Expression of the sacPA and sacB genes of Bacillus subtilis is positively modulated by transcriptional regulatory proteins encoded by the sacT and sacY genes, respectively. Previous genetic studies led to the suggestion that SacT and SacY function as nascent mRNA binding proteins preventing early termination of transcription at terminators located in the leader regions of the corresponding genes. Here we report the overproduction, purification to near homogeneity, and characterization of the two antiterminators, SacT and SacY. Using mRNA band migration retardation assays and a reconstituted transcriptional antitermination system, the mRNA binding functions and antitermination activities of purified SacT and SacY are demonstrated under in vitro conditions. The results establish for the first time that members of the BglG family of antiterminators function in antitermination in the absence of other proteins in vitro. Purified SacT is shown to be phosphorylated by phosphoenolpyruvate in a phosphotransferase-catalyzed reaction dependent on Enzyme I and HPr. Unexpectedly, the purified SacT is shown to be functional in mRNA binding and in transcriptional antitermination independently of its phosphorylation state.

Gene expression is generally regulated at two different levels, control of transcriptional initiation and control of transcriptional termination. In bacteria, termination-antitermination controls viral development (Ptashne, 1992), amino acid biosynthesis (Babitzke et al., 1995), tRNA synthase production (Henkin, 1994), ribosomal RNA and protein production (Heinrich et al., 1995), and carbohydrate utilization (Amster-Choder and Wright, 1993). Moreover, RNA-protein interactions are proving important for numerous biological processes such as intron splicing, enzyme catalysis, and protein synthesis and secretion (Béfourt et al., 1995; Wiedmann et al., 1994; Nagai and Mattaj, 1994).

Induction of the sac (sucrose utilization) genes of Bacillus subtilis and the bgl (β-glucoside utilization) operon of Escherichia coli is controlled by transcriptional antitermination (Cruzzet et al., 1990; Débarbouillé et al., 1990; Amster-Choder and Wright, 1993). Within the B. subtilis sac system is the sacB gene encoding levansucrase and the sacA operon encoding the sucrose permease and a phosphoehosphorase. In these systems, transcription is initiated at constitutive promoters, and expression is regulated by controlled read-through at transcriptional terminators located between the promoters and the first structural genes of the operons. Gene-specific antiterminators are required to prevent early transcriptional termination.

Three genes within the bgl operon of E. coli, bglG, bglF, and bglB, are involved in aromatic β-glucoside utilization (Schnetz et al., 1987; Schnetz and Rak, 1988). BglG is a positive regulator that recognizes a specific RNA sequence located upstream of the terminator of the bgl operon. Binding of BglG to a specific secondary structural element on the bgl mRNA prevents transcriptional termination by blocking the formation of a terminator (Houman et al., 1990). BglF, the β-glucoside permease, is an Enzyme I of the phosphoenolpyruvatesugar phosphotransferase system (PTS) with a IIABC domain structure (Saier and Reizer, 1992). It also functions as a negative transcriptional regulator, controlling the activity of BglG by phosphorylation-dephosphorylation in response to the external level of inducer. The degree of phosphorylation of BglG in vivo is believed to be dependent on the cellular concentration of BglF, which serves as the direct phosphoryl donor and acceptor in the antitermination kinase-phosphatase-catalyzed reactions. The antiterminator is active in the nonphosphorylated state because this form of the protein exhibits a dimeric structure while the phosphorylated form is monomeric and therefore inactive (Amster-Choder et al., 1989; Amster-Choder and Wright, 1992).

In B. subtilis two regulatory genes, sacT and sacY, encode proteins that respectively control transcription of the sacPA and sacB genes. Their protein products are homologous to BglG and apparently function by an analogous antitermination mechanism (Houman et al., 1990; Débarbouillé et al., 1990; Aymerich and Steinmetz, 1992). The PTS has been shown to regulate both the sacPA and sacB genes in vivo, and unphosphorylated SacY appears to be responsible for high level expression of these genes in mutants lacking Enzyme I (pstI) or HPr (pstH). Conversely, in pstH and pstI mutants, SacT is nonfunctional, indicating that phosphorylation is required for its activity (Arnaud et al., 1992).

On the basis of these observations, we have postulated that SacT may possess two phosphorylation sites (Arnaud et al., 1992). HPr, the second phosphocarrier protein of the PTS, is phosphorylated by Enzyme I, and HPr(His−P) possibly phosphorylates the antiterminator SacT on one site as a prerequisite for its activity. Then, if SacT is phosphorylated at a second site in the absence of sucrose, inactivation may result as pro-

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‡ To whom correspondence should be sent. Fax: 33-1-45688938; E-mail: mdebarbo@pasteur.fr.
posed for BglG and SacY (Schnetz and Rak, 1990; Amster-Choder et al., 1989; Crutz et al., 1990). This second phosphorylation event would depend on the presence of an as yet unidentified sucrose sensor protein, functionally similar to the sucrose-specific permease of the PTS. In the presence of sucrose, only this second site in SacT would become dephosphorylated, and SacT would thereby be activated when sucrose is translocated into the cell. Alternative mechanisms have been proposed (Arnaud et al., 1992).

Several transcriptional regulatory proteins homologous to BglG, SacT, and SacY have been identified. These proteins include AbgG of Clostridium ljungdahlii (GenBank accession number L49336), BlgR of Lactococcus lactis (Bardowski, 1994), LicT from B. subtilis (Schnetz et al., 1996), Arbg from Erwinia chrysanthemi (El Hassouni et al., 1992), the multidomain proteins of B. subtilis, CelR (SWISSPROT identifier P46321), and LevR (Débarbouillé et al., 1991), each which contains a BglG/SacT-like module. Levr, like BglG of E. coli, has been shown to be phosphorylated by PEP in a PTS-catalyzed reaction (Stülke et al., 1995). Nevertheless, none of these proteins has been purified and used in vitro to demonstrate its antitermination activity.

In this report we describe the overproduction, purification, and characterization of SacT and SacY. We showed that purified SacT and SacY bind to a specific sacPA mRNA sequence, and we describe a reconstituted SacY- or SacT-dependent transcriptional antitermination system which is functional in vitro. Finally, we demonstrate that SacT is phosphorylated by PEP in a process that depends only on Enzyme I and HPr as previously deduced from genetic studies. Altogether, the data presented herein serve to establish that both SacT and SacY serve as transcriptional antiterminators in the absence of auxiliary proteins.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Plasmids, Growth Conditions, and Preparation of Cell Extracts—**Intermediate stages of plasmid cloning and routine plasmid DNA propagation were carried out in E. coli strains TG1 (F' trdA36 lacI4ΔlacZM15 proA-1 thi/ supE44 hsdM-mcrB15 rpsL-15 leuB6) and BL21 (F-ompT hsdS-de3 recA1) (Codexis, CA). Purification of HPr (Thoma et al., 1989) was performed essentially as described previously (Sutrina et al., 1992). Bacterial strain TG1 or BL21 were grown in LB medium (at 37°C) with 50 μg/mL ampicillin and incubation was then continued for an additional 3 h. Cells were then harvested by centrifugation, washed twice with 20 mM Tris-HCl buffer, pH 7.9, containing 10 mM EDTA, 5 mM MgCl2, and 2 mM dithiothreitol, and finally incubated at 0°C for 10 min. Glass-distilled glycerol (37%) was added to the bacterial pellets to a final concentration of 20%, and bacteria were stored at −70°C until used.

**In Vitro RNA Synthesis—**The two plasmids, pPA2 and pPA3, which code for the sacPA promoter region (see Fig. 3), were used as templates for RNA probe synthesis. The RNA probes were linearized with EcoR1 and HindIII, respectively, and extracted with phenol before conducting in vitro RNA synthesis experiments. RNA synthesis was performed using the paired promoter T7 system kit (Amersham Corp.) following the manufacturer’s instructions. Transcription mixtures used for probe synthesis contained 100 μCi of α-[35S]UTP and 12.5 μM UTP. The RNA probes were purified after migration on 8% denaturing polyacrylamide gels and resuspended in 10 mM Tris, pH 7.5, containing 1 mM EDTA and 25 μg/ml yeast tRNA.

**Phosphorylation of SacT—**Assay mixtures for the PTS-dependent phosphorylation of SacT contained, unless otherwise indicated, 4 μg of SacT, 2 μg of HPr, 1.6 μg of Enzyme I, and 12.5 mM MgCl2. The final volume of 20 μl of binding buffer (10 mM HEPES, pH 7.6, containing 1 mM EDTA, 5 mM MgCl2, and 2 μM dithiothreitol) was adjusted to 10 μM Tris, pH 7. For phosphorylation reactions, the reaction mixtures were incubated at 80°C for 3 min and transferred to an ice bath. They were used within a few minutes for binding assays.

**RNA Binding Assays—**The RNA transcripts (100 μg/ml in 5 μl of binding buffer) with 1 unit of human placenta Factor Xa were incubated with 2 μl of sacT containing 50 pmol of labeled RNA. The reaction mixtures were incubated at 80°C for 3 min and transferred to an ice bath. The native or phosphorylated SacT or SacY (in 10 μl of phosphorylation reaction mixture) was mixed with the RNA probes at 4°C, incubated at 16°C for 10 min, and finally incubated at 0°C for 10 min. Glass-distilled glycerol (final concentration 10%, Eastman Kodak Co.) was added before loading the samples onto an 8% nondenaturing polyacrylamide gel with a cross-linking ratio of 29:1. The gels were run for 2 h at 4°C. Electrophoresis for 2 h at 4°C was carried out at 300 V using 0.25 μM

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SacT and BamHI sites of pAM18. The Xmn-I-Sau3A DNA fragment was isolated from plasmid pBSG8-34. In this plasmid, the original Sau3A restriction site was replaced by a BamHI site (Arnaud et al., 1992).

Previously described growth conditions of E. coli MZ1 containing the appropriate pRE1 derived overexpression vector were used to overproduce SacT, SacY, the PTS proteins, Enzyme I and HPr, and the IAA⁺-binding protein domain of B. subtilis (Sutrina et al., 1990). For overproduction of SacT and SacY in E. coli BL21(DE3) bearing the overexpression vector pET19b-T1 or pET19b-Y1, 1 mM isopropyl-1-thio-β-D-galactopyranoside was added at A₅₀₀ of 0.4–0.7 to cultures grown in LB medium (at 37°C), and incubation was then continued for an additional 3 h. Cells were then harvested by centrifugation, washed twice with 200 mM Tris-HCl buffer containing 0.1 mM phenylmethylsulfonyl fluoride and 10% glycerol (TPG buffer), and ruptured by two passages through an Aminoxy French pressure cell at 10,000 p.s.i.

**Protein Purification—**Initial attempts to purify SacT and SacY from E. coli MZ1 containing pRE1-T1 or pRE1-Y1, respectively, resulted in precipitation of the partially purified antiterminators. Precipitation usually occurred during or after concentration by ultrafiltration (YM10, Amicon, Inc.) of the protein preparations that had been partially purified by ion-exchange chromatography using a DEAE-Sephasil column (see below). Because a similar concentrating step was not required during purification of the two antiterminators from extracts of E. coli BL21(DE3) bearing the pET19b-derived plasmids, subsequent purification of these proteins was carried out following overproduction of SacT and SacY in E. coli BL21(DE3) bearing pET19b-T1 and pET19b-Y1, respectively. The following purification protocol was applied to both SacT and SacY. A crude extract derived from cells grown in 6–8 liters of culture medium was loaded onto a column of DEAE-Sephasil (200 ml bed volume) which had been pre-equilibrated with TPG buffer. The column was washed with 600 ml of the same buffer, and proteins were then eluted with a linear salt concentration gradient (2000 ml of 0–1 M NaCl) in TPG buffer. Fractions (15 ml) were collected and assayed by SDS-polyacrylamide gel electrophoresis for the presence of SacT or SacY which eluted as a broad peak at about 0.35–1 M NaCl. Fractions containing His-tagged SacT or His-tagged SacY were pooled and loaded onto an immobilized Ni⁺⁺ column (His-Bind resin, Novagen) pre-equilibrated with 20 mM Tris-HCl buffer, pH 7.9, containing 0.5 M NaCl, 10% glycerol, and 5 mM imidazole. The column was washed with 20 ml of Tris-HCl buffer, pH 7.9, containing 0.5 M NaCl, 10% glycerol, and 60 mM imidazole, and the His-tagged antiterminator was eluted with the same buffer with the imidazole concentration increased to 1 M. The purified proteins were then dialyzed against 50 mM Tris-HCl buffer, pH 8.5, containing 20% glycerol, 0.5 mM EDTA, and 0.5 M NaCl and stored at −70°C until used.

Enzyme I, HPr, and the recombinant IIA graft protein domain of the B. subtilis Enzyme I subunit were overproduced and purified as described previously (Reizer et al., 1989, 1992; Sutrina et al., 1990).
and the amounts of proteins as follows: crude extracts, 33 and 35 μg; lanes 1 and 5, respectively; DEAE-Sephadex pools, 13 and 11 μg, lanes 2 and 6, respectively; His-Bind resin pools, 2.8 and 3 μg, lanes 3 and 7, respectively; His-Bind resin pool 7 and 6 μg, lanes 4 and 8, respectively. B, mobility of purified SacT (8 μg) in SDS-sample buffer lacking a reducing agent (lane 1), in SDS-sample buffer containing 1 mM dithiothreitol (lane 2), in 6 mM dithiothreitol (lane 3), and in 10 mM dithiothreitol (lane 4). Positions of molecular mass markers (in kilodaltons) are indicated on each panel.

Tris-HCl containing 0.2 mM glycine as running buffer.

In Vitro Transcription Assay—Transcription was assayed in a reaction mixture containing 40 mM Tris-HCl, pH 7.9, 10 mM MgCl₂, 100 mM KCl, 1 mM dithiothreitol, 5 mM template DNA, 0.2 mM UTP, and 5 nM template DNA, 0.2 M glycine as running buffer. Assay mixtures were then incubated at 65°C for 10 min and loaded onto a 7% polyacrylamide sequencing gel (Sanger and Coulson, 1978). Radioactivity in bands was quantified using a PhosphorImager (Molecular Dynamics).

Other Methods—Polyacrylamide gel electrophoresis (SDS-polyacrylamide gel electrophoresis) was performed with a Pharmacia Phast System or as described before (Reizer et al., 1983). Proteins labeled with [³²P]IPEP were separated by SDS-polyacrylamide gel electrophoresis and detected by autoradiography as described previously (Reizer et al., 1989). Protein was determined by the Lowry method (Lowry et al., 1951) with bovine serum albumin as the standard protein.

RESULTS

Overproduction and Purification of SacT and SacY—As shown in Fig. 1A, the overproduced SacY or SacT represents the major protein band in an extract of E. coli BL21(DE3) bearing pET19b-Y1 or pET19b-T1, respectively. Following a two-step purification protocol that consisted of (a) ion-exchange chromatography on a DEAE-Sephadex column and (b) affinity chromatography of the His-tagged proteins on a His-Bind metal (e.g. Ni²⁺) chelation resin (see “Experimental Procedures”) nearly electrophoretically pure preparations of SacT and SacY were obtained (Fig. 1A). The single faint band that migrated faster than SacT or SacY is most likely a proteolytic product of the major protein band, since its mobility relative to SacT remained unchanged under modified polyacrylamide gel electrophoresis (Fig. 1B), and it was phosphorylated by the PTS as was SacT (see below). The purified SacY® migrated on SDS-polyacrylamide gels as a band with an apparent molecular mass of ~34,000 Da, in agreement with the molecular mass of 32,465 Da calculated from the deduced amino acid sequence of the protein. Interestingly, purified SacT migrated on SDS-polyacrylamide gels as a band with an apparent molecular mass of ~30,000 Da, although the molecular mass of SacT calculated from the deduced amino acid sequence (32,074 Da) is similar to that of SacY. One additional prominent band, corresponding to the homodimeric form of SacT (~68,000 Da), was detected in SDS-polyacrylamide gels if the purified SacT protein was not boiled before loading the gels and the reducing agent β-mercaptoethanol was omitted from the sample buffer (Fig. 1B). At increasing concentrations of dithiothreitol (1–10 mM) in the sample buffer the amounts of monomeric SacT gradually increased at the expense of its homodimeric form (Fig. 1B). These findings provide evidence for a dimeric SacT.

PTS-dependent Phosphorylation of SacT—An autoradiogram showing the phosphorylation of SacT by purified Enzyme I and HPr in the presence of [³²P]IPEP is shown in Fig. 2. In lane 1, the purified Enzyme I, HPr, and IIA Glc of B. subtilis were incubated with [³²P]IPEP, and only these three proteins were labeled. In lane 2, Enzyme I and SacT were incubated with [³²P]IPEP, and only the former protein was labeled. By contrast, SacT was readily phosphorylated when incubated in the presence of Enzyme I, HPr, and [³²P]IPEP (lane 3). As
expected for a histidyl or cysteyl phosphorylated protein, the $^{32}$P-labeled SacT was labile under acidic conditions but stable under alkaline conditions (data not shown). These observations establish that SacT can serve as a phosphoryl acceptor with the general energy-coupling protein, HPr(His-P) serving as the phosphoryl donor. Although the phosphorylation of SacT by HPr(His-P) is in agreement with previously published genetic data, we do not ignore the possibility that an additional site in native SacT. Results presented in Figs. 4 and 5 demonstrate that both the native and the phosphorylated SacT can bind to RNA targets and that the SacT-dependent function (Debbarbouillé et al., 1990; Aymerich and Steinmetz, 1992). Indeed, the formation of mRNA-SacT complexes was observed after heat denaturation of the probes at 80 °C as described under "Experimental Procedures." As shown in Fig. 4 (lanes 1–5), two distinct mRNA-protein complexes were clearly detected when a high concentration of SacT was incubated with probe 1. An mRNA-protein complex was also observed with probe 2 (lanes 6–10), and heat denaturation of the probe was similarly required as had been observed with full-length probe 1. Competition experiments were then carried out by addition of unlabeled RNA probe 2 to a binding assay mixture containing the same radiolabeled probe. As shown in Fig. 4 (lanes 10–15), when the concentration of the unlabeled competitor was increased, the labeled probe was released progressively from the RNA-protein complex. The same experiment was carried out with unlabeled probe 1 leading to the release of the corresponding labeled probe 1 (data not shown). In order to test whether the in vitro phosphorylation of SacT was required for RNA binding, gel shift experiments were performed using the native SacT. Results presented in Figs. 4 and 5 demonstrate that both the native and the phosphorylated SacT can bind both probes.

Previous studies suggested that SacT and SacY have similar mRNA targets and that the sacPA operon is constitutively expressed in a $\Delta sacT, \Delta pts(GH1)$ double mutant due to a SacY-dependent function (Debbarbouillé et al., 1990; Aymerich and Steinmetz, 1992). Similar binding assays were performed using purified SacY. Increasing amounts of SacY were incubated with either labeled probe 1 or labeled probe 2 (Fig. 6). Two
complex was observed in the presence of irrelevant proteins, activities of the purified proteins, since no ribonucleoprotein complexes were observed using either probe 1 (lanes 1–5) or probe 2 (lanes 6–10). Retardation of the two RNA probes by SacT or SacY is due to specific RNA binding activities of the purified proteins, since no ribonucleoprotein complex was observed in the presence of irrelevant proteins, i.e. Enzyme I and HPr (Fig. 5, lanes 5 and 6), and all band migration retardation assays were performed in the presence of excess amounts of nonspecific tRNA. Altogether, the data demonstrate that the reported mRNA migration retardations represent specific recognition of the probes by SacT and SacY. They further indicate that PTS-catalyzed phosphorylation of SacT is not required for interaction in vitro with the two RNA probes used.

SacT and SacY Are Transcriptional Antiterminators in Vitro—SacY and SacT are homologous to BglG (see Introduction), and genetic evidence strongly suggests that all three proteins are transcriptional antiterminators (Aymérich and Steinmetz, 1987; Zuzowski et al., 1990; Débarbouillé et al., 1990). The availability of purified SacT and SacY allowed direct determination of their transcriptional antitermination activities in vitro. Plasmid pTP7 contains a DNA fragment of 305 base pairs bearing the upstream region of the sacPA operon including the promoter and the SacT-regulated terminator (Débarbouillé et al., 1990). In this plasmid, a single BamHI restriction site is located about 80 base pairs downstream of the transcriptional terminator in the sacP coding sequence. The DNA of pTP7 was linearized with BamHI and then used as template in the in vitro transcription assays as described under “Experimental Procedures.” As expected for RNA transcriptional termination at the palindrome, a short transcript of 121 bases was observed in the presence of RNA polymerase core enzyme associated with σ^A from B. subtilis. By contrast, addition of either SacT or SacY to the in vitro transcription mixture readily promoted the synthesis of a long, full-length transcript (212 bases) corresponding to the full length of the sacPA DNA template (Fig. 7, left panel). These experiments clearly show that both SacT and SacY are transcriptional antiterminators that can function under in vitro conditions independently of their modification by phosphorylation.

The latter conclusion was supported by findings demonstrating the lack of a significant effect on the antitermination activity following phosphorylation of SacT by Enzyme I, HPr, and PEP (data not shown). Since this finding is in apparent conflict with the previously published model deduced from in vivo genetic data (Arnaud et al., 1992), we considered the possibility that SacT had been phosphorylated during overproduction by the resident PTS of E. coli. To examine this possibility, the putative SacT-P was preincubated with approximately 20-fold excess of purified HPr and subsequently assayed for transcriptional antitermination activity. Since the characterized HPr-
mediated phosphorylation reactions are all reversible, it is probable that excess HPr would effect removal of phosphate from the putative SacT-P. Nevertheless, the results obtained with the HPr-treated antiterminator were similar to those obtained with the untreated SacT (Fig. 7, right panel). While the apparent conflict between the presently demonstrated antitermination activity of unphosphorylated SacT and the previously deduced phosphorylation-dependent antitermination mechanism is not yet resolved, our data clearly establish that the PTS exerts dual control over the antitermination activity of this protein as compared to PTS-mediated control of the two homologues, SacY and BglG (Arnaud et al., 1992). While evidence was previously presented demonstrating that the activities of SacY and BglG are negatively modulated by the PTS due to their phosphorylation by the respective sugar specific permeases, SacX and BglF, the activity of SacT was deduced to be under positive control by the general energy coupling protein HPr or a phosphorylated protein thereof (see Introduction and references cited therein). Our data establish that SacT is phosphorylated by HPr(His→P) by the sequential relay of phosphate through the PTS. We emphasize, however, that reversible phosphorylation of SacT by this general energy coupling protein of the PTS is not sufficient to confer sucrose-specific induction of the sacPA operon.

The N antiterminator protein of coliphage lambda, similarly to SacT and SacY, recognizes a sequence in the nascent RNA. This protein interacts with RNA polymerase to suppress transcriptional termination. In this case, control signals are transmitted to RNA polymerase via protein-protein interactions and RNA looping (Greenblatt et al., 1993; Friedman and Court, 1995). In addition to N, four host factors, NusA, NusB, NusE, and NusG, facilitate antitermination in vivo. Interestingly, only one factor, NusA, is required for in vitro antitermination at moderate N concentrations, while at high N concentrations NusA is not required (Rees et al., 1996). The results reported here contrast therefore with those obtained for the coliphage N protein in that no additional factors proved to be required for in vitro antitermination. To the best of our knowledge, this is the first report of a reconstituted in vitro antitermination system using a BglG homologue.

An unexpected finding reported in the present communica-
Using the reconstituted antitermination assay system, our current studies are designed to examine these possibilities and to reconcile the genetic and biochemical data with respect to the physiologically relevant form of SacT.

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