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

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The efficient expression in *Escherichia coli* of the Tn*9*-derived chloramphenicol acetyltransferase (EC 2.3.1.28) gene fused distal to the promoter and N terminus of the *Bacillus subtilis aprA* gene was dependent on the initiation of translation from the ribosome-binding site in the *aprA* gene.

Translational coupling (14) in *Escherichia coli* (1, 3, 15, 16) and in *Bacillus subtilis* (18, 23) has been observed. In the usual case of translational coupling, the expression of a distal gene in an operon is dependent on the translation of a proximal gene which has a translation stop codon close to or even overlapping the initiation codon of the distal gene. The exact mechanism of translational coupling is not clearly understood, although several models have been proposed (14).

We found that a inefficiently used gram-negative ribosome-binding site (RBS) could be used much more efficiently in *B. subtilis* if translation of a *B. subtilis-E. coli* fusion mRNA was initiated at the *B. subtilis* RBS and if the translation of the proximal *B. subtilis* gene terminated within the gram-negative RBS of the distal *E. coli* gene (23).

Using the same *B. subtilis-E. coli* gene fusions, we investigated whether translation coupling occurred in *E. coli* and whether the mechanism was similar to that found in *B. subtilis*. Since the distal Tn*9*-derived chloramphenicol acetyltransferase (*cat* [*ptsG*]) gene has a gram-negative RBS (6), the RBS should be used efficiently in *E. coli* and not be dependent on translation of the proximal *B. subtilis* gene unless some type of transcription-translation coupling also occurs.

The *B. subtilis-E. coli* gene fusion was constructed between the N terminus of the *B. subtilis* subtilisin gene (S fragment) (7, 22) and the promoterless Tn*9*-derived *cat* gene to form the shuttle plasmid pGR71S (5, 7, 21, 23). The S fragment contains the promoters, RBS, signal peptide sequence, propeptide sequence, and the N-terminal 49 amino acids of the mature subtilisin protein (22). When this shuttle plasmid was transformed into *E. coli* HB101(λ) (10), the specific activity of chloramphenicol acetyltransferase (CAT) (9, 17) remained constant during all phases of growth (data not shown).

Translation initiation in pGR71S can occur either from the gram-positive RBS of subtilisin and continue into the gram-negative RBS of the *cat* gene in frame with two translation stop codons or from the gram-negative RBS of the *cat* gene (23). The product made from this construction is a native-sized CAT product, and the activity is low when compared with that found in *B. subtilis* (Fig. 1) (23).

To see whether translational coupling was occurring in this gene fusion, we deleted a 178-base-pair (bp) *Hpa*I fragment from the S fragment (pGR71SΔ3), which simultaneously created a translational termination codon 230 bp upstream of the gram-negative RBS of the *cat* gene (23). The presence of this deletion resulted in the lowering of *cat* gene expression by about 50% (Fig. 1, line 2). To test whether this lower activity was the result of disruption of translational coupling, we inserted a 10-base *Bam*HI linker into the *Hpa*I deletion site (pGR71SΔ4), which simultaneously restored the open reading frame, removed the translation stop codon, and allowed the translation to continue to the translation termination codon in the RBS of the *cat* gene (Fig. 1, line 3) (23). When CAT activity was tested, a fivefold higher activity was observed for pGR71SΔ4 than for pGR71SΔ3 (Fig. 1; compare lines 2 and 3).

As a control, a fragment containing only the promoter of the subtilisin gene (*aprA*) was inserted in front of the *cat* gene (pGR71Papr) (23). In this construct, translation could be initiated only from the *cat* gene RBS. The CAT activity from this construction (Fig. 1, line 4) was higher than that found with pGR71S and pGR71SΔ3 but lower than that with pGR71Δ4. In all of the constructs, only native-sized CAT was produced as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (8) and Western blot analysis (data not shown) (23).

To show that the differences in *cat* gene expression were not due to variations in the level of CAT mRNA, we measured the amounts of mRNA being transcribed from the fusion genes with an anti-CAT mRNA probe. The probe was constructed by inserting the *Hin*dIII-*Sal*I *cat* gene (5) into the *Sal*I-*Hin*dIII site of plasmid pSP65 (13), followed by in vitro transcription of this template plasmid with SP6 RNA polymerase (13). As an internal control for the amount of mRNA being made from the constructs, a Km^r mRNA probe (Km^r mRNA is mRNA from the Km^r gene) was used to eliminate differences in plasmid copy number, in mRNA isolation, and in relative decay of different mRNA sizes. The probe was constructed by cloning a 400-bp *Hin*cII-*Bgl*II fragment (this part of the Km^r gene is carried by plasmid pGR71 [5]) in the *Hin*cII-*Bam*HI sites of plasmid pSP65. These probes were used to measure the amounts of CAT mRNA and Km^r mRNA (2, 4, 11, 12, 19) produced by the different pGR71 constructs (23) (Table 1).

The results in the last column of Table 1, which are based on normalized CAT mRNA concentrations, have almost the same relative values as the specific activity data for CAT. These results indicate that transcriptional differences were not the reason for the variations observed in *cat* gene expression. The most significant difference in CAT expression was seen between pGR71SΔ3 and pGR71SΔ4. The translation in pGR71Δ3 is terminated 230 bases upstream of the *cat* RBS, whereas in pGR71SΔ4, the translation is continued to the RBS of the *cat* gene. The only difference

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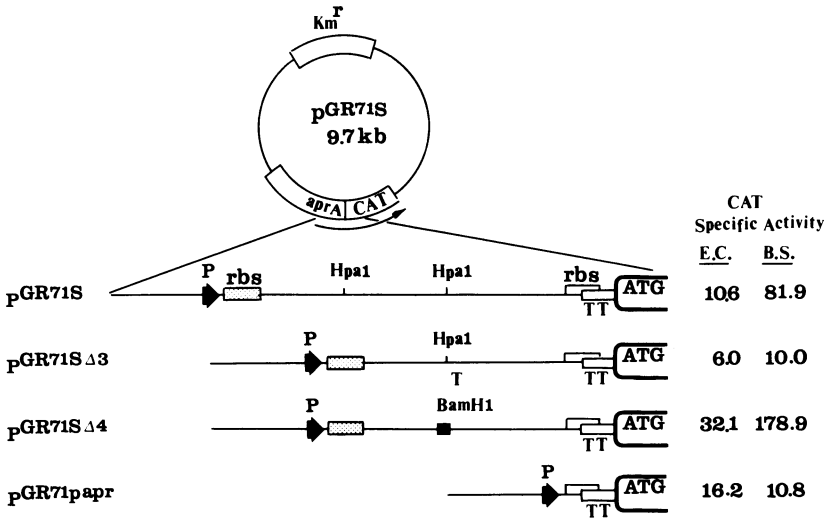

FIG. 1. A schematic representation of the fusion between the N terminus of the *aprA* gene and the *cat* gene present in plasmid pGR71S. The construction of the plasmids has been described by Zaghloul et al. (23). ▭, RBS of *B. subtilis*; ▭, RBS of *E. coli*; TT, translation termination sites in the RBS of the *cat* gene; T, translation termination site resulting from the 178-bp *Hpa*I deletion in pGR71SΔ3; ▬, 10-bp *Bam*HI linker added into the *Hpa*I site, resulting in the elimination of the translational termination site in pGR71SΔ4; ⏵, *aprA* promoter (which is found alone [no *B. subtilis* RBS] in front of the *cat* gene in pGR71Papr). The data for *B. subtilis* CAT-specific activity are from Zaghloul et al. (23). E.C., *E. coli*; B.S., *B. subtilis*.

between these two constructs is the 10-base *Bam*HI insert in pGR71SΔ4, which eliminates the termination codon and restores the open reading frame up to the *cat* RBS. These results strongly suggest that translational coupling, not coupled premature transcription termination, is the major reason for the difference observed between pGR71SΔ3 and pGR71SΔ4.

The three- to fourfold higher activity of pGR71SΔ4 over pGR71S and the mRNA data (Table 1) suggest that the shorter translational distance between the *aprA* and *cat* RBS allows more efficient expression of the *cat* gene. The results do not support the presence of a putative transcriptional pause site in the 168 bp which were deleted.

An enhancing role for multiple RBSs is suggested by the greater CAT activity seen with pGR71SΔ4 than with pGR71Papr, since the pGR71SΔ4 mRNA has both the *aprA* and *cat* RBSs, whereas pGR71Papr mRNA has only the *cat* RBSs.

The CAT activities of the constructs in *E. coli* are lower because *B. subtilis aprA* promoters, which are weakly expressed in *E. coli* (20), control the expression of the heterologous gene fusion.

TABLE 1. Level of CAT expression in *E. coli* cells carrying recombinant plasmids normalized to mRNA content

Plasmid	Relative level of CAT expression				
	Km^r mRNA[a]	CAT mRNA[b]	CAT mRNA/Km^r mRNA	Sp act of CAT	Sp act/ normalized CAT mRNA
pGR71S	2.10	1.36	0.52	10.6	20.4
pGR71SΔ3	1.00	1.00	1.00	6.0	6.0
pGR71SΔ4	2.70	1.00	0.40	32.1	80.2
pGR71Papr	1.04	1.13	1.10	16.1	14.6

[a] Relative level of Km^r mRNA as determined by hybridization to anti-Km^r mRNA probe. Values are relative to that of pGR71SΔ3.

[b] Values derived as described in footnote *a*, except hybridization was to anti-CAT mRNA probe.

Our results indicated that although translation from the gram-negative RBS did occur and a native-sized product was synthesized as expected, the efficiency of translation was greater if translation was initiated from the RBS of the upstream *B. subtilis* gene and if the ribosomes terminated translation in the gram-negative RBS of the *cat* gene. Thus, translational coupling is necessary for efficient expression of the *cat* gene in the heterologous fusion even in the gram-negative host.

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