In Vitro Expression of a Tn9-Derived Chloramphenicol Acetyltransferase Gene Fusion by Using a Bacillus subtilis System

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A coupled in vitro protein-synthesizing system has been developed with components derived totally from Bacillus subtilis. The system synthesizes specific gene products from various exogenous DNA templates, including B. subtilis phage φ29, plasmid pUB110, and a heterologous B. subtilis-Escherichia coli gene fusion containing the transposon Tn9-derived chloramphenicol acetyltransferase (cat) gene. The gene fusion product was able to show CAT activity, bind specifically to a Sephacryl-chloramphenicol column, and react immunologically against anti-CAT antiserum. The fidelity of this in vitro system was demonstrated by the synthesis of gene products identical to that made in vivo. We suggest that this system may be used to study the regulation of gene expression in vitro.

To study the regulation of gene expression in Bacillus subtilis, it is desirable to develop a coupled in vitro protein-synthesizing system derived totally from this organism. The differences in promoters, transcriptional apparatus, transcriptional regulatory mechanisms (7), and translation signals and machinery (19, 26, 32, 30, 31) between gram-positive and gram-negative organisms invalidate the use of the Escherichia coli or any other heterologous in vitro protein-synthesizing system in the analysis of gene expression in B. subtilis.

Thus, although several in vitro protein-synthesizing systems have been described for several Bacillus species (3, 6, 13, 18, 20), these systems used mainly synthetic, endogenous, or exogenous RNA as the translational template. These systems also used several components or fractions derived from E. coli, which could not only complicate the interpretation of the results but also lead to faulty conclusions.

To overcome these difficulties, we have developed a DNA-directed cell-free protein-synthesizing system that is derived totally from B. subtilis. This system contains readily prepared components, takes advantage of added RNA polymerase and tRNA prepared from B. subtilis, and is effective in terms of rate and specificity of synthesis.

This system was able to synthesize an active enzyme by use of one of the transposon Tn9-derived chloramphenicol acetyltransferase (cat) gene fusions, pGR71-43, as the DNA template (9, 10). Furthermore, the product was characterized by its electrophoretic mobility on polyacrylamide gels, its binding characteristics to a specific affinity column, and its interaction with anti-CAT antiserum.

MATERIALS AND METHODS

Bacterial strains and media. B. subtilis 168 cells grown in superrich medium (11) were used to prepare the components for the in vitro system. E. coli HB101 cells carrying plasmid pBR328 were used to purify CAT (28).

Preparation of nucleic acids. Plasmid pGR71 and its derivatives were purified by the procedure described by Lovett and Keggins (21). Phage φ29 was purified by the methods of Kawamura and Ito (15) and Harding and Ito (12). The phage φ29 DNA was purified by the procedures reported by Ito et al. (14). The tRNA was prepared from B. subtilis DB1 by the method of Ehrenstein and Lipmann (8).

Removal of proteases from cell extracts. The hemoglobin-Sepharose affinity column was made by the methods of March et al. (23) and Chua and Bishuk (4) and used to remove protease activity from crude B. subtilis extracts as described by Nakayama et al. (24).

Purification of RNA polymerase. The RNA polymerase carrying the σ70 factor was purified from B. subtilis W168 vegetative cells grown on rich medium to mid-logarithmic phase by the procedure reported by Halling et al. (13).

Preparation of S150 and unwashed polyribosomes. B. subtilis 168 cells were grown in superrich medium (13) at 37°C to mid-log phase. The cells (20 g, wet weight) were suspended in 20 ml of buffer A (10 mM Tris hydrochloride, pH 7.5, 10 mM MgCl₂, 60 mM NH₄Cl, 5 mM EDTA, 5 mM mercaptoethanol, 10% glycercer, 2 mM phenylmethylsulfonyl fluoride, and 0.2 mM diisopropylfluorophosphate [DFP]). Cells were broken by passage through a French press three times at 18,000 lb/in² at 4°C. Hemoglobin-Sepharose (5 g) was added to the crude extract; the mixture was stirred at 4°C for 30 min and then centrifuged at 15,000 × g for 10 min. The supernatant was mixed again with 5 g of fresh hemoglobin-Sepharose, stirred as above, and then centrifuged for 10 min at 15,000 × g. The supernatant was then centrifuged twice at 30,000 × g for 30 min each at 4°C. The upper third of the supernatant was carefully removed and centrifuged immediately at 150,000 × g for 5 h at 4°C. The upper one-third of this supernatant (S150) was dialyzed against buffer A for 4 h at 4°C. The pellets from the above centrifugations were washed several times with small amounts of buffer A and then suspended in a small volume of the same buffer. This suspension was named the unwashed polyribosome fraction.

Coupled in vitro protein-synthesizing system. The final reaction mixture of 125 μl contained 75 mM Tris-acetate buffer, pH 7.9, 15 mM magnesium acetate, 80 mM ammonium acetate, 50 mM potassium acetate, 3 mM ATP, 0.5 mM each GTP, CTP, and UTP, 2 mM dihydrothreitol, 0.2 mM DFP, 0.1 mM folic acid, 20 μg of pyruvate kinase, 10 mM phosphoenolpyruvate, a mixture of 19 amino acids (without methionine) at 0.1 mM each, 0.02 mM unlabeled methionine, 0.04 μCi of [³⁵S]methionine, 5 μg of DNA (exogenous DNA

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template), 20 μg of *B. subtilis* tRNA, and 12 pmol of *B. subtilis* RNA polymerase carrying σ^45^.

A part of the reaction mixture was preincubated at 30°C for 15 min to minimize the endogenous mRNA template. This preincubation reaction mixture contained, in a volume of 60 μl, 40 mM Tris acetate buffer, pH 7.9, 15 mM magnesium acetate, 80 mM ammonium acetate, 1.5 mM dithiothreitol, 0.2 mM DFP, a mixture of amino acids without methionine at 0.04 mM each, 0.05 mM unlabeled methionine, 300 μg of S150, and 150 μg of unwashed polyribosomes. After the preincubation the mixture was placed on ice, and the rest of the reaction components were added immediately. The final reaction mixture was incubated at 37°C for the times indicated in the text.

**Protein assay.** Protein concentration was determined by the method of Lowry et al. (22) with bovine serum albumin as the standard protein.

**Polyacrylamide gel electrophoresis of in vitro-synthesized proteins.** Reactions for protein synthesis were carried out as described above but with 8 μCi of [35S]methionine. The final reaction mixtures were incubated at 37°C for 1 h, after which they were immediately placed on ice. A 25-μl sample was removed from each reaction mixture to check the incorporation, and sample volumes corresponding to equal numbers of acid-precipitable counts from the reactions with and without DNA were precipitated by the addition of 1 ml of cold acetone. The pellets were dried under low pressure and then suspended in 40 μl of water, and approximately 12 μl of 5× sample application buffer was added to each sample. A 1× sample application buffer contained 60 mM Tris hydrochloride, pH 6.8, 2% (wt/vol) sodium dodecyl sulfate, 10% (vol/vol) β-mercaptoethanol, and 0.001% (wt/vol) bromophenol blue. Samples were boiled for 5 min and then applied to 15% polyacrylamide gels made by the method of Laemmli (16) along with molecular weight standards (Bio-Rad kit). The gels were electrophoresed for 4.5 h, after which each gel was cut vertically into two pieces, the piece containing the standards was soaked in 0.1% Coomassie blue in 50% trichloroacetic acid (TCA) for 2 h and then destained overnight. The other gel piece was soaked in 50% TCA for 45 min and then treated as described by Bonner and Laskey (1) and Laskey (17) to visualize the newly synthesized products.

**Radioisotope method to assay for CAT activity.** The procedure of Shaw (28) was used to measure CAT activity.

**Affinity binding of in vitro-synthesized CAT to a Sephacryl- CM resin.** Methods reported by Zaidenzaig and Shaw (34) and Packman and Shaw (25) were used to synthesize the Sephacryl-chloramphenicol (Sephacryl-CM) resin and to bind the fusion CAT to the resin (T. I. Zaghoul, Ph.D. thesis, University of California, Davis, 1986).

**CAT antiserum.** Rabbit anti-CAT antiserum was kindly provided by David S. Goldfarb.

**Western immunological blot.** The Western blot was carried out as reported by Zaghoul et al. (33) to detect the in vitro-synthesized proteins.

**RESULTS**

**Development of a coupled cell-free protein-synthesizing system from *B. subtilis*.** During the development of the coupled cell-free protein-synthesizing system described in Materials and Methods, we tested the concentrations of several key components to maximize the synthetic capacity of the system. The [35S]methionine incorporation increased linearly when 100 to 300 μg of fraction S150 was added to the 125-μl reaction mixture. Maximum incorporation occurred at 300 μg per 125-μl reaction mixture.

The concentration of Mg^{2+} was critical. One has to consider the Mg^{2+} concentration in the polyribosome, S150, DNA, and any other components that are added to the reaction mixture. The optimum concentration for Mg^{2+} was 15 mM, with higher concentrations causing severe inhibition of protein synthesis.

The optimum concentration of DNA depended on the source of the exogenous DNA. For φ29 DNA, only 2 μg per 125-μl reaction mixture was sufficient; more than 6 μg resulted in inhibition of incorporation. For plasmid pGR71-43 DNA, 8 μg was optimum.

To minimize the effects of unlabeled methionine on incorporation, use of 0.03 mM at the preincubation stage and 0.025 mM during the final stage gave optimum results. At these concentrations [35S]methionine incorporation was specific and in reasonable and measurable amounts, e.g., 20 cpm/pmol of methionine incorporated.

**In vitro synthesis of φ29 early gene products.** When φ29 DNA was used as the exogenous DNA, three major products corresponding to 22.0-, 15.0-, and 10.5-kilodalton (kDa) proteins were synthesized (Fig. 1). Three minor bands of 30.0, 26.0, and 13.0 KDa were also apparent. The 13.0-kDa band corresponded electrophoretically to the 13-kDa gene 4 protein. None of these products were made in the absence of φ29 DNA template.

**In vitro synthesis of fusion CAT product encoded by plasmid pGR71-43.** Plasmid pGR71-43 is a derivative of the promoter probe plasmid pGR71 (9, 10) and has an HindIII (1.5 kilobases [kb]) insert called 43 that was isolated from the *B. subtilis* chromosome fused to the *cat* gene present in pGR71 (9). The right one-third of this fragment (the end fused with the *cat* gene) was sequenced by Wang and Doi (32) and contains two overlapping promoters transcribed by σ^45^ and σ^37^ RNA polymerase holoenzymes during growth and the early stationary phase, respectively (32). The N-
terminal region of the fusion CAT (made in vivo) was sequenced by Goldfarb et al. (10).

Plasmid pGR71-43 was used as the exogenous DNA template to see whether this in vitro system would be able to synthesize the fusion CAT protein. Reaction mixtures of 250 μl each were used as described in Materials and Methods. The test reaction mixture contained 16 μg of pGR71-43 DNA, while the control reaction did not contain any exogenous template. The reaction mixture containing pGR71-43 template synthesized several products. One major product corresponding to a protein of 27 kDa had the same electrophoretic mobility as the in vivo-synthesized fusion CAT product reported by Goldfarb et al. (10) (Fig. 2).

A second product corresponding to a protein of 36 kDa had the same electrophoretic mobility as the kanamycin nucleotidyltransferase enzyme encoded in plasmid pUB110. This was not unexpected, since the entire pUB110 plasmid is part of pGR71-43 (9). A third major product of 11 kDa encoded by pUB110 was evident. In addition, two minor products appeared at 25 and 26 kDa (Fig. 2) that were encoded by pUB110 (29).

Identification and characterization of the in vitro-synthesized fusion CAT. Several additional experiments were carried out to confirm that the 27-kDa product was actually the fusion CAT product. Reaction mixtures containing either pGR71, pGR71-43, or no exogenous DNA were assayed for CAT activity by the radioisotope method. The reaction mixture containing pGR71-43 DNA had significantly higher CAT activity (3,312 cpm) than those containing pGR71 or no exogenous DNA (175 and 0 cpm, respectively). Similar results were obtained when pUB110 plasmid was used as the template for the synthesis of kanamycin nucleotidyltransferase (data not shown). CAT activity was also determined by the rate of acetylation of 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) the results with pGR71-43, pGR71, and no exogenous DNA were 0.95, 0.05, and 0 nmol of DTNB acetylated per min, respectively.

Since CM is a substrate for CAT, the Sephacryl-CM resin binds CAT or CAT derivatives (25, 28, 34). When the reaction contained pGR71-43 as the template, a protein was synthesized that was capable of binding to the Sephacryl-CM resin. This protein had a slower electrophoretic mobility than native CAT and had a mass of 27 kDa (Fig. 3). This is the expected mobility and mass for the fusion CAT product. The reaction mixtures containing pGR71 or no exogenous DNA did not produce any proteins that were capable of binding to the Sephacryl-CM resin.

Identification and characterization of the in vitro-synthesized fusion CAT. Reaction mixtures contained pGR71 DNA (lane A), pGR71-43 DNA (lane B), or no exogenous DNA (lane C). The products were bound to the Sephacryl-CM column, eluted from the column, and then analyzed by gel electrophoresis. The solid arrowhead points to the fusion CAT, and the open arrowhead indicates the normal location of native CAT (no band appears at this location, since native CAT was not synthesized in these experiments).

Identification of the in vitro-synthesized fusion CAT by the Western blot technique. Anti-CAT antiserum was used to identify the fusion CAT product. The reaction mixtures contained pGR71 DNA (lane A), pGR71-43 DNA (lane B), or no exogenous DNA (lane C). The solid arrowhead points to the fusion CAT product. The open arrowhead points to the usual location of native CAT.
The product from the reaction mixture containing pGR71-43 reacted immunologically with anti-CAT antiserum. This product also migrated more slowly electrophoretically than native CAT, at the 27-kDa position (Fig. 4). No immunologically reacting products were synthesized when pGR71 or no exogenous DNA was present in the reactions.

**DISCUSSION**

A DNA-directed cell-free protein-synthesizing system was developed for studying the expression of *B. subtilis* genes. It differs from previous *Bacillus* systems in several significant ways. This system is fully derived from *B. subtilis* cells; it contains components that are readily prepared without using DNase I; it takes advantage of added RNA polymerase $\sigma^B$ holoenzyme and tRNA; it is very effective in terms of the net amounts of synthesis, because of the removal of proteases in the crude extract; it has the ability to synthesize catalytically active proteins; and it has the fidelity to yield proteins similar to those made in vivo.

Several different exogenous DNA templates were used to direct the synthesis of specific gene products. The $\phi 29$ DNA directed the synthesis of products that corresponded to in vitro (5) and in vivo (2) products of the early genes. Highly purified plasmid pUB110 DNA directed the synthesis of kanamycin nucleotidyltransferase enzyme (data not reported). Plasmid pGR71-43 DNA promoted the synthesis of a protein with a mass of 27 kDa and an electrophoretic mobility identical to that of the fusion CAT protein observed in vivo (10). The difference between the fusion CAT product and the native CAT is 21 amino acids, i.e., 8 amino acids (the N-terminal fusion peptide) fused in translational phase upstream of CAT, plus 13 amino acids coded by the CAT leader region (Fig. 5) (10). The size of the in vitro-synthesized product is in agreement with the in vivo results reported previously. Native CAT was not observed as a product when plasmid pGR71-43 was used as the template. This indicates that the *B. subtilis* ribosomes were either not able to efficiently use the *E. coli* ribosome-binding site (RBS) of the cat gene (Fig. 5) or that translational occlusion occurred from efficient use of the upstream *B. subtilis* RBS in insert 43. The in vivo results suggest that the *cat* gene RBS is not used efficiently (10, 33).

Zaghoul et al. (33) showed that the gram-negative rbs of the Tn9-derived cat gene, in another heterologous gene fusion (pGR71S), was utilized in vivo very weakly or not at all by the translational machinery of *B. subtilis*. However, the same RBS was fully utilized by the *B. subtilis* translational machinery when translation was initiated from a *B. subtilis* RBS upstream of the gram-negative RBS by a translational coupling mechanism (33). Thus, under very specific conditions, gram-negative RBSs can be utilized efficiently by the gram-positive translation machinery.

The development of this system occurs at a propitious time, since a number of regulated *B. subtilis* genes have been cloned recently and this system should allow an analysis of their regulatory mechanisms.

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**LITERATURE CITED**