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Involvement of CysB and Cbl Regulatory Proteins in Expression of the *tauABCD* Operon and Other Sulfate Starvation-Inducible Genes in *Escherichia coli*

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Starvation for sulfate results in increased synthesis of several proteins in *Escherichia coli*. Among these Ssi (sulfate starvation-induced) proteins are the products of the *tauABCD* genes, which are required for utilization of taurine as sulfur source for growth. In this study, the role of the *cbl* gene in expression of *tauABCD* and other *ssi* genes was investigated. The protein encoded by *cbl* shows high sequence similarity to CysB, the LysR-type transcriptional activator of the genes involved in cysteine biosynthesis. Strain EC2541, which contains an internal deletion in *cbl*, was unable to utilize taurine and other aliphatic sulfonates as sulfur sources. Two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed that many of the Ssi proteins were not synthesized in EC2541. Expression of a translational *tauD′-′lacZ* fusion required the presence of both *cbl* and *cysB*. The interactions of CysB and Cbl with the promoter region of *tauABCD* were studied by using gel mobility shift experiments and DNase I footprinting. CysB occupied multiple binding sites, whereas Cbl occupied only one site from 112 to 68 bp upstream of the transcription start site. Acetylserine, the inducer of transcription of CysB-regulated genes, stimulated binding of CysB but not of Cbl. Sulfate had no effect on binding of both proteins to the *tauABCD* promoter region. These results indicate that Cbl is a transcription factor for genes required for sulfonate-sulfur utilization and maybe for other genes whose expression is induced by sulfate starvation.

Cysteine biosynthesis via sulfate assimilation in plants and microorganisms represents the predominant mechanism by which inorganic sulfur is reduced and incorporated into organic compounds. This pathway has been characterized in detail in *Escherichia coli* and *Salmonella typhimurium* (see reference 24 for a recent review). Most of the genes involved are coordinately regulated as the cysteine regulon—high-level expression of these genes requires sulfur limitation, the LysR-type (11, 34) transcriptional regulator CysB, and the presence of the inducer molecule *N*-acetyl-L-serine. Transcription from *cys* gene promoters is initiated by CysB protein binding to activation sites positioned just upstream of the −35 regions of these promoters, and inducer facilitates interaction of CysB with these sites (15, 23, 28). Sulfur limitation is a necessary condition for derepression of the *cys* regulon because L-cysteine inhibits the serine transacetylase enzyme required for inducer synthesis. In addition, sulfide and thiosulfate act as anti-inducers of CysB-dependent transcription (13, 23). Maximal expression of *cys* genes is seen during growth with limiting sulfur sources such as glutathione or L-djenkolic acid, whereas growth with sulfate, sulfite, or thiosulfate leads to partial repression of these genes, and they are fully repressed during growth with sulfide, L-cysteine, and L-cystine (22, 24). The partial repression of *cys* gene expression by sulfate is ascribed to the inhibitory effect of sulfide generated during sulfate reduction, rather than to a direct effect of sulfate itself.

Laboratory studies on the *cys* regulons of *E. coli* and *S. typhimurium* have elucidated in detail the mechanisms of assimilation of sulfur from inorganic sulfate, thiosulfate, sulfite, and sulfide. However, in nature the levels of these compounds may be low, especially in soil (2), and bacteria that spend at least part of their lifetime outside the gut have to rely on organosulfur compounds such as sulfate esters, sulfamates, and sulfonates as sulfur sources. Sulfonate sulfur is of particular interest, as it accounts for over 40% of the total sulfur in the soil (2) and is also released into the environment in the form of man-made pollutants (20). *E. coli* is able to utilize several aliphatic sulfonates as sulfur sources (36, 37). During growth with ethanesulfonate or glutathione, the organism produces a set of proteins which are absent during growth with either cysteine or sulfate (21, 30). Two of these proteins have recently been found to be required for the utilization of taurine (2-aminoethanesulfonate) as a sulfur source. The operon in which these proteins are encoded, *tauABCD*, has been cloned and sequenced (37). Results from sequence alignments indicate that the operon encodes components of an ABC-type transporter (TauABC), whereas it has been demonstrated experimentally that TauD is an α-ketoglutarate-dependent dioxygenase involved in the oxygenolytic release of sulfite from taurine (7). Expression of the *tauD* gene was shown to depend on functional CysB, and it was fully repressed in the presence of cysteine or cystine and also during growth with sulfate (37). Utilization of sulfate-sulfur in preference to taurine-sulfur (36) is thus due to repression of *tauABCD* expression in the presence of sulfate.

This tight repression of *tauABCD* by sulfate is different from the regulation reported for CysB-dependent genes of the *cys* regulon and may suggest involvement of a different transcription factor. *E. coli* also possesses the *cbl* gene, whose product shows 41% amino acid sequence identity with CysB (17). It has been shown that the *cbl* gene is not essential for assimilation of

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TABLE 1. *E. coli* strains and plasmids used in this study

Strain or plasmid	Genotype or relevant features	Reference or source
Strains		
MC4100	F$^-$ *araD139* Δ(*argF-lac*)*U169 rpsL150 relA1 deoC1 ptsF25 rbsR flbB5301*	4
DH5α	*supE44* Δ*lacU169*(ϕ80 *lacZ*Δ*M15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Laboratory collection
JC7623	F$^-$ *thr-1 ara-14 leu-6 proA2 lacY1 sbcC201 tsx-33 galK2 sbcB15 his-4 recC22 recB21 rpsL31 xyl-5 mtl-1 argE3 thi-1*	*E. coli* Genetic Stock Center
EC1250	MC4100 *trp-1*	18
EC2275[a]	EC1250 *cysB*	This study
EC2524	EC1250 Δ*cbl* Cm^r	This study
EC2541	EC1250 Δ*cbl* Kan^r	This study
MW108	MC4100 Φ(*tauD*::*lacZ*) (λp*lac*Mu9)	37
MW108*cysB*	MW108 *cysB trpB*::Tn*10*	37
MW108*cbl*	MW108 Δ*cbl* Cm^r	This study
Plasmids		
pTrc99A	Expression vector, Ap^r, *trc* promoter, pBR322 ori	Pharmacia
pBR325	Cloning vector, Ap^r Tet^r Cm^r, pBR322 ori	Gibco-BRL
pUC4KIXX	Kan^r Ble^r cartridge	Pharmacia
pME4101	Ap^r, *tauABC′* in pUC18	37
pME4131	Ap^r Kan^r *tauA′*::*′lacZ*	37
pMH121	Ap^r, *cbl* on 2.65-kb *Pvu*II fragment of λ347 (Kohara library) cloned in pBR322 *Ssp*I-*Eco*RV	This study
pMH128	Tet^r, *cbl* on 1.6-kb *Sma*I-*Dra*I fragment of pMH121 cloned in pBR322 *Sca*I	17
pMH139	Cm^r Δ*cbl*, obtained by excision of 1-kb *Mun*I-*Eco*RV fragment of pMH121 and insertion of *cat* gene on 1.3-kb *Bst*UI fragment from pBR325	This study
pMH149	Kan^r Δ*cbl*, obtained by excision of 1-kb *Mun*I-*Eco*RV fragment of pMH121 and insertion of *kan* cartridge as a 1.2-kb *Sma*I fragment from pUC4KIXX	This study
pMH176	Ap^r p*trc-cbl*, *Bsp*HI/*Bam*HI-digested 1036-bp PCR product obtained with primers MH1 and MH2 from template pMH121 inserted in pTrc99A *Nco*I/*Bam*HI	This study
pMHK28	Ap^r p*trc-cysB*, *Eco*RI/*Sst*I-digested PCR fragment obtained with primer MH3 and universal forward M13/pUC primer from template pTEC30 cloned in pTrc99A	This study
pJOH1	Ap^r *cysB* (*E. coli*) cloned in pBR322	27
pTEC30	Ap^r *cysB* (*S. typhimurium*) cloned in pT7T3	N. M. Kredich

[a] *cysB* mutation was P1 transduced to EC1250 from strain NK1 (6).

sulfate in wild-type *E. coli*, although a *cbl* mutant grows somewhat more slowly with sulfate than does the wild-type strain (17). Since expression of *cbl* is CysB dependent, it seems likely that *cbl* also plays a role in sulfur metabolism.

Here we report that the *cbl* gene product is an essential positive factor for expression of the *tauABCD* operon and for utilization of several alkanesulfonates whose genes have not yet been identified. We also show that both the CysB and Cbl proteins interact with the *tauA* promoter region. The interrelationship of CysB and Cbl in *tauABCD* expression is discussed on the basis of in vitro binding studies of both proteins to the *tauA* promoter region.

MATERIALS AND METHODS

Chemicals. Restriction endonucleases and T4 DNA ligase were obtained from Gibco-BRL Life Technologies (Paisley, United Kingdom) or MBI Fermentas (Vilnius, Lithuania). DNase I came from Gibco-BRL, and *Taq* polymerase was from MBI Fermentas or from Promega (Madison, Wis.). [γ-^{32}P]ATP was supplied by Amersham Buchler (Braunschweig, Germany). *O*-Acetyl-L-serine (OAS) was from Sigma-Aldrich (Poznan, Poland). All other chemicals were from Aldrich (Buchs, Switzerland), Fluka (Buchs, Switzerland), Acros (Basel, Switzerland), or Merck (Zürich, Switzerland) and were of the highest purity grade available.

Bacterial strains, plasmids, and growth conditions. Strains of *E. coli* and plasmids used in this study are listed in Table 1. All strains were grown at 37°C either in Luria-Bertani (LB) medium (32) or sulfur-free minimal medium (37) supplemented with 0.2% glucose and the desired sulfur source to a final concentration of 0.25 mM. When required, growth media contained ampicillin (100 μg/ml), tetracycline (15 μg/ml), chloramphenicol (20 μg/ml), or kanamycin (25 μg/ml).

Construction of *cbl*-null mutants. Deletions within the *cbl* gene were created by excision of the 1-kb *Mun*I-*Eco*RV fragment of plasmid pMH121, encompassing the promoter region and 94% of the *cbl* open reading frame. The deletion was tagged by insertion of either the *cat* gene excised from pBR325 as a 1.3-kb *Bst*UI fragment or the *kan* cassette excised from pUC4KIXX as a 1.2-kb *Sma*I fragment. The resulting plasmids (pMH139 and pMH149, respectively) were linearized and transformed into *E. coli* JC7623. Cm^{r}Ap^s or Kan^{r}Ap^s transformants were selected, and correct replacement of the *cbl* gene was confirmed by Southern hybridization of genomic DNA with *cbl*-, *cat*-, or *kan*-specific probes and by PCR analysis using *cbl*-specific primers. The Δ*cbl*::*cat* and Δ*cbl*::*kan* mutations were then transferred to strain EC1250 by P1-mediated transduction to give, respectively, strains EC2524 and EC2541. P1 transduction of Δ*cbl*::*cat* from EC2524 to strain MW108 gave strain MW108*cbl.*

Oligonucleotides. Oligonucleotides were synthesized at the Institute of Biochemistry and Biophysics (Warsaw, Poland) or were obtained from Microsynth (Balgach, Switzerland). MH1 (5′-TTA**TCA**TGAATTTCCAACAACT-3′ [bp 284 to 305 of the *cbl* gene sequence]) (17) and MH2 (5′-**CGGGA**TCCGTATGTCA CTGGTT-3′ [complementary to bp 1297 to 1320 of the same sequence]) were used as the forward and reverse primers, respectively, for amplification of the *cbl* gene for cloning in the pTrc99A expression vector. MH3 (5′-**GGAATT**CTAAT CTGGATGATGTATT-3′ [bp −19 to −1 of the *cysB* region sequence]) (27) and M13/pUC universal forward primer (Pharmacia) were used as the forward and reverse primers, respectively, for PCR amplification of the *S. typhimurium cysB* gene from pTEC30. JP8 (5′-TGTG**GGATC**CGATGAAATT-3′ [complementary to bp 33 to 51 of the sequence shown in Fig. 5]) and JP9 (5′-GATTGAGAAT **TC**TACAGTGA-3′ [bp −266 to −247 of the same sequence]) served for amplification of the *tauA* promoter region. Nucleotides that have been changed to introduce restriction sites are shown in boldface.

Two-dimensional PAGE of total cell proteins. For two-dimensional polyacrylamide gel electrophoresis (PAGE) of cell proteins, *E. coli* MC4100 or EC2541 cells were grown in minimal medium and harvested in the mid-exponential phase (A_{650} = 0.7). Cell pellets were prepared for electrophoresis by lysis in sodium dodecyl sulfate (SDS) buffer followed by nuclease treatment and saturation with urea (12). Two-dimensional PAGE was done as previously described (21), and the proteins were visualized by silver staining (1).

β-Galactosidase assays. β-Galactosidase activities were assayed in cells taken from mid-log-phase cultures either by the method of Miller (26) with *o*-nitrophenylgalactoside (ONPG) as a substrate or fluorometrically with 4-methylumbelliferyl-β-D-galactopyranoside as a substrate as previously described (37).

Preparation of extracts containing Cbl and CysB. *E. coli* extracts for gel mobility shift assays and DNase I footprinting were prepared from strain DH5α harboring pMH176 (*cbl*$^+$), pMHK28 (*cysB*$^+$), or pTrc99A (vector only). Transformed cells were grown in LB-ampicillin, and isopropylthiogalactopyranoside (IPTG) was added (0.5 mM, final concentration) in early exponential phase (A_{600}

= 0.15). Growth was continued for a further 2 h, and cells were then harvested by centrifugation and resuspended in buffer A (50 mM Tris-Cl [pH 7.5], 1 mM Na_2EDTA, 1 mM phenylmethylsulfonyl fluoride). Cell suspensions were sonicated on ice, and cellular debris was removed by centrifugation at 16,000 × *g* for 30 min at 4°C. Ammonium sulfate (229 mg/ml) was added to the supernatants, and the precipitated proteins were collected by centrifugation and redissolved in buffer A. These extracts were then used for gel mobility and DNase protection studies. For the CysB preparation, the ammonium sulfate step was preceded by a streptomycin sulfate precipitation step as described by Miller and Kredich (25); for other preparations, this step was omitted. The amount of CysB and Cbl proteins in the extracts was estimated by SDS-PAGE and Coomassie blue staining to be not less than 50% of total protein.

Highly purified CysB protein of *S. typhimurium* was a generous gift of N. M. Kredich. Attempts to purify Cbl to homogeneity were unsuccessful due to rapid aggregation of protein into insoluble material. Since highly purified CysB and a CysB-enriched extract gave identical results in DNA binding assays with *tauA* and *cysP* promoters, we assume that our Cbl preparation also retained at least 50% of the specific binding activity of the pure protein. In most experiments described below we used highly purified CysB protein, which is referred to as CysB, and an extract with overexpressed Cbl protein containing about 50% Cbl protein, referred to as Cbl. The amounts of each protein used in the binding experiments reflect this difference.

DNA manipulations. Standard procedures (1) were used for transformation, plasmid isolation, restriction enzyme digestion, 5′ labeling of DNA fragments, and Southern hybridization. PCR was carried out in a Perkin-Elmer Cetus Gene Amp System 2400. Reaction mixtures contained 1 to 2 ng of template, 50 pmol of each primer, 10 nmol of each deoxynucleoside triphosphate, and 2 U of *Taq* polymerase (Promega) in a final volume of 50 μl. Amplification was performed by 25 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 30 s to 1 min, depending on the length of the amplified fragment.

DNA fragments containing the *tau* promoter region were generated either by PCR (317-bp fragment obtained with primers JP8 and JP9 and plasmid pME4101 as a template) or by restriction cleavage of plasmid pME4101 and isolation of the 506-bp *Mun*I-*Bgl*II fragment. DNA fragments were 5′ labeled with [γ-^{32}P]ATP, using T4 polynucleotide kinase (Promega). For the DNase I protection assay, the 317-bp DNA fragment was labeled on either the transcribed or the nontranscribed strand by using 5′-labeled JP8 primer or 5′-labeled JP9 primer, respectively, for the PCR amplification. Labeled DNA fragments were purified by QIAquick spin columns (Qiagen).

DNA binding assays. The gel mobility shift method (9, 10) was used to study the binding of CysB and Cbl proteins to the *tau* control region. Reaction mixtures (20 μl) contained approximately 10 ng of labeled DNA fragment and 2 μg of sonicated calf thymus DNA per ml to reduce unspecific binding, in a buffer consisting of 40 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 100 mM KCl, 1 mM dithiothreitol, and 100 μg bovine serum albumin per ml. The binding proteins added were highly purified CysB, CysB-enriched extract, or Cbl-enriched extract at various final concentrations. An extract obtained from cells containing the expression vector pTrc99A served as a control for binding specificity, since this vector was used for CysB and Cbl overexpression. Some samples also contained OAS, sodium thiosulfate, or sodium sulfate at specified concentrations. Samples were incubated at 37°C for 5 min and then separated on a 5% acrylamide-bisacrylamide (82:1) nondenaturing gel in 0.05 M Tris-borate-EDTA buffer (pH 8.3) for 1.5 h at 10 V/cm. Radiolabeled bands were visualized by autoradiography.

Although *N*-acetyl-L-serine is thought to be the true inducer of CysB-dependent transcriptional activation (28, 35), OAS was shown to function better in gel mobility shift experiments and equally well in DNase I protection assays (13, 28). The differences between the two are mostly operational and have been discussed previously (23). In this study, OAS was used as an inducer with CysB and is referred to in the text as acetylserine.

DNase I protection assay. Incubation mixtures were similar to those used for DNA binding studies except that approximately 100 ng of 5′-labeled DNA fragment (0.5×10^5 to 1×10^5 dpm) and 2 mM $MgCl_2$ were included. DNase I digestions were done as described earlier (13). The products of DNase digestion were analyzed on 6% polyacrylamide–7 M urea sequencing gels alongside a sequencing reaction (33) performed with the same fragment as a standard.

RESULTS

The *cbl* gene is required for growth with organic sulfonates. *E. coli* MC4100 is able to utilize a number of organic sulfonates, including taurine, *n*-alkanesulfonates (C_2 to C_6), isethionate, sulfoacetate, 3-morpholinopropanesulfonate, and piperazine-1,4-bis-2-ethanesulfonate, as sole sulfur sources. To study the effect of the *cbl* gene on sulfonate utilization, we generated a deletion in the *cbl* gene, with concurrent insertion of a kanamycin resistance cassette, and recombined this allele onto the chromosome of strain EC1250 to give strain EC2541. This strain was unable to utilize any of the sulfonates listed above as a sole sulfur source, though growth with other sulfur sources was unaffected. Transformation of strain EC2541 with plasmid pMH128, containing the wild-type *cbl* gene, or with plasmid pMH176, expressing low levels of Cbl from the *trc* promoter (without addition of IPTG), restored the ability to grow with all the tested sulfonates to the same level as seen for the wild-type strain. This finding indicates that the *cbl* gene product is required for taurine utilization and also for utilization of other sulfonates as sulfur sources.

In contrast to *E. coli*, *S. typhimurium* cannot utilize sulfonates as sulfur sources (36). To test for the presence of the *cbl* gene in *S. typhimurium*, we probed genomic DNA isolated from *S. typhimurium* LT2 by Southern hybridization with a DNA fragment internal to the *cbl* coding sequence. The *E. coli cbl*-specific probe gave no signal with *Salmonella* DNA (data not shown) at the same stringency of wash conditions as used previously to clearly detect the *E. coli* chromosomal *cbl* sequence with a *Klebsiella aerogenes cbl*-specific probe (17). This result as well as the comparison of the gene order at corresponding positions in gene maps of *E. coli* (17) and *S. typhimurium* (5; see also the sequence deposited in GenBank under accession no. L35477) suggest that the *cbl* gene is absent from the *S. typhimurium* genome.

Cellular protein pattern of a *cbl*-null mutant. An additional indication that the *cbl* gene product is required for the expression of multiple genes was obtained by comparison of the two-dimensional electrophoretic pattern of protein extracts prepared from strain EC2541 with that of the wild type-strain (MC4100). The results of this analysis are presented in Fig. 1. During growth with lanthionine or glutathione as the sulfur source, wild-type *E. coli* cells showed upregulation of the sulfate starvation-induced proteins (Fig. 1A), which have previously been identified (30). The protein pattern observed for the *cbl* mutant under these sulfate starvation conditions resembled the pattern normally seen for sulfate-grown wild-type cells (30): the sulfate starvation-induced proteins TauA, TauD, Ssi4, Ssi6, and CysK were no longer present, and the level of the sulfate-binding protein Sbp was greatly reduced (Fig. 1B). The *cbl* gene product is therefore clearly required for synthesis of sulfate-regulated proteins. Interestingly, although the *cbl* mutant can grow with sulfate (17), the *cbl* gene knockout evidently affects the synthesis of two proteins, Sbp and CysK, involved in the sulfate assimilatory pathway. The ability of the *cbl* mutant to assimilate sulfate may be explained by the fact that *E. coli* contains the functional backups for Sbp and CysK, namely, CysP and CysM, respectively (24).

During growth with lanthionine or glutathione, several proteins were observed to be upregulated in the *cbl* mutant in comparison to the wild-type strain, but these proteins have not been further investigated.

Regulation of *tauA* promoter in vivo. Evidence that Cbl is involved in regulation of expression of the *tauABCD* genes was provided by measurement of β-galactosidase activities in strains containing *tauA′-′lacZ* or *tauD′-′lacZ* protein fusions (Table 2). The chromosomal *tauD′-′lacZ* fusion was not expressed in strain MW108*cbl*, and the *tauA′-′lacZ* fusion on plasmid pME4131 was not expressed in strain EC2541 (Δ*cbl* Kan^r [Table 1]). Introduction of plasmid pMH176, containing *cbl* under control of the *trc* promoter, could restore expression of *tauD′-′lacZ* in MW108*cbl* but not in MW108*cysB* (Table 2). From these results, we conclude that Cbl is a positive factor required for *tauABCD* expression. We further conclude that CysB, in addition to its role as a positive factor required for *cbl* expression, has a second function that is critical for controlling *tauABCD* expression. Given the known role of CysB as a tran-

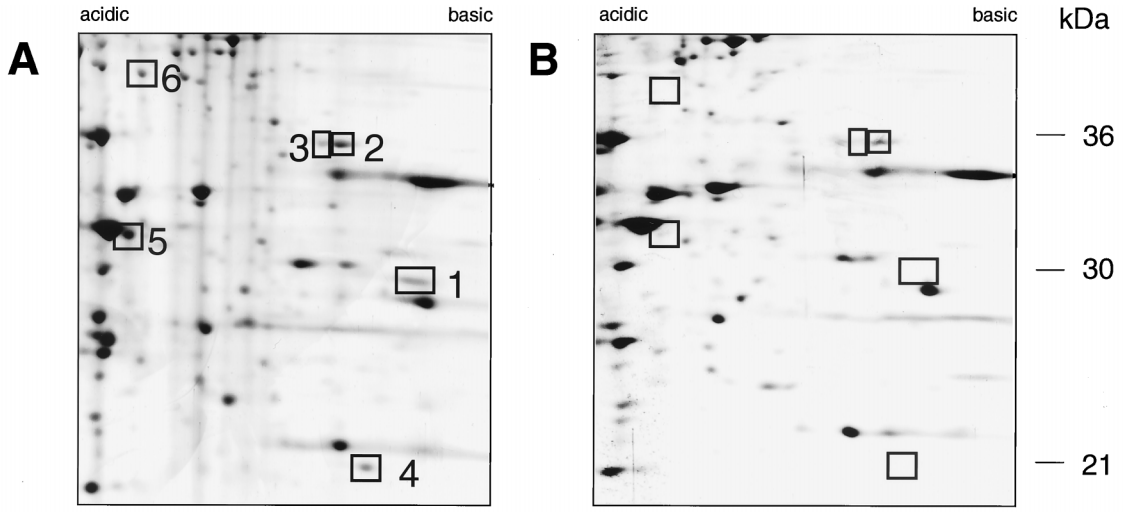

FIG. 1. Two-dimensional PAGE separation of total cell extracts from strains MC4100 (A) and EC2541 (B) after growth in minimal salts medium with lanthionine as the sole sulfur source. The sulfate starvation-induced proteins indicated are as follows: 1, TauA; 2, sulfate-binding protein Sbp; 3, TauD; 4, Ssi4; 5, cysteine synthase CysK; and 6, Ssi6 (30). These proteins are absent from gels prepared from extracts of either strain after growth with sulfate (not shown).

scriptional activator of genes in the sulfate starvation regulon, we formulated the hypothesis that Cbl and CysB together directly function as transcriptional activators of *tauABCD*.

Binding of CysB and Cbl proteins to the *tau* regulatory region. We first tested the potential interaction of the CysB and Cbl proteins with the *tau* promoter region by a gel mobility shift assay, using a radiolabeled 317-bp fragment extending from positions −266 to +51 relative to the major transcription start site of *tau* operon (37) (see Fig. 5). Incubation of purified CysB protein with this fragment resulted in formation of the complex, designated C1, at levels proportional to the CysB concentration added (Fig. 2, lanes 1 to 4). Acetylserine (OAS; 1 to 10 mM, Fig. 2, lanes 5 to 7) stimulated formation of this complex and slightly increased its mobility, and also led to the appearance of more slowly migrating complexes, C2 and C3. Incubation of partially purified Cbl extract (Cbl overexpressed from cells transformed with plasmid pMH176 [see Materials and Methods]) with the *tau* promoter fragment resulted in formation of a single complex (Fig. 2, lanes 8 to 11) whose mobility was comparable to that of the C1 complex formed with CysB. The amount of the Cbl-DNA complex increased proportionally to the amount of Cbl preparation added and was not affected by the presence of acetylserine.

The appearance of multiple complexes of the *tau* promoter fragment with CysB (Fig. 2), but only a single complex formed with Cbl, suggested the presence of more that one binding site for CysB but a unique binding site for Cbl. The 317-bp *tau* promoter fragment was cleaved with *Mun*I (see Fig. 5) to give two fragments, the 141-bp upstream fragment and the 176-bp downstream fragment. The 141-bp upstream fragment was able to bind CysB but did not form a complex with Cbl, whereas the 176-bp downstream fragment formed complexes with both CysB and Cbl proteins (data not shown). For further binding studies with the promoter-proximal region of *tau*, we used the 506-bp *Mun*I-*Bgl*II fragment extending from bp −126 to +380 relative to the *tau* transcription start site (*Bgl*II site within the *tauA* coding region [37]). Binding of CysB to this fragment (Fig. 3) in the presence of acetylserine gave C1 and C2 complexes (lane 6), suggesting that the sequence located downstream of the *Mun*I site may still contain more than one binding site for CysB.

Previous studies on the mechanism of activation of *cys* gene

TABLE 2. β-Galactosidase activities in strains containing fusion *tauA'-'lacZ* on a plasmid (pME4131) or chromosomally encoded fusion *tauD'-'lacZ* (MW108)[a]

Sulfur source for growth	β-Galactosidase activity						
	Miller units		pmol of MUF/ml/min/OD_{600}				
	EC1250(wt) (pME4131)	EC2541(*cbl*) (pME4131)	MW108	MW108*cbl*	MW108*cysB*	MW108*cbl* (pMH176)	MW108*cysB* (pMH176)
Sulfate	9	1.8	<5	<5	NG[b]	<5	NG
Cystine	2	<1	<5	<5	<5	<5	<5
Glutathione	454	4.6	84	<5	<5	105	<5
Methionine	201	<1	100	<5	<5	49	<5
Taurine	1,633	NG	NG	NG	NG	NG	NG
Butanesulfonate	ND[c]	NG	2,801	NG	NG	1,876	NG
Lanthionine	672	<1	748	<5	<5	1,594	<5

[a] Chromosomally encoded β-galactosidase in strain MW108 was measured with the MUF (4-methylumbelliferone) assay, whereas plasmid (pME4131)-encoded activity was measured with ONPG (see Materials and Methods). In our hands, 1 Miller unit corresponded to approximately 100 pmol of MUF/ml/min/unit of optical density at 600 nm (OD_{600}). wt, wild type.

[b] NG, no growth.

[c] ND, not determined.

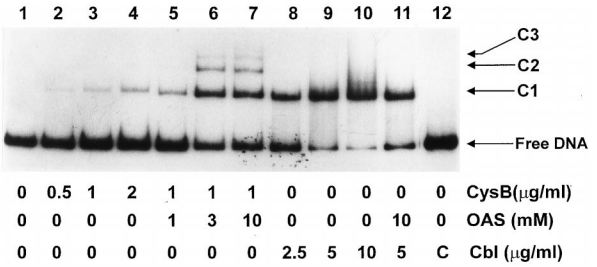

FIG. 2. Binding of CysB and Cbl proteins to the *tau* regulatory region in a gel mobility shift assay. The 5′-labeled fragment extending from positions −266 to +51 relative to the *tau* transcription start site was preincubated with purified CysB or Cbl crude extract (at protein concentrations indicated), OAS (where indicated), and 2 μg of sonicated calf thymus DNA per ml to reduce unspecific binding. Mixtures were run on a 5% polyacrylamide gel as described in Materials and Methods. The protein extract prepared from cells containing the vector used for Cbl overexpression was used at 20 μg/ml as a control for Cbl binding specificity (designated C in lane 12). The most rapidly migrating primary complexes, (C1) and higher-order complexes (C2 and C3) are discussed in the text.

promoters by CysB have shown that thiosulfate is a potent anti-inducer of CysB-dependent transcription (13), but the effect of thiosulfate on binding of CysB to these promoter regions was not evaluated. Unexpectedly, addition of 1 mM thiosulfate did not inhibit the interaction between CysB and the *tau* promoter fragment but actually stimulated binding (Fig. 3; compare lanes 2 and 4 as well as lanes 5 and 7). Sulfate, which has no effect on CysB-dependent in vitro transcription of *cys* genes (13), also did not affect binding of CysB to this fragment (Fig. 3; compare lanes 5 and 8). In the presence of Cbl (5 to 10 μg/ml), a single DNA-protein complex was formed with the *Mun*I-*Bgl*II fragment. The presence of thiosulfate did not influence the amount of this complex formed or its mobility (Fig. 3, lanes 9 to 11).

Binding sites for CysB and Cbl in the *tau* promoter region. To identify which parts of the *tau* control region are responsible for binding of CysB and Cbl, DNase I footprinting assays were performed. Protection against DNase I digestion was studied with the 317-bp *tau* promoter fragment, extending from positions −266 to +51, labeled on either the transcribed or the nontranscribed strand. In the presence of CysB protein alone, protection of the transcribed strand (Fig. 4A) is evident in the region between positions −224 to −183 and also, although barely detectable, in the region from bp −120 to −11. Protection of the downstream region (bp −120 to −11) was more pronounced in the presence of acetylserine. Only one

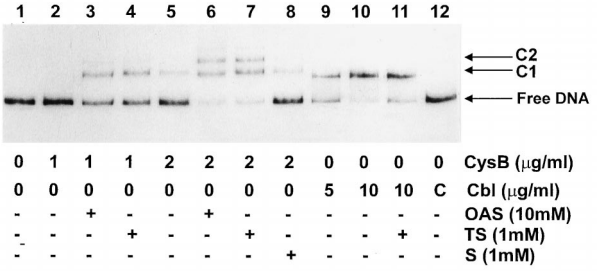

FIG. 3. Binding of CysB and Cbl proteins to the *tau* promoter fragment starting at the *Mun*I site. Reaction mixtures contained 5′-labeled 506-bp restriction fragment *Mun*I-*Bgl*II (extending from positions −126 to +380 relative to the transcription start site of *tauABCD*), sonicated calf thymus DNA at 2 μg/ml, and the indicated amounts of purified CysB, crude Cbl, OAS, sodium thiosulfate (TS), and sodium sulfate (S). Control protein extract (C; lane 12) was used at 20 μg/ml. DNA-protein complexes designated C1 and C2 are described in the text.

distinct region was protected by the Cbl protein, between bp −112 and −68 of the transcribed strand.

Footprinting analysis of the nontranscribed strand (Fig. 4B) confirmed the results obtained with the transcribed strand and provided additional information on DNase I-hypersensitive sites. In the −221 to −187 CysB protection region, a DNase-hypersensitive site was seen at position −206; this site was not observed in the presence of acetylserine. In the downstream region (−120 to −14), two sites (−105 and −71) appeared more sensitive to DNase in the absence of acetylserine. The data obtained by footprinting analysis are summarized in Fig. 5.

DISCUSSION

The *tauABCD* operon is required for the utilization of the natural sulfonate taurine as a sulfur source by *E. coli* and represents a class of genes whose members are expressed only in the absence of sulfate. Proteins encoded by members of this *ssi* (sulfate starvation-inducible) gene class have been characterized by two-dimensional electrophoresis of *E. coli* extracts from cells starved for sulfate (21, 30). We report here that the *cbl* gene product, a LysR-type transcriptional regulator that is closely related to CysB (17), is required for utilization of taurine and other sulfonates as sulfur sources and also plays a role in expression of several proteins induced by sulfate starvation, including TauA and TauD, the sulfate-binding protein Sbp, and cysteine synthase CysK. These findings make it attractive to speculate that Cbl is a general transcription factor for genes related to sulfonate-sulfur utilization and possibly for *ssi* genes in general. In this report, we have focused on the regulation of the *tauABCD* operon as a possible paradigm for other *ssi*-type genes.

It has previously been reported that the *tauABCD* operon is not expressed in vivo in the presence of a *cysB* mutation (37). We found that a *cbl* mutation also completely abolishes the expression of *tauABCD*, assayed as β-galactosidase activity from either *tauA′-′lacZ* or *tauD′-′lacZ* fusions. The situation is complicated by the fact that expression of the *cbl* gene itself is CysB regulated (17). However, when the Cbl protein was provided in the absence of functional CysB (by expressing the *cbl* gene from a *trc* promoter), the *tauD′-′lacZ* fusion was still not expressed. These results led us to conclude that Cbl is an essential transcription factor for *tauABCD* expression and, furthermore, that activity of the *tau* promoter region also requires the direct involvement of the CysB protein.

To gain further insight into the interrelationship between CysB and Cbl in activation of *tauABCD* expression, we undertook in vitro studies of binding of both proteins to the *tau* promoter region and found that a 317-bp DNA fragment containing 266-bp region preceding the transcription start site of *tau* bound both CysB and Cbl proteins in a specific manner. Although binding stoichiometry was not investigated in this study, we believe that the most rapidly migrating bands (C1 in Fig. 2) correspond to primary complexes containing a single molecule of each protein bound to the *tau* control region. From earlier studies on the interaction of CysB with *cysJ*, *cysK*, and *cysP* control regions, it is known that CysB binds to DNA as a tetramer, giving a primary complex with a mobility of 0.6 to 0.7 relative to uncomplexed DNA fragments of about 320 bp (14). The primary complexes (C1) obtained with CysB and Cbl with the 317-bp *tau* promoter fragment show similar relative mobilities, and since CysB and Cbl monomers are similar in size (324 and 316 amino acids, respectively) (17, 27), it is likely that Cbl, like CysB, binds to DNA as a tetramer.

A single binding site for the Cbl protein (Cbl-BS) in the *tau*

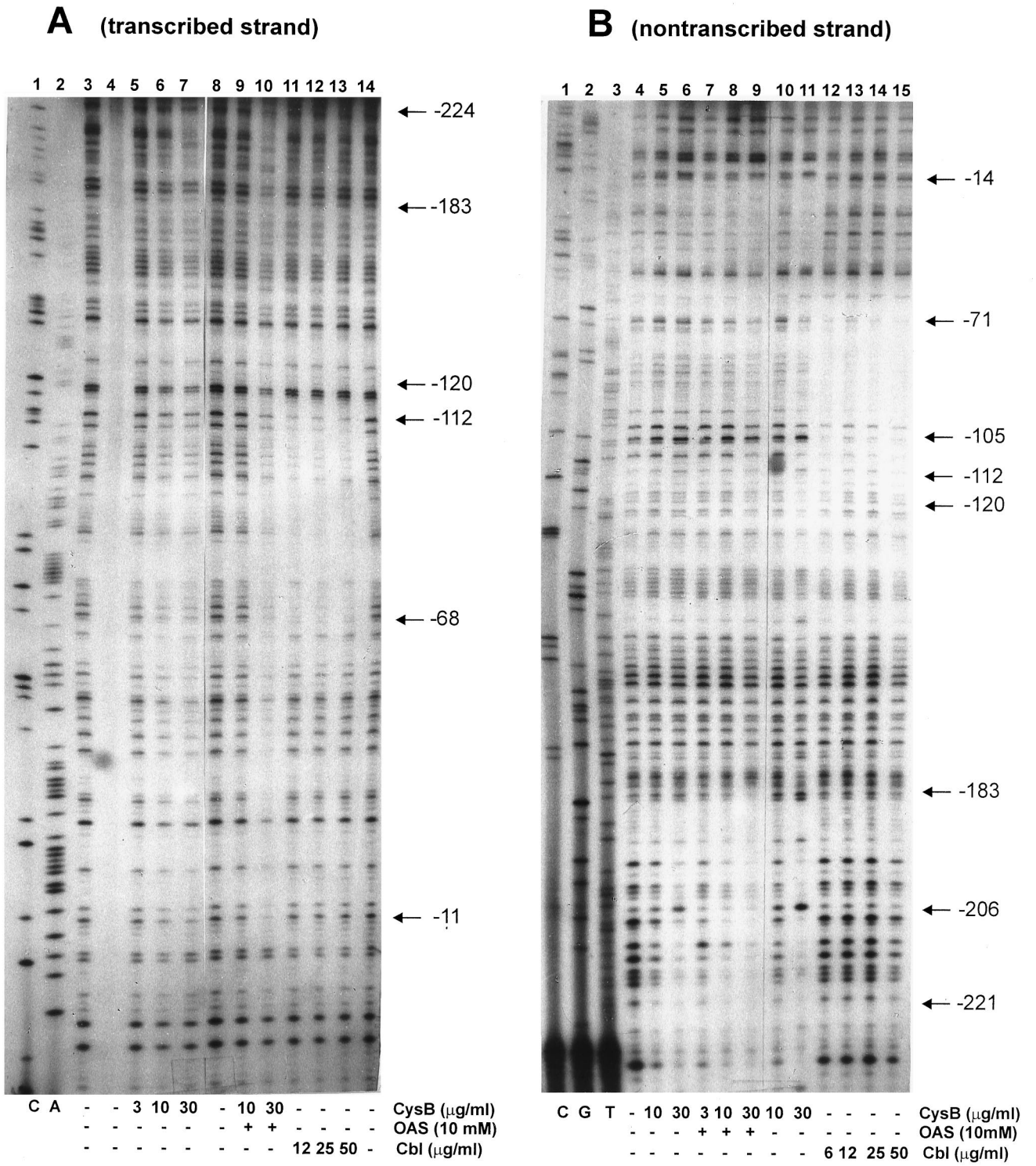

FIG. 4. DNase I protection of the *tau* promoter region by CysB and Cbl proteins. The 317-bp DNA substrate (extending from positions −266 to +51 relative to the transcription start site of *tau*) was generated by PCR and was labeled on either the transcribed or the nontranscribed strand. CysB protein, Cbl protein (crude extract), and acetylserine (OAS) were present as indicated. CysB was included as purified protein except in reaction mixtures 10 and 11 in panel B (nontranscribed strand), where it was present as a crude extract. In panel A, reaction mixture 4 contained no DNase and reaction mixture 14 contained control protein extract at 200 μg/ml. Sequencing reactions (C and A for the transcribed strand and C, G, and T for the nontranscribed strand) performed with DNA fragments used as substrates for DNase are shown in the leftmost lanes of each panel. The numbers in the margins refer to positions relative to the *tau* transcription start site.

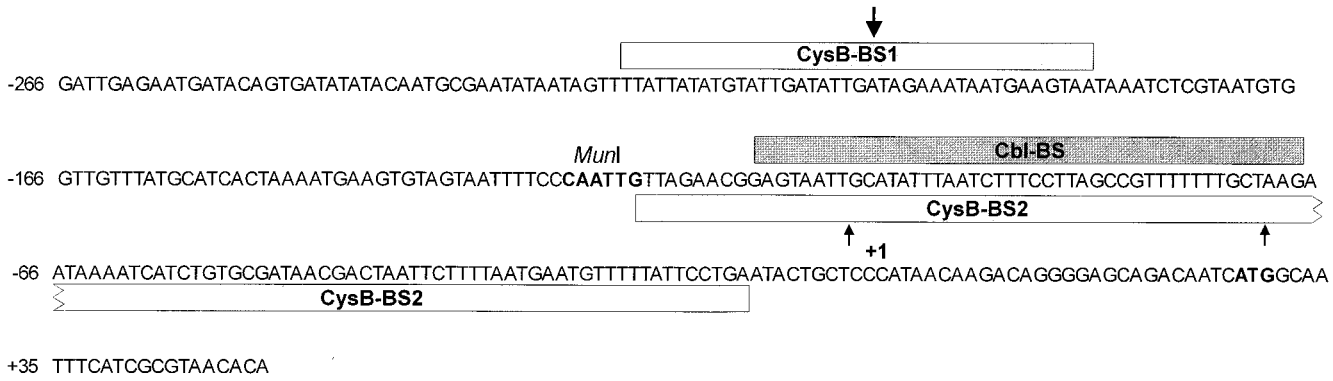

FIG. 5. Binding sites for CysB and Cbl proteins in the *tau* regulatory region identified by DNase I footprinting. The sequence is shown as that of the nontranscribed strand only. The vertical arrows represent sites that are made strongly (thick arrow) or moderately (thin arrows) oversensitive to DNase digestion by CysB in the absence of acetylserine. Positions of the *Mun*I site and *tauA* start codon are boldfaced; the transcription start site is indicated as +1.

control region was identified between bp −68 and −112. The length of Cbl-BS (about 44 bp) is not unusual for binding sites of LysR family members, but its position relative to the transcription start site is atypical. Most characterized LysR-type proteins, including CysB, exert their activatory effects on transcription initiation when bound just upstream of the −35 regions of the cognate promoters; binding in such regions seems to be required for direct contacts with RNA polymerase (see reference 34 for a review).

Our studies on the interaction of CysB with the *tau* regulatory region revealed the presence of two CysB-binding regions designated CysB-BS1 and CysB-BS2. The CysB-BS1 region, by its length and sequence, resembles activating CysB-binding sites in *cysJ*, *cysK*, and *cysP* promoter regions (15, 23). CysB-BS1 is probably a high-affinity binding site that can be occupied by CysB alone even in the absence of acetylserine but whose protection is markedly increased in the presence of inducer. We believe that the C1 complex observed with CysB alone in Fig. 2 represents binding of CysB to CysB-BS1. Interaction of CysB with CysB-BS1 may involve DNA bending and increased susceptibility of particular DNA region to DNase digestion (DNase-hypersensitive site at position −206). The presence of acetylserine leads to a complex of slightly higher mobility and lacking the DNase hypersensitive site; this complex likely contains unbent DNA. The position of CysB-BS1 (between bp −221 and −183) contrasts with the positions of activating CysB-BSs upstream of the *cys* genes, where they lie just upstream of the −35 boxes of the respective promoters. Since truncation of the *tau* promoter fragment to remove the region including CysB-BS1 did not reduce expression of a *tauA′-′lacZ* fusion in vivo (37), it seems unlikely that CysB bound to CysB-BS1 has a direct activating effect on transcription from the *tau* promoter.

The second CysB-binding region, CysB-BS2, lies between bp −122 and −11 relative to the *tau* transcription start site and, as judged from its length, probably allows binding of more than one CysB molecule. The C1 and C2 complexes formed by CysB with a *tau* fragment truncated to bp −126 and containing only CysB-BS2 but not CysB-BS1, may represent binding of one and two molecules of CysB to CysB-BS2, respectively. The overall affinity of CysB to CysB-BS2 seems to be low, as protection of this region was barely detectable in the absence of acetylserine, but binding of CysB to this region is probably cooperative, as both C1 and C2 complexes were always seen together. Surprisingly, we noticed that binding of CysB to CysB-BS2 was stimulated not only by acetylserine but also by thiosulfate. Thiosulfate has been shown to act as a potent anti-inducer of CysB- and acetylserine-dependent transcription initiation in *cys* gene promoters (13), but its effect on binding of CysB to DNA had not been previously evaluated. On the basis of the known interactions of CysB with the *cysP* promoter (14) and our own unpublished experiments (16), we hypothesize that the effect of thiosulfate observed in this study results from locking CysB in a conformation which is favorable for multiple contacts with large regions involving DNA bending but is unfavorable for binding to unique CysB activating sites.

One simple model that explains the function of Cbl and CysB proteins in *tauABCD* expression postulates that Cbl is the primary and essential positive regulator of *tau* promoter and that involvement of CysB represents cross talk of this protein with a particular portion of the CysB-BS2/Cbl-BS region to positively affect the overall promoter efficiency. Speaking in favor of this model, we note that CysB and Cbl have exceptionally high amino acid sequence similarity, including their helix-turn-helix DNA-binding motifs (17), and hence such cross talk is not unlikely. Several other pairs of homologous LysR-type regulators, including CatR-ClcR (29), NocR-OccR (38) GcvA-AmpR (8), and GltR24-GltC (3), have been shown to mutually recognize their binding sites in cognate promoter regions and, in some cases, cross-activate their target genes in vivo.

However, the results presented here speak against this simple model. We have shown that both Cbl and CysB are required for expression of *tauABCD*. We therefore propose that the presence of Cbl alone is not sufficient to activate the *tau* promoter, due to the unfavorable position of its binding site relative to the −10 region. Cbl bound to Cbl-BS would help to recruit CysB (or reposition CysB within the CysB-BS2 region) to the activating-type binding site, favorable for optimal contacts with the transcriptional apparatus. This model of concurrent interaction of two regulatory proteins is supported by examples of CRP-MalT in the *malK* (31) regulatory region and FNR-CRP in the *ansB* regulatory region (19).

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