

Catabolic Repression of *secB* Expression Is Positively Controlled by Cyclic AMP (cAMP) Receptor Protein-cAMP Complexes at the Transcriptional Level

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SecB, a protein export-specific chaperone, enhances the export of a subset of proteins across cytoplasmic membranes of *Escherichia coli*. Previous studies showed that the synthesis of SecB is repressed by the presence of glucose in the medium. The derepression of SecB requires the products of both the *cya* and *crp* genes, indicating that *secB* expression is under the control of catabolic repression. In this study, two *secB*-specific promoters were identified. In addition, 5' transcription initiation sites from these two promoters were determined by means of *secB-lacZ* fusions and primer extension. The distal P1 promoter appeared to be independent of carbon sources, whereas the proximal P2 promoter was shown to be subject to control by the cyclic AMP (cAMP) receptor protein (CRP)-cAMP complexes. Gel-mobility shift studies showed that this regulation results from direct interaction between the *secB* P2 promoter region and the CRP-cAMP complex. Moreover, the CRP binding site on the *secB* gene was determined by DNase I footprinting and further substantiated by mutational analysis. The identified *secB* CRP binding region is centered at the -61.5 region of the *secB* gene and differed from the putative binding sites predicted by computer analysis.

An *Escherichia coli* global regulatory protein, cyclic AMP (cAMP) receptor protein (CRP), is involved in the regulation of transcription, either positively or negatively, in many genes involved in carbon metabolism (4, 18). CRP, a homodimer, undergoes a conformational change when complexed with its allosteric effector cAMP and binds to a specific sequence located near or within target promoters to regulate transcription (18). The cellular level of cAMP is controlled by carbon sources, in part by the inhibition of adenylate cyclase activity by glucose (36). CRP is present predominantly in the cAMP-complexed, active conformation under non-catabolite-repressed conditions (e.g., growth with glycerol as the carbon source). The level of CRP-cAMP complex is reduced under catabolite-repressed conditions (e.g., growth with glucose), decreasing the activities of CRP-dependent promoters (4, 28).

The CRP binding sites lie at different locations with respect to transcriptional initiation sites of various promoters (44). Simple CRP-dependent promoters in which CRP-cAMP alone is sufficient for activation have been grouped into two classes based on the location of the recognition site for CRP and the corresponding mechanisms for activation (2, 13). For class I CRP-dependent promoters, such as *lacZ* and *malT* promoters, the CRP binding site is located upstream of the -35 region (2, 12). For class II CRP-dependent promoters like *galP1*, the CRP binding site is centered near the -35 region (7). The interaction between CRP and RNA polymerase has been shown to play a critical role in the activation of transcription in CRP-dependent promoters (6, 18).

SecB, an export-specific molecular chaperone in *E. coli*, promotes protein export across cytoplasmic membranes for a subset of precursor proteins. SecB binds to these precursors, thus keeping them in loosely folded translocation-competent conformations (22, 35, 46). Subsequently, SecB-precursor com-

plexes target the membrane translocase composed of SecYEG and SecDF-YajC complexes via interaction through a soluble and membrane-associated translocation ATPase, SecA protein (5, 10, 16, 35).

Our previous studies showed that the synthesis of SecB is controlled by carbon nutrients, in contrast to those of other Sec proteins, which are growth rate dependent (39). The cellular amount of SecB is reduced in the presence of glucose. CRP-cAMP has also been shown to be involved in SecB synthesis. Exogenous cAMP partially compensates for the repressed level of SecB in the presence of glucose, and the compensatory recovery by the addition of cAMP is lost in cells lacking functional production of CRP from a *cya* and *crp* mutant strain. When plasmids carrying the wild-type *crp* gene were reintroduced into this *cya* and *crp* mutant strain, cells were shown to restore the response to the exogenous cAMP. Moreover, deletion studies on the upstream portion of *secB* suggest that the expression of *secB* is controlled by more than one promoter (39). In this study, we report the identification of two *secB*-specific promoters and their corresponding 5' transcriptional initiation sites.

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* strains and plasmids used in this study are listed in Table 1.

Cell growth, media, and DNA manipulations. The medium used in this study was the modified minimal medium A (MinA) described by Davis and Mingioli (11) plus CaCl_2 (5 $\mu\text{g}/\text{ml}$), $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (0.25 $\mu\text{g}/\text{ml}$), and thiamine (10 $\mu\text{g}/\text{ml}$). All carbon sources were at 0.5% (wt/vol), and Casamino Acids (Difco) was at 0.1% (wt/vol). Antibiotics were used when required at the following concentrations: 100 $\mu\text{g}/\text{ml}$ (ampicillin); 10 $\mu\text{g}/\text{ml}$ (tetracycline).

In most cases, cells were grown in glycerol minimal medium overnight at 37°C in a rotary shaker water bath and inoculated into the same fresh medium. During the exponential phase of growth, cells were transferred to fresh media containing the indicated carbon sources with or without cAMP, and cell growth was monitored by measuring optical density at 600 nm (OD_{600}).

Plasmid DNA digestion, transformation, and other routine DNA manipulations were performed as described by Sambrook et al. (37). Plasmid DNA for nucleotide sequencing and PCR template was isolated by using plasmid isolation kits (Qiagen Inc., Valencia, Calif.).

Constructions of *secB-lacZ* transcriptional and translational fusion plasmids. To construct transcriptional fusions, restriction enzyme-digested fragments from

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant genotype, phenotype, or description	Reference
Strains		
MC4100	F ⁻ <i>ΔlacU169 araD136 relA rpsL150 fbbB5301 deoC7 ptsF25 thi</i>	8
ZK4	MC4100 <i>recA5</i>	14
RH77	MC4100 <i>Δcya851 Δcrp-zhd732::Tn10</i>	25
BL21λ(DE3)	F ⁻ <i>ompT hsdSB λlscUV5-T7 gene 1</i>	42
Plasmids		
pQF50	Transcriptional fusion vector, Ap ^r	23
pTS series	<i>secB-lacZ</i> transcriptional fusions	This work
pQF52	Translational fusion vector, Ap ^r	23
pTQ series	<i>secB-lacZ</i> translational fusions	This work
pT7-6	Vector with T7 RNA polymerase promoter	43
pHA7E	pBR322 derivative, <i>crp</i> ⁺ Ap ^r	17
pT7-CRP	pT7-6 with <i>crp</i> gene, Ap ^r	This work
pHK205	pBR322 derivative, <i>secB</i> ⁺ Ap ^r	20

cloned *secB* gene plasmid in pHK205 (20) were ligated into appropriate multi-cloning sites of pQF50. For translational fusions, either restriction enzyme-digested fragments or PCR products, with pHK205 as a template with corresponding primers (Table 2), were first cloned into *Sma*I-digested pUC18. Then, fragments between the *Xba*I site of pUC18 and the *secB* internal *Eco*RV site were inserted into *Xba*I-*Sma*I-digested pQF52. All these fusion plasmids were transformed into ZK4, and transformants were selected on 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) plates. All fusion plasmids were reisolated, and DNA sequences were confirmed by DNA sequencing.

β-Galactosidase assay. β-Galactosidase activities were determined in permeabilized cells (29). Cells harboring *lacZ* fusion plasmids were normally grown in glycerol medium to exponential phase (OD₆₀₀, ≈1.2) and washed once with MinA medium. Cells were reinoculated into minimal medium with glycerol, glucose, or glucose plus cAMP (10 mM in final) to an OD₆₀₀ of 0.1. Cells were allowed to grow for 3.5 generations before the β-galactosidase assay. For RH77 cells, cells from overnight culture in the glucose medium with 0.1% Casamino Acids were grown to exponential phase in the presence or absence of cAMP for about 3.5 generations.

RNA preparation and primer extension. Total RNAs were prepared from ZK4 cells harboring pHK205, pTQ17, or pTQ46. Cells grown in glycerol minimal medium were harvested and transferred to minimal medium with either glycerol or glucose as the carbon source and were grown for 3.5 generations. Total RNAs were extracted by the acidic hot phenol method (33); briefly, cell pellets were suspended in lysis buffer containing 0.02 M sodium acetate (pH 5.2), 1 mM EDTA, 0.5% sodium dodecyl sulfate (SDS), and 0.2% diethylpyrocarbonate (DEPC). An equal volume of phenol equilibrated with 0.02 M sodium acetate (pH 5.2) was added, and the mixture was incubated at 65°C for 5 min with gentle shaking. The aqueous phase was reextracted with an equal volume of phenol-chloroform (1:1 [vol/vol]) and precipitated with 3 volumes of 100% EtOH (ethanol). The RNA pellet was dissolved in DEPC-treated deionized H₂O and treated with 10 U of RNase-free DNase I in the presence of 40 U of RNase inhibitor (Boehringer Mannheim, Indianapolis, Ind.). The RNA was precipitated by adding 1/10 of the volume of 3 M sodium acetate and then 2.5 times the volume of 100% EtOH.

Primer extension was carried out by mixing indicated amounts of total RNA and 1 pmol of ³²P end-labeled primer in the reaction mixture containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, and 10 mM dithiothreitol (DTT). The reaction mixture was heated to 96°C for 5 min, incubated at 60°C for 60 min, slowly cooled to 42°C, and then incubated for 10 min. Reverse transcription was done at 42°C for 45 min by the addition of 200 U of reverse transcriptase (Superscript II; GIBCO BRL, Gaithersburg, Md.) and 1 mM deoxynucleoside triphosphate (dNTP). Final products were analyzed on a 6% polyacrylamide-8 M urea sequencing gel (Sequagel; National Diagnostics, Atlanta, Ga.). Reference sequences were carried out with the same primers used in primer extensions and with DNA from corresponding plasmids as templates (38). Primers with sequences complementary to the coding strand 5'-TCACCACGAGTAATCAC CTTCACTTTCG-3' and 5'-TGGAAAACGTGCGGCGGTTTCGGC-3' were used in both primer extensions and reference sequencing ladders for P1 and P2, respectively.

Construction of pT7-Crp and CRP protein purification. To clone the *E. coli* *crp* gene behind bacteriophage T7 RNA polymerase promoter, the *crp* gene from pHA7E (1) was excised with *Bam*HI and *Eco*RI digestions. The 1-kb *Bam*HI-*Eco*RI fragment was inserted in pT7-6 (43) digested with *Bam*HI and *Eco*RI. The resulting plasmid, pT7-Crp, was transformed into BL21(ΔDE3), which carries an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible T7 RNA polymerase gene.

CRP was overproduced from strain BL21(ΔDE3)/pT7-CRP induced with IPTG. Cell extracts were prepared, and CRP was purified by cAMP affinity chromatography followed by ion-exchange chromatography under conditions described previously (47). The identity of CRP protein was confirmed by both amino-terminal peptide sequencing and immunoblotting with anti-CRP sera (15). The purity of CRP was judged by analyzing purified fractions on SDS-12% polyacrylamide gel electrophoresis (PAGE), followed by Coomassie blue staining, and was estimated to be over 98% homogeneous (data not shown).

Site-directed mutagenesis and *secB* DNA fragment preparation. DNA fragments containing various *secB* upstream regions were amplified by PCR with appropriate oligonucleotides shown in Table 2, with pHK205 as the template. Amplified fragments were subcloned into the *Sma*I site of pUC18 cloning vector. Site-directed mutagenesis on the *secB* CRP binding site and putative binding sites was performed by PCR amplification with base pair-substituted primers (shown in Fig. 4A and Table 2). After subcloning into pUC18, the orientation of inserts and sequences of all cloned fragments were verified by DNA sequencing for both coding and template strands (AmpliTag; Applied Biosystems Inc., Foster City, Calif.). DNA fragments were purified from agarose gel after 5'-*Hind*III and 3'-*Eco*RI digestions and labeled with [α-³²P]dATP and dGTP (New England Nuclear, Boston, Mass.) by using Klenow enzyme (Promega, Madison, Wis.).

Gel-mobility shift assay and DNase I footprinting. DNA fragments employed in these experiments are shown in Fig. 4B. ³²P-labeled DNA fragments and purified CRP proteins were incubated in binding buffer (10 mM Tris-HCl [pH 7.8], 50 mM KCl, 1 mM EDTA, 50 μg of bovine serum albumin/ml, 1 mM DTT, 0.05% Nonidet P-40, 50 μM cAMP, 20 μg of salmon sperm DNA [Sigma Chemical Co., St. Louis, Mo./ml] for 30 min in a total volume of 20 μl. Then, 3 μl of loading buffer (binding buffer containing 50% glycerol and 0.1 mg of bromophenol blue/ml) was added, and the samples were immediately loaded on a 5% polyacrylamide gel (Protogel; National Diagnostics), with current applied. Then electrophoresis was initiated at a low voltage and progressively increased to 150 V over 60 to 75 min. The electrophoresis buffer contained 10 mM Tris-HCl (pH 7.8), 1 mM EDTA, and 50 μM cAMP and was replaced with fresh buffer a couple of times during the run.

DNase I footprinting experiments were carried out as previously described (32). Reactions were performed in a total volume of 100 μl of CRP binding buffer as described above with the addition of 2.5 mM MgCl₂ and 1 mM CaCl₂. ³²P-labeled DNA fragments (0.1 nM) were incubated with CRP proteins (0 to 6.4 × 10⁻⁹ M) in the presence of cAMP for 30 min. Then, pancreatic DNase I (0.2 μg; Boehringer Mannheim) was added, followed by 2 min of digestion. After

TABLE 2. Oligonucleotide primers used to construct probes for CRP binding assays

Primer	Probe(s) ^a	Relative description ^b	Sequence of oligonucleotide (5' to 3') ^c
42	Pr-46, -47, -102, -78, -80	wt <i>secB</i> TS	(+174) TGGAAAACGTGCGGCGGTTTCGGC
46	Pr-46	wt <i>secB</i> upstream CS	(-134) ATGGCAACGC CGCCAAGCGTGAAG
47	Pr-47	wt <i>secB</i> upstream CS	(-89) GCACCACGGTTCCCCAGATTTTATT
56	Pr-60	wt <i>secB</i> TS	(+296) ACACAGGAAC CCGGTTCTTCGCCCGGC
60	Pr-60	wt <i>secB</i> upstream CS	(-58) ACAGCACATTGGCGGCTGTGATGACTTGTA
78	Pr-BCRP-M	BCRP mutation CS	(-71) GATCTTTCTTTCAGCAGCGGTTGGCGCTG
80	Pr-BCRP-W	BCRP wt CS	(-76) CTAGAGATTTTTATTGACGCACAGCACAT

^a W, wild type; M, mutations; BCRP, *secB* P2 CRP binding site.

^b wt, wild type; TS, template strand; CS, coding strand.

^c Underlines, CRP binding site or part of it. Italicized letters indicate base pair changes to create restriction sites, and bold letters indicate substituted base pairs for CRP site mutations as shown in Fig. 4. Numbers in parentheses indicated the 5' position of primers relevant to the +1 of the *secB* transcript.

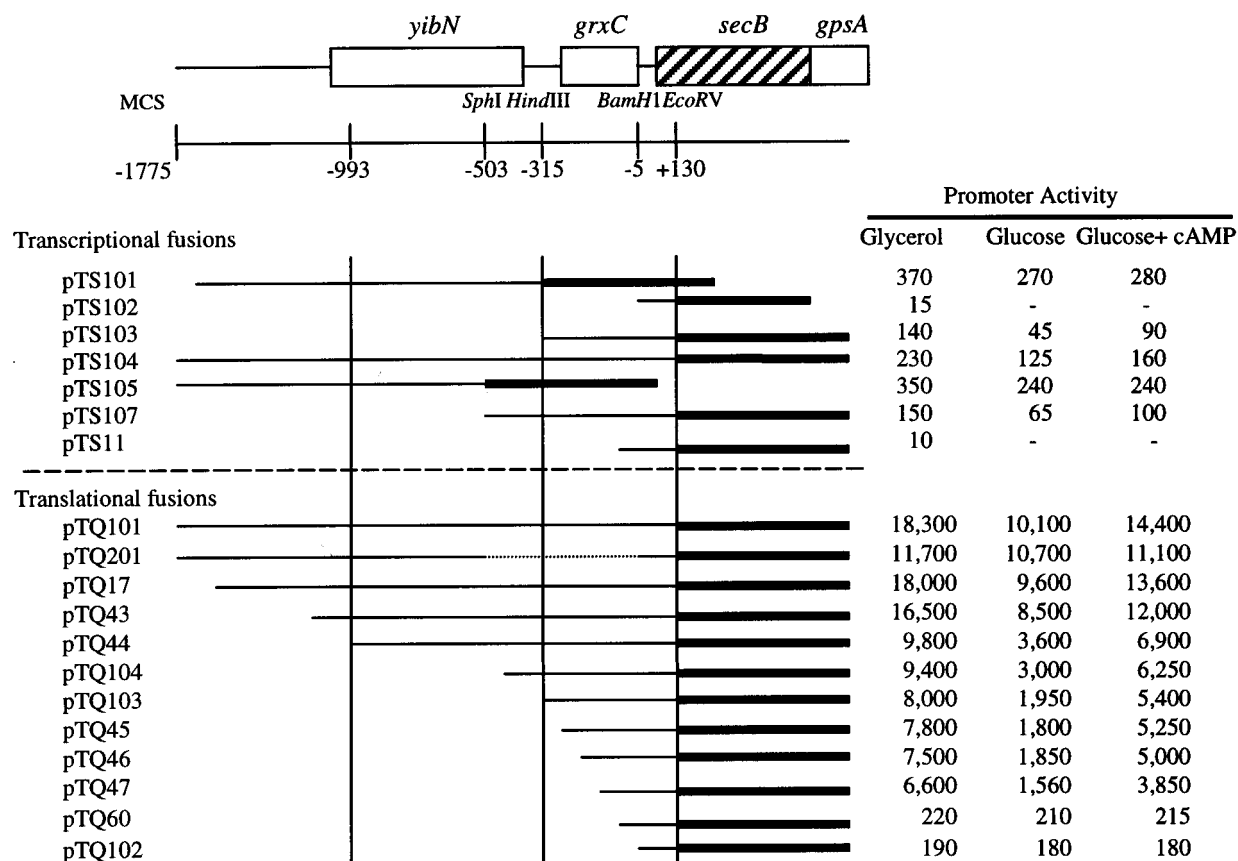


FIG. 1. Overview of *secB* and neighboring genes and diagrams of *secB-lacZ* fusion constructions. Boxes indicate ORFs for *yibN*, *grxC*, *secB*, and *gpsA*. The *yibN* product has not yet been identified; *grxC* has recently been suggested to code for glutaredoxin 3; *gpsA* encodes *sn*-glycerol-3-phosphate dehydrogenase. Solid lines show intergenic regions of these genes. For *secB-lacZ* fusion constructions, solid lines represent *secB* upstream regions fused to the corresponding *lacZ* gene, solid bars represent a promoterless *lacZ* gene or a promoterless *lacZ* gene without a ribosome-binding site, and MCS denotes multicloning sites. Promoter activities were determined by measuring β -galactosidase activities for cells carrying corresponding fusion plasmids with different carbon sources and with the addition of exogenous cAMP. During exponential growth in the glycerol medium, cells were transferred into indicated media. The activities of β -galactosidase (expressed in Miller units) were assayed. Data represent the average of three independent experiments, and standard error was typically within $\pm 10\%$.

precipitation with ethanol, the products were analyzed in a 6% polyacrylamide-8 M urea sequencing gel against a G + A ladder (26).

RESULTS

The expression of the *secB* gene is controlled by two distantly separated promoters, one of which is subjected to catabolic regulation. Previous studies on the SecB synthesis suggested that there might be separate promoters for *secB* expression with different regulatory mechanisms (39). To identify these putative *secB* promoters, *secB-lacZ* transcriptional fusions were constructed (Fig. 1). The presence of promoter activity was examined by measuring β -galactosidase activities from cells carrying these fusion plasmids. The responses to either glycerol or glucose as a sole carbon source and the effect of added cAMP were determined (Fig. 1). Two significant promoter activities were observed, one upstream of the *HindIII* site (at the -315 bp, pTS101), and the other downstream of the *HindIII* site (pTS103). Cells carrying plasmid pTS101 essentially did not respond to different carbon sources or to the addition of cAMP in the presence of glucose. In contrast, β -galactosidase activities in cells carrying plasmid pTS103 were significantly reduced in the presence of glucose, but not as severely if cAMP was also present. These responses were comparable to those of actual SecB synthesis observed with plasmid

pKS101 containing a deletion of the *HindIII* upstream region (39). Plasmid pTS107, carrying a 5' end extended by about 200 bp, did not show significant differences from pTS103.

To determine whether these two promoters are specific to *secB* expression, the 5' end of *secB*, with progressively smaller segments derived from the 1.8-kb upstream region, was fused to a *lacZ* gene that lacks both its own promoter and ribosome-binding site for translation (Fig. 1). SecB translational activities were measured as β -galactosidase activities (Fig. 1). β -Galactosidase activities from fusion plasmids, pTQ101, pTQ17, and pTQ43, were high in glycerol and repressed in glucose. However, there were significant decreases in overall activities when fusion fragments were shortened further. In addition, glucose repression became more prominent as seen in pTQ44 through pTQ47. From these data, we conclude that the expression of *secB* is controlled by two spatially separated promoters. The distal promoter, P1, resides within the fragment between pTQ43 and pTQ44 fusions, and the proximal promoter, P2, resides within a pTQ47 fragment. The P2 promoter likely accounts for the repression by glucose, since the internal deletion of the *SphI-BamHI* fragment from pTQ17 (pTQ201, Fig. 1) resulted in a complete loss of the ability to respond to different carbon sources. On the other hand, pTQ201 was expressed in a constitutive manner under the conditions tested.

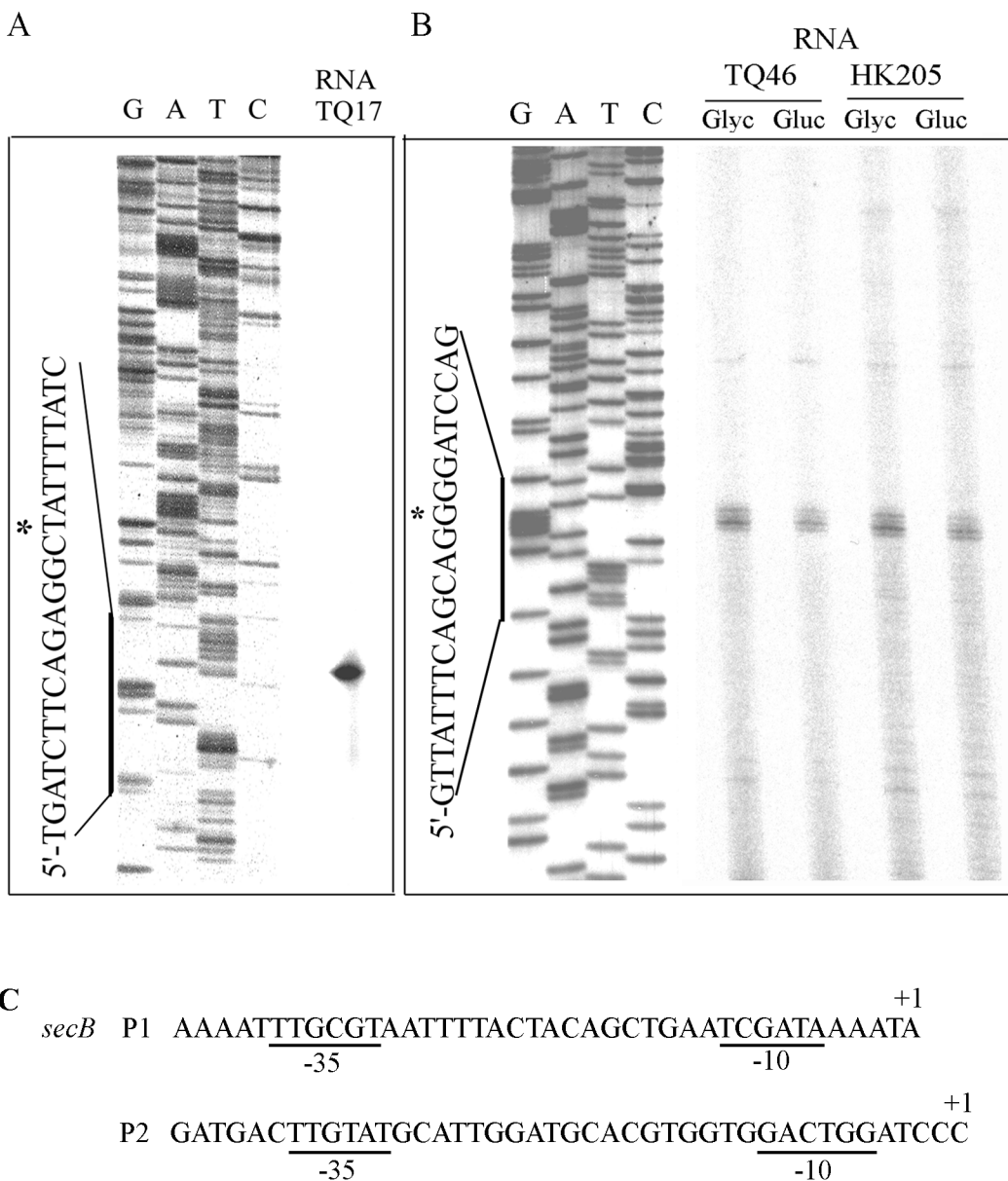


FIG. 2. Primer extension for the identification of 5' ends of transcripts from the *secB* P1 and P2 promoters. Total RNAs were prepared from ZK4 harboring plasmid pTQ17 grown in glycerol or plasmid pTQ46 or pHK205 grown in glycerol or glucose media as indicated. Primers complementary to coding strand were extended at 42°C for 45 min with reverse transcriptase. Reference DNA sequencing was done with the same primers as those in primer extensions. (A) P1 promoter. (B) P2 promoter. Total RNA (2.5 and 5 µg, respectively) was used for panels A and B, respectively. Asterisks indicate 5' positions from each respective promoter. (C) Sequences of the *secB* P1 and P2 promoters.

Determination of 5' ends of *secB* transcripts from the P1 and P2 promoters. The transcriptional initiation sites were determined by primer extension assays (Fig. 2). Total mRNAs were prepared from cells harboring the plasmid-encoded *secB* gene, pHK205 (21) or *secB-lacZ* fusion plasmids, pTQ46 or pTQ17, grown in glycerol or glucose minimal media. The 5' end of the *secB* transcript from the proximal promoter P2 was located 75 bp upstream of the initiation codon of SecB and subsequently designated +1 (Fig. 2B). Upon quantitation of primer extension products with mRNAs from glycerol- or glucose-grown cells, P2 transcripts were consistent with β-galactosidase activities of pTQ46 measured at the time of total RNA

preparations (data not shown). The 5' end of distal promoter P1 transcript was also determined by a separate primer extension experiment with mRNA extracted from plasmid pTQ17 and located at position -988 with respect to the newly identified +1 of P2 transcript (Fig. 2A). Both promoter sequences are shown in Fig. 2C. These 5' transcriptional initiation sites were in agreement with previous fusion studies (Fig. 1).

Characterization of the roles of CRP-cAMP in the *secB* expression: direct interaction between the *secB* and the CRP-cAMP complex. The addition of cAMP in the glucose medium resulted in increased transcriptional activation at the *secB* P2 promoter (Fig. 1). This recovery from catabolic repression

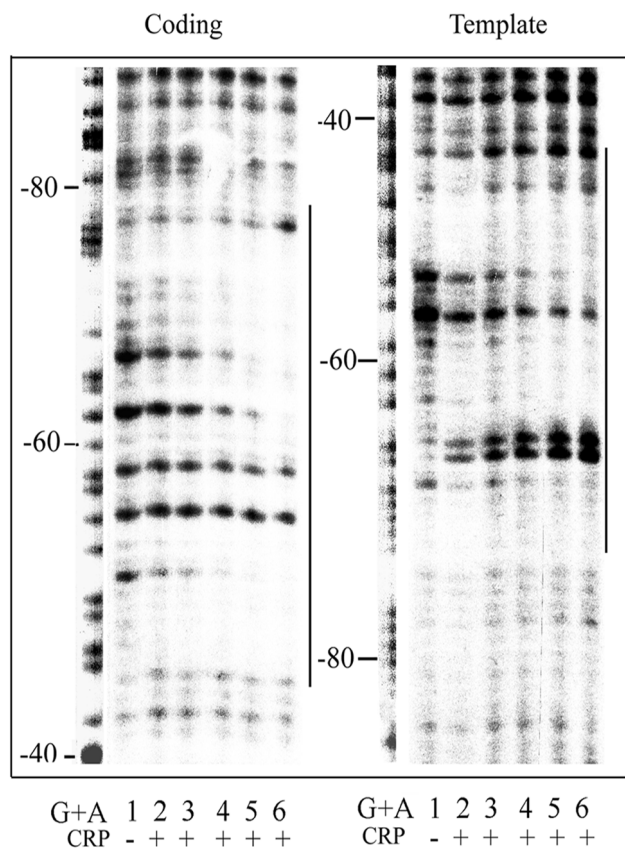


FIG. 3. CRP binding site for the *secB* P2 promoter. DNase I footprinting experiments with DNA fragment containing the *secB* promoter regions were carried out with purified Crp proteins. Final Crp protein concentrations are 0, 0.2×10^{-9} , 0.4×10^{-9} , 0.8×10^{-9} , 1.6×10^{-9} , and 6.4×10^{-9} M in lanes 1 to 6, respectively. Vertical dark bars indicate protected binding regions by Crp proteins, and numbers are in respect to the +1 transcriptional initiation site from the P2 promoter.

requires the presence of the *crp* gene (39). These results suggest the involvement of the CRP-cAMP complex on the expression of *secB*. The possible interaction between the CRP-cAMP complex and the *secB* P2 promoter regulatory region was examined directly, by measuring the binding activity of purified CRP protein on the DNA fragment containing the *secB* P2 promoter. Preliminary results showed that CRP effectively bound to the *secB* P2 promoter region in the gel electrophoretic mobility shift assay (data not shown; see Fig. 5A).

To determine the CRP binding site, DNase I footprinting experiments were performed. A labeled 170-bp DNA fragment containing *secB* regulatory regions was incubated with increasing concentrations of CRP in the presence of cAMP and then treated with DNase I. The products were analyzed on denaturing polyacrylamide gels (Fig. 3). CRP bound and protected the DNA region between -75 and -49 bp from DNase I cleavage. The protected region was centered -61.5 bp from the P2 promoter. Several sites in both coding and template strands showed enhanced cleavages.

A CRP titration experiment was performed by gel electrophoretic mobility shift assay. A 314-bp DNA fragment, probe 46 (Pr-46), corresponding to the same upstream sequences as those in pTQ46 was used (Fig. 1). The affinity of CRP for the *secB* gene, expressed as the apparent K_d value, was estimated to be about 0.8×10^{-9} M, where the ratio of free DNA and

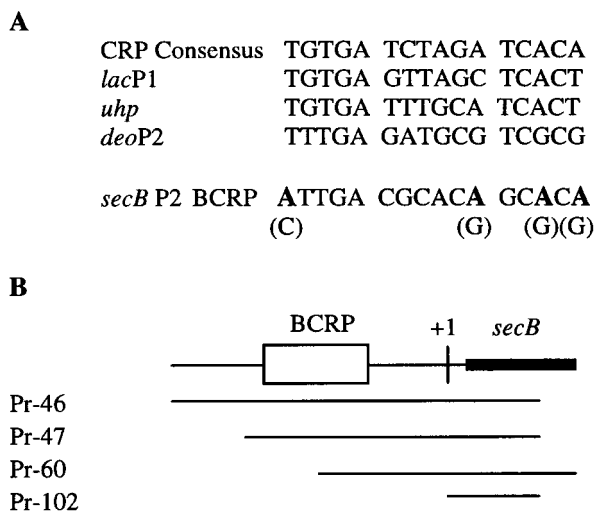


FIG. 4. CRP binding consensus sequence comparison and DNA fragments for CRP binding assay. (A) Bold face letters indicate base pairs replaced by those in parentheses by site-directed mutagenesis. (B) DNA fragments used in the CRP binding assay and their relative positions with respect to the *secB* P2 CRP binding site. The probe numbers correspond to the 5' primers for PCR amplifications, except Pr-102, which has the same 5' end as that in pTQ102 in Fig. 2. Pr, probe; BCRP, *secB* P2 CRP binding site.

bound DNA is 1 (Fig. 5A). Only a single shifted band of the same mobility was observed within the CRP concentrations ranging from 5×10^{-11} M to 6.4×10^{-9} M. Sequence-specific CRP binding for *secB* upstream regions was shown by incubating Pr-46 in the presence of nonreactive control probe 102 (Fig. 4B, Pr-102), from which the 5' regulatory regions were removed from Pr-46 to +1 position, while the same 3' end was retained. No shifted band was observed over the same CRP concentrations tested (Fig. 5A). Moreover, the absence of cAMP in the assay abolished the binding activity of CRP (data not shown).

The effect of the binding site truncation and mutations on CRP binding and *secB* P2 promoter activity. The identified CRP binding site of the *secB* P2 promoter (hereafter designated BCRP) was somewhat surprising in that it is not very similar to previously known CRP binding sites (Fig. 4A) (12, 34). In addition, initial searches with Nucleotide Subsequence Search, MacVector, version 6.0 (Oxford Molecular, Campbell, Calif.), revealed two other potential candidates for CRP binding sites, one located at the -119-bp region and the other at the -44-bp region of the *secB* gene (relative to transcriptional start site +1). To further substantiate the actual BCRP site, deletions and site-directed mutations (Fig. 4A and B) were introduced and compared for CRP binding activities. A site-directed point mutation with a 4-bp substitution in the BCRP site (Fig. 4A) almost completely abolished the CRP binding affinity (Fig. 5B, lanes 5 to 8; Pr-BCRP-M) compared to that without mutations (Fig. 5B, lanes 1 to 4; Pr-BCRP-W). In addition, deletion upstream of BCRP, as in probe Pr-47, showed the same affinity for CRP (Fig. 5C, lanes 4 to 6; Pr-47) as in Pr-46 (Fig. 5C, lanes 1 to 3). However, when the left half of BCRP binding sequences was truncated (probe Pr-60), the ability of this fragment to bind CRP was almost completely abolished (Fig. 5C, lanes 7 to 9). Moreover, mutations at the -44-bp consensus sequence, TGTGA, did not have any effect on CRP binding (data not shown), and this site is not involved in CRP binding, even when the real BCRP site is altered or deleted (Fig. 5B and C). These data provide further evidence

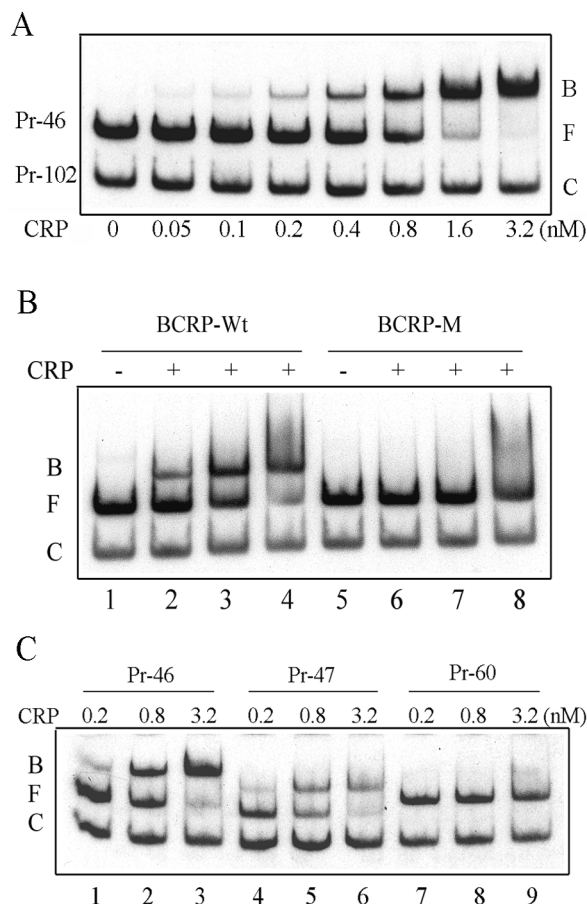


FIG. 5. CRP titration and mutational analysis on *secB* CRP binding sites. CRP binding affinities were determined with various *secB* DNA fragment probes carrying mutations on the CRP binding site as described in the legend for Fig. 4. (A) Binding of CRP protein on the *secB* gene. Gel-mobility shift assays were carried out with *secB* P2 containing DNA fragments and purified CRP. 32 P end-labeled *secB* DNA probes were incubated with increasing amounts of purified Crp protein in the presence of 50 μ M cAMP. DNA-protein complexes were analyzed in 5% native PAGE. (B) Site-directed mutagenesis on the BCRP site (*secB* P2 CRP). CRP proteins are 0, 0.2, 0.8, and 3.2×10^{-9} M in lanes 1 and 5, 2 and 6, 3 and 7, and 4 and 8, respectively. (C) Deletion and truncation on the BCRP site (*secB* P2 CRP). Abbreviations: B, bound-DNA probe; F, free-DNA probe; and C, unbound negative control DNA probe.

that the BCRP site is, indeed, the real CRP binding site for the *secB* gene, as shown by DNase I footprinting.

The effects of CRP binding site mutations on the expression of *secB* were further examined in vivo by *secB-lacZ* fusions in conjunction with *cya* and *crp* mutations. *secB-lacZ* fusions with mutations on the CRP binding site were constructed from the DNA fragments used in gel-mobility shift assay (Fig. 6). Translational activities of *secB* promoters were compared with or without the addition of cAMP in both wild-type and *cya* and *crp* mutant backgrounds (Fig. 6). As expected, fusion plasmids carrying both the P1 and the P2 promoters, pTQ17 and pTQ43, showed moderate responses to the addition of cAMP (less than a twofold increase), while plasmids with P2 promoter only (pTQ45, pTQ46, and pTQ80) had a more pronounced increase by the addition of cAMP (about three- to fourfold). Fusion plasmids carrying either deletion or mutations on the CRP binding site lost almost all promoter activity (pTQ60 and pTQ78) and showed little response to the cAMP. When examined in *cya* and *crp* mutant background (RH77), all these

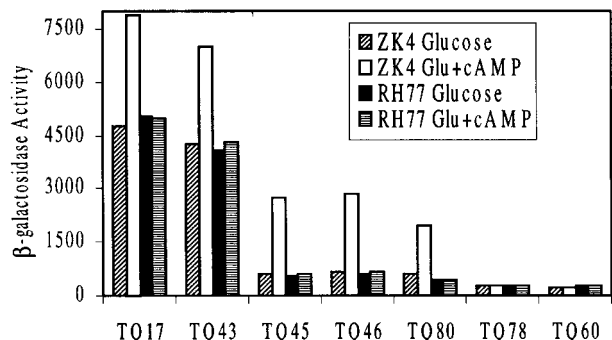


FIG. 6. The effect of CRP binding site mutations on *secB* promoter activity. Promoter activities and their responses to cAMP were determined from wild-type ZK4 cells as well as in *cya* and *crp* mutant background RH77 cells carrying *secB-lacZ* fusion plasmids with mutations on the CRP binding site. Fusion plasmids pTQ60, pTQ78, and pTQ80 were constructed by subcloning of DNA probes used in the experiments shown in Fig. 5B and C into translational fusion vector pQF52 as described in Materials and Methods. All other fusion plasmids were described in the legend for Fig. 1. Cells carrying indicated fusion plasmids were grown overnight in minimal medium containing 0.5% glucose and 0.1% Casamino Acids and were transferred to the same fresh media by dilutions with or without cAMP. Activities were expressed in Miller units, and averages of two independent cultures and standard error were within $\pm 10\%$.

plasmids lost their ability to respond to the exogenously added cAMP.

DISCUSSION

In this study, two spatially separated promoters for *secB* were identified by primer extension as well as by transcriptional and translational fusions. These results were in agreement with suggestions from previous physiological studies (39) as well as with *E. coli* genomic DNA sequence analysis (39, 41). Although these two promoters are separated by almost 1 kb, both lead to specific *secB* expression as determined by translational fusions (Fig. 1). The removal of the distal P1 promoter from fusion constructs results in a reduction of about twofold in overall *secB*- β -galactosidase expression, in agreement with the previous deletion analysis (39). Only the proximal P2 promoter is under the control of different carbon sources, while the distal P1 promoter appears to mediate expression in a constitutive manner under the conditions tested. Furthermore, glucose repression at the P2 promoter is shown to be regulated at the transcriptional level as evidenced by both *secB-lacZ* fusions and primer extension analysis (Fig. 1 and 2). Regulation at the transcriptional level was further substantiated by the finding that the stability of the SecB protein was unchanged in media containing different carbon sources (data not shown).

The catabolic repression at the *secB* P2 promoter involves the CRP-cAMP complex, since the addition of cAMP partially relieves glucose repression, and this compensatory recovery requires the presence of the *crp* gene (39). Gel-mobility shift assays with a DNA fragment containing the *secB* P2 promoter region and purified CRP protein clearly demonstrate that the effect of CRP-cAMP on *secB* expression results from a direct interaction between CRP and *secB* (Fig. 3). It also appears that there is only a single binding site for CRP on *secB*, since a single shifted band was observed over the range of CRP concentrations tested. The finding that the CRP binding site to the *secB* P2 promoter is centered at about -61.5 bp upstream of the $+1$ transcriptional initiation site indicates that it may be a class I CRP-dependent promoter like the *lacP1* promoter (12). The estimated CRP binding affinity of *secB* P2 (0.8×10^{-9} M)

is also comparable to that of *lacP1* (0.328×10^{-9} M [45]), even though the *secB* site deviated more than *lacP1* from the well-defined consensus sequences of CRP binding sites (Fig. 4A). Mutational analyses substantiate the finding that the site newly identified by DNase I footprinting is the real CRP binding site. Moreover, the consensus sequence (TGTGA) near -44 bp does not contribute or function, even in the absence of the real CRP binding sequences.

Two open reading frames (ORFs) upstream of *secB* also appear to be under the control of the distal P1 promoter. The distal putative ORF to *secB* is designated *yibN*, and the proximal ORF has recently been identified as a homologue to *grxC*, a minor glutaredoxin 3 (3). No other significant promoter activities have been identified for these ORFs, and SecB was expressed from the P1 promoter as efficiently as from the P2 promoter despite the distance between *secB* and the P1 promoter (Fig. 1). Therefore, it is likely that these four genes, i.e., the two upstream ORFs' genes, *secB*, and *gpsA*, form an operon from the P1 promoter whose regulation differs from that of P2. In contrast, the expression from P2 covers only *secB* and downstream *gpsA* with carbon source-dependent expression. The physiological and functional relationships among these genes are unclear.

Since the only known physiological function of the SecB protein is as a molecular chaperone in protein export, it was previously assumed that the production of SecB may depend on growth rates of cells to support the increased need for protein translocation in faster-growing cells. Nonviability of *secB* null strains on Luria-Bertani plates and their viability on the glycerol minimal plate may also be partially explained by growth rate-dependent needs of the SecB protein (20). However, our previous studies showed that SecB synthesis is not related to the growth rates of cells (39). Moreover, Shimizu et al. (40) found that the nonviability of *secB* null strains on Luria-Bertani plates is due to the insufficient expression of the *gpsA* gene that is located immediately downstream from *secB* and encodes biosynthetic *sn*-glycerol-3-phosphate dehydrogenase. In addition, the *gpsA* gene is translationally coupled to *secB*, as the start codon of GpsA overlaps with the stop codon of SecB (41), a finding we have confirmed. The *secB* P2 promoter region is required for the efficient expression of the *gpsA* gene (40). We conclude that the expression of *secB* does not depend on the growth rate of cells; rather, it is related to carbon sources in the media by means of catabolic repression mediated by the CRP-dependent P2 promoter.

The unique regulation of *secB* expression may be related to the fact that SecB is involved in the export of only a subset of precursor proteins. Thus, the export defect of OmpA caused by SecB deficiency is not as severe as other SecB-dependent precursors such as MBP (maltose binding protein) and LamB (20, 27). The expression of MBP and LamB is also activated by the CRP-cAMP complex (31), whereas the expression of OmpA is constitutive and thus affected little by CRP (30). Since SecB is present in relatively low amounts compared to SecB-dependent precursors (typically 100- to 400-fold in excess of SecB [39]) and SecB binds transiently to these precursors (19), small variations in SecB quantities (twofold overall) may be sufficient to modulate relatively large variations in precursors. Thus, it is possible that CRP-cAMP-activated expression of SecB from the proximal promoter P2 is designed to serve for similarly activated expression of precursors such as pLamB and pMBP. Another scenario, however, is also possible. Precursors of exported proteins are synthesized mainly from membrane-bound polysomes in fast-growing cells. They are synthesized equally from free polysomes and membrane-bound polysomes in slower-growing cells (9). Thus, in faster-growing cells in the pres-

ence of glucose, there may be less need for a chaperone for export. Accordingly, the requirement for and the production of SecB are less. Alternatively, other chaperones, such as trigger factor and GroEL (24), can function under these conditions in place of SecB.

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