hawkes et al. (8). Anti-CAT antibody was obtained from D. Goldfarb (5).

Insertion of the *Bam*HI 10-mer linker. *Bam*HI 10-mer linker was purchased from Boehringer Mannheim Biochemicals, Indianapolis, Ind. The linker was phosphorylated by the procedure described by Maniatis et al. (16). Ligation was done as recommended by Bethesda Research Laboratories, Inc., Gaithersburg, Md., with T4 DNA ligase. All restriction enzymes were purchased from either Bethesda Research Laboratories or Boehringer Mannheim.

RESULTS

Sequence of S-CAT fusion. The analysis of the fusion between the S fragment and the CAT gene (S-CAT fusion gene) in plasmid pGR71 revealed that the open reading frame from the *aprA* gene in the S fragment would continue briefly into the leader region of the CAT gene but would terminate at two translation stop codons (Fig. 1). Therefore, no fusion protein product could result between the S-CAT fusion. The production of the native CAT gene product in this fusion (27) indicated that translation must have initiated at the CAT ribosome binding site. This could have occurred in two possible ways. (i) The fusion between the S fragment and the CAT gene resulted in some favorable sequence or conformation that made the usually inactive CAT ribosome binding site accessible to the *B. subtilis* translation system, or (ii) the translation was initiated from the ribosome binding site of the *aprA* gene in the S fragment, was terminated in the CAT ribosome binding site, and was reinitiated from the CAT ribosome binding site by translational coupling to produce a native-sized CAT.

To distinguish between these two possibilities, we made a deletion within the truncated *aprA* gene, which would result in a translation termination codon far upstream of the CAT ribosome binding site, to see whether this would affect the translation of the CAT gene. For this purpose the *Hind*III S fragment was cloned into plasmid pUBH1 to form plasmid pUBHS1 (Fig. 2) (9). pUBHS1 was cleaved with *Hpa*I to delete a 178-base-pair (bp) fragment from the original S fragment. *Hpa*I cleaved the *aprA* gene 13 amino acids into

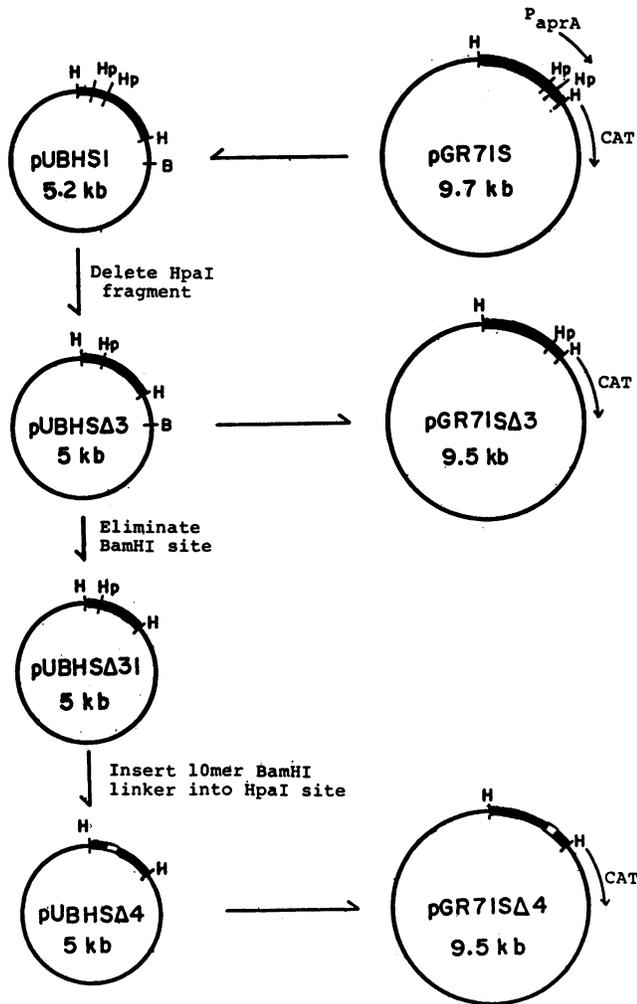


FIG. 2. Construction of the deletion mutants of S fragment. Plasmid pGR71SA3 has a 178-bp *HpaI* deletion within the signal peptide and propeptide regions of *aprA* gene. Plasmid pGR71SA4 has a *BamHI* 10-mer insertion in the deletion site of pGR71SA3. See text and the legend to Fig. 3 for further details on these constructs. Abbreviations: H, *HindIII*; Hp, *HpaI*; B, *BamHI*. Arrows indicate the directions of transcription.

the signal sequence and part of the propeptide of the *aprA* gene (Fig. 3). The plasmid containing the deletion was called pUBHSA3 (9). The *HindIII* SA3 fragment was then transferred back to plasmid pGR71, and this plasmid was called pGR71SA3 (Fig. 2). This plasmid now contained an SA3-CAT fusion gene.

The deletion in the SA3 fragment created a stop codon just 12 codons downstream from the deletion site which led to translation termination. This stop codon was 230 bases upstream from the CAT ribosome binding site (Fig. 3). The analysis of CAT expression in this deletion mutant showed that very little or no expression of the CAT gene occurred (Table 1). This indicated that the CAT ribosome binding site was not being used efficiently in the SA3-CAT fusion and suggested that CAT expression may be dependent on continued translation from the S fragment into the CAT ribosome binding site.

If this were indeed the case, then the insertion of a small 10-mer fragment into the deletion site which restored the

open reading frame and removed the termination codon after the deletion site should again have resulted in translation to the CAT ribosome binding site and in continued translation of the CAT gene into a native-sized product by translational coupling.

The insertion of the 10-mer fragment into the *HpaI* site in pUBHSA3 and the resulting sequence are illustrated in Fig. 3. To construct this insertion, a *BamHI* site in pUBHSA3 was eliminated by cleaving pUBHSA3 with *BamHI* and by filling in the site with the Klenow fragment of DNA polymerase I which resulted in the construction of plasmid pUBHSA31 (Fig. 2). This plasmid was cleaved with *HpaI*, and then the *BamHI* (10-mer) linker was inserted into this site to form plasmid pUBHSA4. The pUBHSA4 plasmid was recut with *BamHI* to make sure that only one copy of the linker was inserted; this was repeated three times (data not shown). The *HindIII* SA4 fragment was cleaved and cloned back into plasmid pGR71 to form plasmid pGR71SA4 (Fig. 2).

The insertion of the 10-mer linker into the *HpaI* site restored the open reading frame and eliminated the translation stop codon in the *aprA* gene. The pGR71SA4-CAT construction was then identical to the native S-CAT fusion except that approximately 170 bp was missing in the signal sequence-propeptide region of the *aprA* gene (Fig. 3).

When CAT activity was tested for the cells containing pGR71SA4, CAT activity was restored by a factor of 17 upon the insertion of the 10-mer linker which restored translation, once again, to the CAT ribosome binding site (Table 1, pGR71SA3 versus pGR71SA4). Most significantly, these data indicate that translation from the upstream *aprA* cistron was necessary for efficient expression of the CAT gene.

Another interesting point concerning these studies is that the activity in the SA4-CAT fusion gene was even higher than the activity of native S-CAT fusion (compare pGR71S versus pGR71SA4 in Table 1). The only difference between these two fusion genes was that the SA4-CAT fusion had fewer bases between the *aprA* and the CAT ribosome binding sites than did the native S-CAT fusion.

Measurement of CAT activity in plasmid constructions. To determine whether the gram-negative CAT ribosome binding site was active in *B. subtilis*, only the promoter for the *aprA* gene was obtained on a suitable fragment by cleaving between the *aprA* promoter and the *aprA* ribosome binding site. The *aprA* promoter placed in plasmid pGR71 would thus transcribe the CAT gene, and any expression would have to occur from translation that was initiated at the gram-negative CAT ribosome binding site. This value would serve as a control value for the expression of CAT from its own gram-negative ribosome binding site in *B. subtilis*. The *HindIII* S fragment (1.25 kilobases) was cleaved with *AhaIII* and *EcoRI* (the *EcoRI* site was 200 bp from the left *HindIII* end, whereas the *AhaIII* site was 480 bp from the right *HindIII* end; the *AhaIII* cleaved precisely between the *aprA* promoter and the *aprA* ribosome binding site [28]). The *EcoRI* site of the 580-bp *EcoRI*-*AhaIII* fragment was subjected to the fill-in reaction with the Klenow fragment of DNA polymerase I. *HindIII* linkers, after a phosphorylation step, were ligated to the ends of the blunt-ended, 580-bp fragment. The resulting fragment was cleaved with excess *HindIII* enzyme, and the fragment was purified by use of a 1% agarose gel. The *HindIII* 580-bp fragment was inserted into the *HindIII* site of plasmid pGR71 to form plasmid pGR71-Papr. This plasmid carried the *HindIII* insert with the *aprA* promoter at its right end in the direction for transcribing the CAT gene. When CAT activity was tested for cells

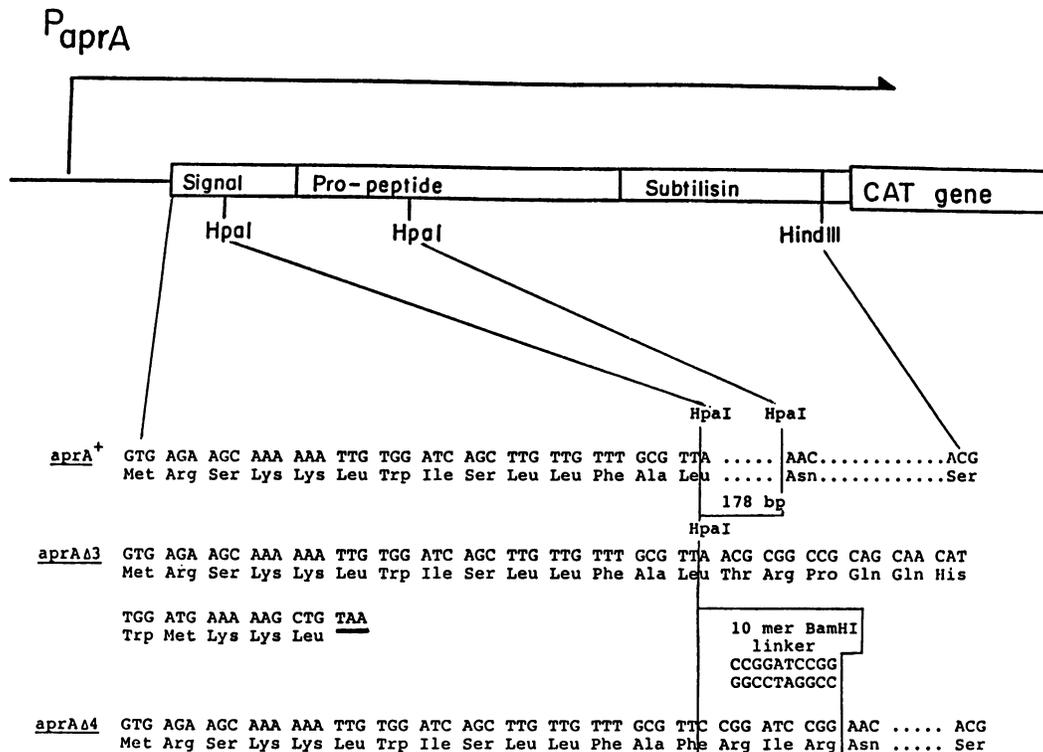


FIG. 3. The sequences of the open reading frames in plasmids pGR71S, pGR71SΔ3, and pGR71SΔ4. The *HpaI* deletion removed 178 bp from the *aprA* gene found in plasmid pGR71S creating plasmid pGR71SΔ3. The deletion created a translation stop codon 12 codons downstream from the deletion site. The 10-mer *BamHI* linker inserted into the *HpaI* site restored the open reading frame and removed the translation stop codon. This allowed translation to proceed to the CAT ribosome binding site and allowed translation termination to occur within the CAT ribosome binding site.

carrying pGR71-Pap_r, little or no activity was detected 3 h after the end of the log phase of growth (*T*₃) (Table 1). Thus, the gram-negative ribosome binding site of the CAT gene was not used efficiently by the *B. subtilis* translation system, and these results confirmed previous findings (6). Since *B. subtilis* DB104 carrying pGR71-Pap_r gave approximately the same specific activity of CAT as *B. subtilis* DB104 carrying pGR71SΔ3, translational readthrough probably did not occur between the *aprA* and CAT genes in the pGR71SΔ3 clone.

In measurements of CAT gene expression, CAT activity was measured during early log, mid log, late log, and at *T*₃. *B. subtilis* DB102 and DB104 (9) were used and gave the same results with recombinant plasmids pGR71, pGR71-Pap_r, pGR71S, pGR71SΔ3, and pGR71SΔ4, indicating that recombination between the S fragment in the pGR71 derivatives and the chromosome was negligible. Since the *aprA* gene on the S fragment is only expressed during the stationary phase (7), no CAT activity was detected during the early-growth phases. Activity values in Table 1 are therefore from *T*₃ cells.

Immunological detection of CAT produced by the recombinant plasmids. By using the immunological (Western) blot method (3, 25) with anti-CAT antibody, we tested extracts of strain DB104 carrying recombinant plasmids pGR71S, pGR71SΔ3, and pGR71SΔ4 for the sizes of their CAT products (Fig.4). All of the clones showed expression of a native-sized CAT product, which confirmed our previous observations (27). Even pGR71SΔ3 produced a small but detectable amount of native-sized CAT, which may have been due to weak utilization of the CAT ribosome binding site in *B. subtilis*.

Effect of high copies of S fragment on sporulation. There are several reports concerning the inhibition of sporulation by high copies of certain *B. subtilis* genes. No inhibition of sporulation was observed for clones containing pGR71S, pGR71SΔ3, or pGR71SΔ4. All of these clones sporulated normally. Thus, the presence of the promoter and N-terminal regions of the *aprA* gene did not inhibit the sporulation process.

DISCUSSION

The results suggest strongly that translational coupling occurred between the truncated *aprA* gene and the Tn9-derived CAT gene, since the Tn9-derived CAT gene ribosome binding site is not utilized efficiently by the *B. subtilis*

TABLE 1. CAT activity of cells containing recombinant plasmids

Plasmid ^a	CAT sp act ^b (nmol of DTNB/min per mg)
pGR71.....	0.0
pGR71-Pap _r	10.8
pGR71S.....	81.9
pGR71SΔ3.....	10.0
pGR71SΔ4.....	178.9

^a Each plasmid was carried by *B. subtilis* DB104 and DB102. No differences were observed in CAT activities when different hosts were used. Cells were harvested at *T*₃.

^b The rate of acetylation by CAT is equal to the chloramphenicol-dependent 5,5-dithiobis-2-nitrobenzoic acid (DTNB) reaction rate (21). Each value is an average of four assays.

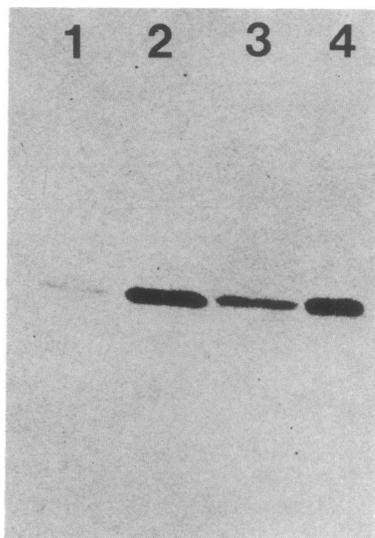


FIG. 4. Immunological identification of the CAT gene products from *B. subtilis* DB104 cells containing plasmids pGR71S Δ 3 (1), pGR71S Δ 4 (2), and pGR71S (3). The control lane (4) contains 1.5 μ g of purified CAT isolated from *E. coli* HB101 cells containing plasmid pBR328. Lanes 1 through 3 contain 100 μ g of total cellular protein from stage T₃ cells containing the recombinant plasmids.

translation system (6). These results suggest that translation initiated at the *B. subtilis* ribosome binding site in the *aprA* gene and terminated within the CAT gene ribosome binding site. The presence of the terminated ribosomes must have facilitated the utilization of the CAT gene ribosome binding site by the *B. subtilis* translation system, since a native-sized CAT was produced. The mechanism underlying the translational coupling of two adjacent genes in an operon is not well understood.

Translational coupling in other systems has been reported where the expression of a distal gene in an operon is dependent on the translation of the proximal gene. In some cases, the translation stop signal of the proximal gene was very close to or overlapped the initiation codon of the distal gene in an operon (1, 2, 4, 17–19). This suggested the possibility that a ribosome could terminate and initiate without leaving the mRNA or that a ribosome could dissociate and rapidly reassociate with the initiation codon of the distal cistron. It has also been suggested that translation coupling occurs by a mechanism in which a translating ribosome rearranges or exposes an otherwise hidden translation initiation signal of the distal gene (17).

In a previous report with a heterologous fusion gene in *B. subtilis*, it was suggested that translational coupling occurred to allow the expression of the gram-negative neomycin phosphotransferase gene (*neo*) from Tn5 (24). Most efficient translational coupling occurred when the translation terminator and initiator codons overlapped, and the reinitiation appeared to be independent of the sequence context and possibly also of ribosomal initiation factors (24). The results in our paper indicate that translation termination and reinitiation occurred within the ribosome binding site of the distal CAT gene; thus, we do not rule out the possibility that the weak CAT ribosome binding site played a significant role in the reinitiation process.

The most likely explanation for the coupling effect in our gene fusion is that ribosomes terminating translation of the

upstream *aprA* cistron in the CAT ribosome binding site reinitiated translation at the CAT ribosome binding site without being released from the mRNA or that the terminating ribosomes were released from the mRNA and led to an increased localization of the ribosomal subunits at the CAT ribosome binding site which overcame the normally poor utilization of this gram-negative ribosome binding site by the *B. subtilis* translation initiation mechanism. Both interpretations are consistent with our results and with the previous results of others (19).

Moreover, the more efficient utilization of a heterologous ribosome binding site which is normally recognized very poorly by the *B. subtilis* translation system was a special feature of this gene fusion. One of us has reported previously on another mechanism by which the CAT gene was utilized by *B. subtilis* (14). In this case, translation was initiated when a tandem duplication or triplication of a 55-bp region that encompassed the CAT ribosome binding site was present. We proposed in that case that a stem and loop structure resulting from the duplication and triplication exposed the ribosome binding site and the start codon AUG in the CAT mRNA in such a manner that duplex formation between the CAT mRNA and the Shine-Dalgarno sequence (22) of the *B. subtilis* 30s ribosomal subunit was facilitated thermodynamically; by combining the free energy of formation of the stem structure with that of the Shine-Dalgarno-mRNA interaction, a stable initiation complex formed between the 30 s ribosome and the mRNA. This type of stem and loop structure has in fact been observed in the ribosome binding region of a *B. subtilis* gene which is expressed efficiently (26). Thus, it appears that different mechanisms may exist for facilitating the interaction of ribosomes and mRNA during initiation of protein synthesis.

The increased expression of the CAT gene in pGR71S Δ 4 was intriguing, since the only sequence differences between this construction and that of pGR71S were the 178 bp missing from the signal and the propeptide regions of the *aprA* gene and the *Bam*HI 10-mer insert. The construction of plasmid pGR71S Δ 4 diminished the distance between the ribosome binding sites of the *aprA* and CAT genes by approximately 170 bp. This enhancement of CAT gene expression in pGR71S Δ 4 may have been the result of a closer ribosome "loading zone" (i.e., the *aprA* ribosome binding site) for the CAT gene or of an elimination of a transcription or translation pausing site in the 170-bp deleted portion of the *aprA* gene. Further studies are necessary to explain this increased rate of CAT expression.

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