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In Vitro Interactions of CysB Protein with the *cysK* and *cysJIH* Promoter Regions of *Salmonella typhimurium*

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The cysteine regulons of *Salmonella typhimurium* and *Escherichia coli* are positively regulated by CysB protein and either *O*-acetyl-L-serine or *N*-acetyl-L-serine, both of which act as inducers. Gel mobility shift assays and DNase I footprinting experiments showed that CysB protein binds to the *S. typhimurium cysK* promoter at two sites, one, designated CBS-K1, at positions −78 to −39 relative to the major transcription start site, and the other, designated CBS-K2, at positions −115 to −79. The *S. typhimurium cysJIH* promoter was found to contain a single binding site, designated CBS-JH, at positions −76 to −35. Acetyl-L-serine stimulated binding to CBS-K1 and CBS-J and inhibited binding to CBS-K2. In the absence of acetyl-L-serine, CysB protein bound to both CBS-K1 and CBS-K2 and gave a complex that migrated more slowly during gel electrophoresis than did that formed in the presence of acetyl-L-serine, in which case CysB protein bound only to CBS-K1. Complexes formed with DNA containing the two binding sites either at the middle or at one end of the fragment migrated differently, suggesting that DNA was bent in the slow complex formed in the absence of acetyl-L-serine and that DNA in the fast complex was less bent or not bent at all. An analysis of upstream deletions of the *cysK* promoter showed that only CBS-K1 is required for in vivo promoter activity. CBS-J is analogous in position to CBS-K1 and is probably also required for activity of the *cysJIH* promoter. CBS-K2 has no known function but may help sequester CysB protein at the *cysK* promoter.

Genes of the cysteine regulon in *Salmonella typhimurium* and *Escherichia coli* are positively regulated by the CysB protein (13, 15, 16), a tetramer of identical 36-kDa subunits (20, 24) that is a member of the LysR family of bacterial activator proteins (9). In addition to requiring CysB protein, derepression of this biosynthetic pathway requires sulfur limitation and the L-cysteine precursor *O*-acetyl-L-serine, which acts as an inducer (14, 15). *N*-Acetyl-L-serine is also an inducer (25) and is derived nonenzymatically from *O*-acetyl-L-serine by an intramolecular *O*-to-*N*-acetyl shift (6). The requirement for sulfur limitation appears to be due to an anti-inducer effect of sulfide, which interferes with the ability of CysB protein and inducer to activate gene expression both in vivo and in vitro (14, 15, 26).

In vitro studies have shown that transcription initiation from the *cysJIH* promoter requires CysB and either *N*-acetyl-L-serine or *O*-acetyl-L-serine (25) and that CysB protein binds to DNA containing the *cysJIH* promoter region (26). Binding occurs in the absence of inducer but is stimulated severalfold by inducer and is inhibited by sulfide (26). *cysK*, which encodes the enzyme *O*-acetylserine (thiol)-lyase A (11), also requires CysB protein and inducer for maximal expression. The *cysK* genes from both *S. typhimurium* and *E. coli* have been cloned and sequenced, and their in vivo transcription initiation sites have been identified by primer extension and S1 nuclease protection techniques (4). In this report, we demonstrate that CysB protein binds to DNA sequences immediately upstream of the −35 regions of both the *cysK* and *cysJIH* promoters and that there are two binding sites for the *cysK* promoter and a single site for the *cysJIH* promoter. Analyses of *cysK* promoter deletions show that only the downstream binding site is required for promoter activity. Evidence is also presented that indicates that binding of CysB protein to the *cysK* promoter induces DNA bending in the absence of inducer but little or no bending in the presence of inducer.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and media. *E. coli* JM105 was the transformation recipient for isolating pUC19 derivatives (36) and the host for M13 phage propagation. NM522 [*hsdΔ5* Δ(*lac-pro*)(F′ *pro*$^+$ *lacI*^{q}*ZΔM15*)] was the host for pT7T3 phagemid derivatives. *S. typhimurium* strains included the wild-type LT2 strain, *cysB403*, DW46 (*cysB484 trpA160*), and DW48 (*cysB1352 trpA160*) (15). *S. typhimurium* LB5000 (*metA222 metE551 trpD2 leu hsdLT hsdSB hsdSB*) is r$^-$ m$^+$ for all three *S. typhimurium* restriction-modification systems (3) and was the initial recipient for transferring plasmids from *E. coli* by transformation. Phage P22HT lysates of these transformants were then used to transfer plasmids to other strains by transduction (22). pRSM28 is a pBR322 derivative that contains *cysZ*, *cysK*, *ptsH*, and a portion of *ptsI* from *S. typhimurium* LT2 (4) and served as the source of the *cysK* promoter. *cysJIH* promoter fragments were derived from the *S. typhimurium* LT7 *cysJIH* insert of pGBK5 (25).

Medium E (34) prepared with an equimolar amount of $MgCl_2$ in place of $MgSO_4$ served as our minimal medium and was supplemented with 0.5% glucose and either 1 mM Na_2SO_4, 0.5 mM L-cystine, or 1 mM L-djenkolic acid as a sulfur source. L-Tryptophan at 0.2 mM was included for auxotrophs. Double-strength YT (21) was used as the rich medium. Where required, chloramphenicol and ampicillin were added at 20 and 25 μg/ml, respectively.

Recombinant DNA, sequencing, and polymerase chain reaction (PCR) methods. Most recombinant DNA methods were those described by Maniatis et al. (17), using enzymes and reagents purchased from Bethesda Research Laboratories, International Biotechnologies, Inc., New England Bio-

labs, and Pharmacia-LKB Biotechnology Inc. Oligodeoxynucleotides were prepared on an Applied Biosystems model 380A automated DNA synthesizer and 5′ labeled with [γ-^{32}P]ATP (3,000 Ci/mmol) and T4 polynucleotide kinase (18). Fragments from restriction digests were treated with *E. coli* alkaline phosphatase prior to 5′ labeling. DNA was sequenced by the dideoxy method (30) as described previously (4, 25) with single-stranded templates derived from derivatives of M13 phage or pT7T3 phagemids (19).

PCR was carried out with a DNA amplification reagent kit from Perkin Elmer Cetus. Reaction mixtures contained 1 to 10 ng of template DNA, 100 pmol of each oligonucleotide, one of which was 5′ labeled with ^{32}P, and 2.5 U of *Taq* polymerase in 100 μl of 10 mM Tris hydrochloride (pH 8.3)–50 mM KCl–1.5 mM $MgCl_2$–0.2 mM each dATP, dGTP, dCTP, and TTP–0.01% gelatin. The mixture was incubated for 36 cycles of 95°C for 1 min, 55°C for 2 min, and 72°C for 2 min. For the final cycle, the 72°C incubation was carried out for 10 min. Following extraction of the mixture with phenol-chloroform-isoamyl alcohol (25:25:1), DNA was precipitated with ethanol, and radiolabeled fragments were identified by agarose gel electrophoresis and radioautography.

Construction of 5′ deletions of the *cysK* promoter region. A promoterless chloramphenicol acetyltransferase gene (*cat*) from pCM7 (Pharmacia-LKB Biotechnology) was inserted as a 0.79-kb fragment into the *Hin*dIII sites of pUC19 and M13mp19 with an orientation away from the multicloning site. pRSM106-A was obtained from the pUC19 derivative by digestion with *Eco*RI and religation to remove a 0.3-kb fragment extending from the multicloning *Eco*RI site to an *Eco*RI site within *cat*. A 175-bp *Fnu*DII-*Hae*III fragment containing the *cysK* promoter region from pRSM28 was inserted into the *Sma*I site of the M13mp19 derivative to give M13mp19-1C, in which the *cysK* promoter was oriented toward *cat* (Fig. 1). This promoter fragment extends from a position 3 bp preceding the *cysK* start codon to an upstream point that includes the last 14 bp of an open reading frame tentatively identified as *cysZ* and is presumed to contain the entire *cysK* promoter (4). The coordinates relative to the major transcription start site are −144 to +31. Deletions originating from the multicloning *Eco*RI site of M13mp19-1C and extending into the upstream portion of the *cysK* promoter were then generated by the method of Dale and Arrow (5), and the extent of each deletion was determined by DNA sequencing. These *cysK* promoter derivatives together with the amino-terminal portion of *cat* were obtained as *Eco*RI fragments and inserted into the *Eco*RI site of pRSM106-A (Fig. 1) to obtain plasmids suitable for studies of *cysK* promoter function. Recombinants containing a reconstituted *cat* gene were selected by resistance to 20 μg of chloramphenicol per ml (a level of resistance provided by an intact promoterless *cat* gene) and were also analyzed by restriction digests to verify the orientation of the *cysK* promoter.

Generation of 5′-labeled promoter fragments. pRSM40 contains *S. typhimurium cysK* with its promoter on a 1.4-kb *Dra*I-*Cla*I fragment inserted between the *Sma*I and *Acc*I sites of pT7T3 19U (Fig. 2A). An *Eco*RI digest was 5′ labeled with ^{32}P and digested with *Nar*I and *Bst*NI to give a 496-bp *Eco*RI-*Nar*I *cysK* promoter fragment, used for gel mobility shift assays, and a labeled 80-bp *Bst*NI-*Eco*RI fragment from the vector, which served as an internal control for nonspecific binding (Fig. 2A). For DNase I footprinting, an *Eco*RI digest of pRSM42, which contains the *cysK* promoter region on a 175-bp *Fnu*DII-*Hae*III fragment inserted in the *Sma*I site of pT7T3 19U, was 5′ labeled and digested with *Bam*HI and *Cla*I to give the *cysK* promoter on a 197-bp *Eco*RI-*Bam*HI fragment labeled on the nontranscribed strand and a labeled 35-bp *Eco*RI-*Cla*I fragment from pT7T3 19U (Fig. 2B). PCR was used with 5′-labeled synthetic oligodeoxynucleotides to amplify the 175-bp *cysK* promoter region of pRSM42 labeled on the transcribed strand and to prepare other labeled *cysK* and *cysJIH* promoter fragments, which are described in Results.

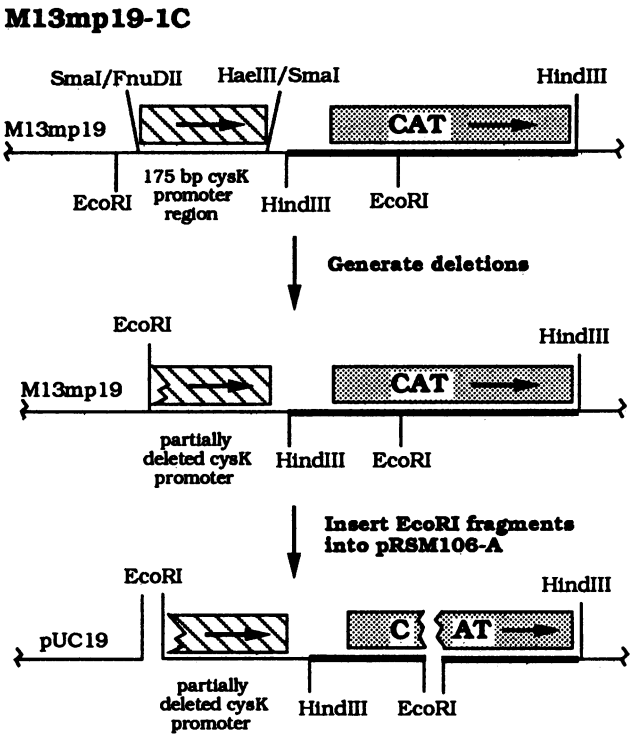

FIG. 1. Construction of upstream deletions in the *cysK* promoter region. M13mp19-1C is an M13mp19 derivative containing a promoterless *cat* gene at the *Hin*dIII multicloning site and a 175-bp *cysK* promoter fragment from pRSM28 (4) at the *Sma*I site. This derivative was used to generate nested deletions (5) beginning at the *Eco*RI multicloning site and extending into the upstream portion of the *cysK* promoter. Partially deleted *cysK* promoters together with the upstream portion of the *cat* gene were excised by *Eco*RI digestion and inserted into the pUC19 derivative pRSM106-A, which contains the downstream portion of *cat*. Recombinants were selected by resistance to 20 μg of chloramphenicol per ml.

DNA-binding assays. DNA binding was studied by the gel mobility shift method (7, 8). Reaction mixtures contained approximately 20 ng of 5′-labeled DNA fragment (1×10^5 to 2×10^5 dpm), various amounts of purified CysB protein, and either *O*-acetyl-L-serine or *N*-acetyl-L-serine in a 20-μl solution of 40 mM Tris hydrochloride (pH 8.0), 10 mM $MgCl_2$, 0.1 M KCl, 1 mM dithiothreitol, and 100 μg of bovine serum albumin per ml. In some experiments, sonicated calf thymus DNA was included at 2 to 5 μg/ml to reduce nonspecific binding. After incubation for approximately 4 min at 37°C (identical results were obtained with incubation times varying between 0.5 and 15 min), 2 μl of 10× loading dye (80 mM Tris hydrochloride [pH 8.0], 0.2 M KCl, 2 mM dithiothreitol, 50% glycerol, 200 μg of bovine serum albumin per ml, 0.1% bromophenol blue) was added, and mixtures were immediately loaded onto a 5% polyacrylamide (acrylamide/bisacrylamide, 82:1) horizontal gel prepared in 10 mM Tris hydrochloride (pH 8.0)–1 mM disodium EDTA that had been prerun for 30 min at 3 V/cm. Following electrophoresis for

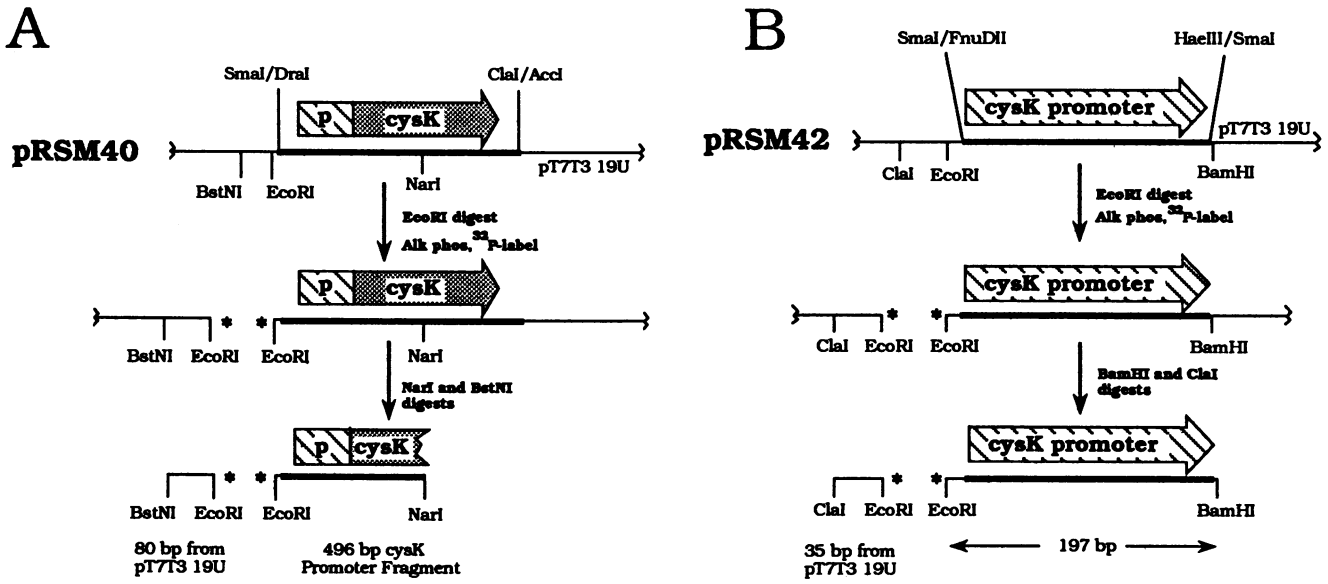

FIG. 2. Generation of 5′-labeled *cysK* promoter fragments by restriction endonuclease digestion. (A) pRSM40, containing *cysK* with its promoter in pT7T3 19U, was digested with *Eco*RI and alkaline phosphatase and 5′ labeled with ^{32}P (*). Digestion with *Nar*I and *Bst*NI gave a 5′-labeled 496-bp *Eco*RI-*Nar*I fragment containing the *cysK* promoter plus a portion of the coding region and an 5′-labeled 80-bp *Bst*NI-*Eco*RI fragment from pT7T3 19U. (B) pRSM42 contains the *cysK* promoter as a 175-bp insert in the *Sma*I site of pT7T3 19U. Following treatment with *Eco*RI and alkaline phosphatase, the DNA was 5′ labeled with ^{32}P and digested with *Bam*HI and *Cla*I to give a 197-bp *cysK* promoter fragment labeled on the nontranscribed strand and a 5′-labeled 35-bp fragment from pT7T3 19U.

0.5 to 1 h at 10 V/cm, the gel was dried onto backing paper, and radiolabeled bands were visualized by radioautography.

DNase I protection assay. DNase I footprinting experiments were performed by using a modification of the procedure of Shi et al. (32). Assay mixtures were similar to those used for DNA-binding studies and contained approximately 20 ng of 5′-labeled DNA fragment (1×10^5 to 2×10^5 dpm), various amounts of purified CysB protein, and either *O*-acetyl-L-serine or *N*-acetyl-L-serine in a 50-μl solution of 40 mM Tris hydrochloride (pH 8.0), 10 mM $MgCl_2$, 0.1 M KCl, 1 mM dithiothreitol, and 100 μg of bovine serum albumin per ml. After preincubation for 5 min at 37°C in a 1.5-ml microfuge tube, 2 μl of DNase I (0.1 μg/ml), 2 μl of sonicated calf thymus DNA (1 mg/ml), and 5 μl of 0.1 M $CaCl_2$ were added to the wall of the tube and spun down to mix. The reaction was carried out at 37°C for 4 min and stopped by the addition of 3.5 μl of 0.25 M disodium EDTA. Following extraction with phenol-chloroform-isoamyl alcohol (25:25:1) and addition of 2 μg of yeast tRNA, DNA was ethanol precipitated, dissolved in water, and analyzed on a sequencing gel.

Other methods. Our methods for the preparation of bacterial extracts (15) and assays for *O*-acetylserine (thiol)-lyase (1) and NADPH-sulfite reductase (33) have been described elsewhere. *S. typhimurium* CysB protein was purified through the methyl agarose step described by Miller and Kredich (20) and was estimated to be 85 to 90% pure. Chloramphenicol acetyltransferase was assayed by the spectrophotometric method of Shaw (31). For all three enzymes, 1 U of activity is that amount catalyzing the formation of 1 μmol of product per min. *O*-Acetyl-L-serine (29) and *N*-acetyl-L-serine (23) were synthesized as described previously. Protein concentrations were determined by the dye-ligand method (2), with bovine serum albumin as a standard.

RESULTS

Binding of CysB protein to the *cysK* promoter region. We initially evaluated the interaction of CysB protein with the wild-type *cysK* promoter by the gel mobility shift assay (7, 8), using a radiolabeled 496-bp *Eco*RI-*Nar*I fragment from pRSM40 (Fig. 2A), which contains the *cysK* promoter region from positions −307 to +170 relative to the major transcription start site (4). This DNA preparation also contained a labeled 80-bp fragment from pT7T3 19U, which served as an internal control for nonspecific binding.

Preincubation with 0.5 μg of CysB protein per ml caused approximately 50% of the *cysK* promoter fragment to migrate as a band with a mobility of 0.52 relative to that of uncomplexed DNA (Fig. 3, lanes 1 and 2). Complex formation was proportional to CysB protein concentration (not shown), and a K_D of approximately 2×10^{-9} M was estimated, assuming that CysB protein binds as a single 144-kDa tetramer. CysB protein did not bind to the 80-bp DNA fragment from pT7T3 19U at concentrations as high as 3 μg/ml. At 0.5 μg of CysB protein per ml, the addition of *O*-acetyl-L-serine stimulated complex formation and also increased the electrophoretic mobility of complexed DNA. Small increases in mobility occurred with as little as 0.1 mM *O*-acetyl-L-serine and reached a limiting value of 0.72 at 10 to 100 mM (Fig. 3, lanes 8 to 10). We refer to this more rapidly migrating band as the fast complex and the band obtained with CysB protein alone as the slow complex. Nonsaturating concentrations of *O*-acetyl-L-serine always gave a single, somewhat broad band with a relative mobility between 0.72 and 0.52. A mobility midway between those of the slow and fast complexes was observed at about 1 mM *O*-acetyl-L-serine. A combination of two discrete bands corresponding to these complexes was never observed.

Addition of *N*-acetyl-L-serine to preincubation mixtures

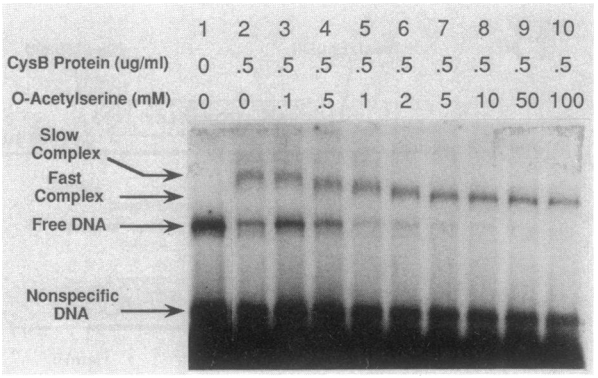

FIG. 3. Binding of CysB protein to a *cysK* promoter fragment in a gel mobility shift assay (7, 8). A 496-bp 5′-labeled *Eco*RI-*Nar*I fragment, containing the *cysK* promoter region from positions −307 to +170 relative to the major transcription start site, was preincubated at 1 μg/ml with a subsaturating concentration (0.5 μg/ml) of CysB protein and various amounts of *O*-acetyl-L-serine for 4 min at 37°C. Mixtures were then run in a 5% polyacrylamide gel as described in Materials and Methods. The DNA preparation also contained an 80-bp *Eco*RI-*Bst*NI fragment from pT7T3 19U, which served as an internal control for nonspecific binding. CysB protein alone gave a band designated the slow complex. *O*-Acetyl-L-serine increased the mobility of complexed DNA and stimulated total binding to the *cysK* promoter fragment. The most rapidly migrating complex is designated the fast complex. The high intensity of the free DNA band in lane 1 is due to a printing artifact and was not noted in the original radioautograph.

also increased the mobility of DNA complexes, but only at concentrations that were 100-fold higher than those required with *O*-acetyl-L-serine (not shown). This differed from our findings with the *cysJIH* promoter, which indicated that the two compounds were equally active in stimulating binding (26). We reasoned that rapid anodal migration of the negatively charged *N*-acetyl-L-serine away from the CysB protein-*cysK* promoter complex during electrophoresis might eliminate any effects on binding, whereas *O*-acetyl-L-serine would not be so affected because it has no net charge at a neutral pH. Indeed, unbound *O*-acetyl-L-serine would probably remain at the origin and slowly isomerize to *N*-acetyl-L-serine (6), thereby providing a steady flow of *N*-acetyl-L-serine toward migrating complexes. In support of this explanation, we observed only the fast complex in a gel run in electrophoresis buffer containing 1 mM *N*-acetyl-L-serine (not shown). These results suggest that although free acetyl-L-serine is not necessary to maintain increased stability once the *cysJIH* promoter-CysB protein complex enters the gel, it is required to stabilize the fast complex of the *cysK* promoter and CysB protein during electrophoresis.

In vitro studies of the *cysJIH* promoter have shown that the anti-inducer sulfide interferes with the ability of acetyl-L-serine to stimulate CysB protein binding (26). Although sulfide also acts as an anti-inducer of *cysK* in vivo (15), it had no consistent effects on gel mobility shifts with *cysK* promoter fragments (not shown). This discrepancy may be related to the results obtained with *N*-acetyl-L-serine, since anodal migration of sulfide during electrophoresis probably exceeds that of *N*-acetyl-L-serine.

DNase I protection of *cysK* and *cysJIH* promoter regions by CysB protein. DNase I footprinting of the *cysK* promoter region was carried out with a 175-bp PCR-generated fragment extending from positions −144 to +31 relative to the major transcription start site, which was 5′ labeled on the transcribed strand, and with a 197-bp *Eco*RI-*Bam*HI fragment from pRSM42 containing the identical region labeled on the nontranscribed strand (Fig. 2B). CysB protein at 25 μg/ml partially protected the transcribed strand from DNase I digestion between positions −110 and −56 upstream of the major transcription start site and also created hypersensitive sites at positions −92 and −82 (Fig. 4, lane 2). In the presence of *O*-acetyl-L-serine, protection from −110 to −80 and formation of the hypersensitive sites were lost, while protection in the region between −78 and −56 was enhanced and extended downstream to position −43 (Fig. 4, lanes 3 to 8). Effects of *O*-acetyl-L-serine were observed at concentrations as low as 0.1 mM, and virtually identical results were obtained with *N*-acetyl-L-serine (not shown). Protection by CysB protein alone was barely detectable at concentrations less than 15 μg/ml but was easily demonstrated for the downstream region at 10 μg/ml in the presence of *O*-acetyl-L-serine (not shown). Similar results were obtained with the nontranscribed strand (Fig. 4, lanes 10 to 13); CysB protein alone at 25 μg/ml partially protected between position −115 and −49 and created hypersensitive sites at positions −87 and −86. As with the transcribed strand, *O*-acetyl-L-serine prevented protection of the upstream region and the formation of hypersensitive sites but stimulated protection of the downstream region and extended it to position −39. These data indicate the presence of two CysB protein-binding sites in the *cysK* promoter region: one, designated CBS-K1, at approximately positions −78 to −39, where binding is stimulated by acetyl-L-serine, and the other, designated CBS-K2, between positions −115 and −79, where binding is inhibited by acetyl-L-serine.

O-Acetyl-L-serine and *N*-acetyl-L-serine also stimulate binding of CysB protein to the *cysJIH* promoter region but do not alter the electrophoretic mobility of such complexes (26). To further characterize the *cysJIH* promoter, DNase I protection was performed with a 221-bp 5′-labeled PCR fragment extending from positions −218 to +3 relative to the major transcription start site (4, 25), which was 5′ labeled on either the transcribed or nontranscribed strand. CysB protein alone at 20 μg/ml had no discernible effect on DNase I digestion, but in the presence of 1 to 10 mM *N*-acetyl-L-serine, protection was observed from positions −71 to −39 on the nontranscribed strand and from −76 to −35 on the transcribed strand (Fig. 5). Virtually identical results were obtained at the same concentrations of *O*-acetyl-L-serine (not shown). In some experiments, CysB protein alone at 40 μg/ml gave slight protection in the region from −70 to −50 but did not protect upstream of position −75 and did not give rise to hypersensitive sites. The binding region between positions −76 and −35 was designated CBS-J and is analogous to CBS-K1 in position and by the fact that its binding to CysB protein is stimulated, rather than inhibited, by acetyl-L-serine.

Characterization of CBS-K1 and CBS-K2 by deletion analyses. To evaluate the functional roles of CBS-K1 and CBS-K2, we generated a series of deletions in the upstream portion of the *cysK* promoter region and inserted these constructs in front of a promoterless *cat* gene in a pUC19 vector for measurements of in vivo promoter strength. The method used to create these deletions (5) resulted in the replacement of upstream promoter sequences by pUC19 sequences ending in 5′-GAATT$(C)_{2-7}$ (Materials and Methods; Fig. 1 and 6). Plasmids were introduced into wild-type *S. typhimurium* LT2, *cysB403*, DW48, and DW46, which were grown on minimal medium containing either L-cystine or the limiting sulfur source L-djenkolic acid and assayed for

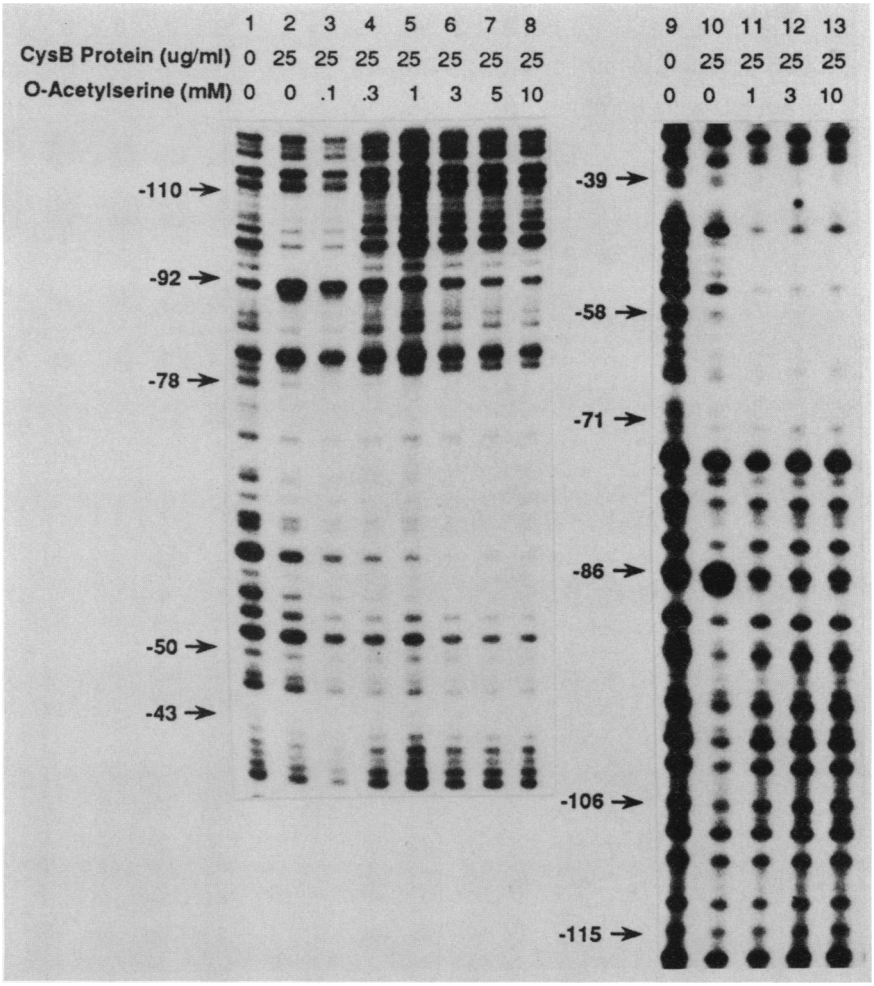

FIG. 4. DNase I protection of the transcribed (lanes 1 to 8) and nontranscribed (lanes 9 to 13) strands of the *cysK* promoter region by CysB protein and *O*-acetyl-L-serine. The 5′-labeled substrate for the transcribed strand consisted of a 175-bp *cysK* promoter fragment extending from positions −144 to +31 relative to the major transcription start site, which was generated by PCR. The 5′-labeled substrate for the nontranscribed strand was a 197-kb *Eco*RI-*Bam*HI fragment of pRSM42, which contained the same 175 bp of *cysK* promoter sequence as well as 22 bp of pT7T3 19U. Following digestion with DNase I, the products were analyzed on a DNA sequencing gel as described in Materials and Methods. CysB protein and *O*-acetyl-L-serine were present as indicated. Numbers in the margins refer to positions relative to the major transcription start site.

chloramphenicol acetyltransferase as a measure of *cysK* promoter strength. These plasmids were also used as templates to generate 5′-labeled fragments by PCR for binding and footprinting studies. The two oligodeoxynucleotide primers used gave fragments beginning at a position in pUC19 located 93 bp upstream of the *Eco*RI multicloning site and ending at position +31 relative to the major *cysK* transcription start site. Thus, each fragment consisted of approximately 100 bp of nonspecific upstream sequence followed by 158 to 76 bp of *cysK* promoter sequence beginning at positions −127 to −45 and ending at position +31 (Fig. 6). For gel mobility shift experiments, slow-complex formation was determined by incubation with 2 μg of CysB protein per ml alone, and *O*-acetyl-L-serine was added at 1 and 10 mM to estimate fast-complex formation and stimulation of total binding.

Chloramphenicol acetyltransferase levels were very high in L-djenkolate-grown wild-type cells carrying pRSM112, pRSM114, pRSM115, pRSM119, and pRSM120, in which different portions of the sequence upstream of position −75 were deleted (Fig. 6; Table 1). For all five derivatives, fast-complex formation occurred with CysB protein alone and was stimulated by *O*-acetyl-L-serine, but the slow complex was observed only with fragments from pRSM112 and pRSM114 (not shown). Chloramphenicol acetyltransferase levels were reduced to about 20% of control levels with pRSM121 and pRSM122, in which deletions extended to positions −68 and −61, respectively, and were further reduced to those of a promoterless *cat* gene in pRSM123 and pRSM124, which lack sequences upstream of positions −54 and −45 (Table 1; Fig. 6). Fast-complex formation was noted with all fragments tested, even with that derived from pRSM124, which contains only 7 bp of CBS-K1 (Fig. 6). Stimulation of binding by *O*-acetyl-L-serine correlated with measurable in vivo *cysK* promoter activity and did not occur with fragments from pRSM123 and pRSM124. These results indicate that an intact CBS-K1 site is required for full expression from the *cysK* promoter in vivo, although sequences downstream of position −62 are sufficient for at least partial activity. CBS-K2, or at least the downstream

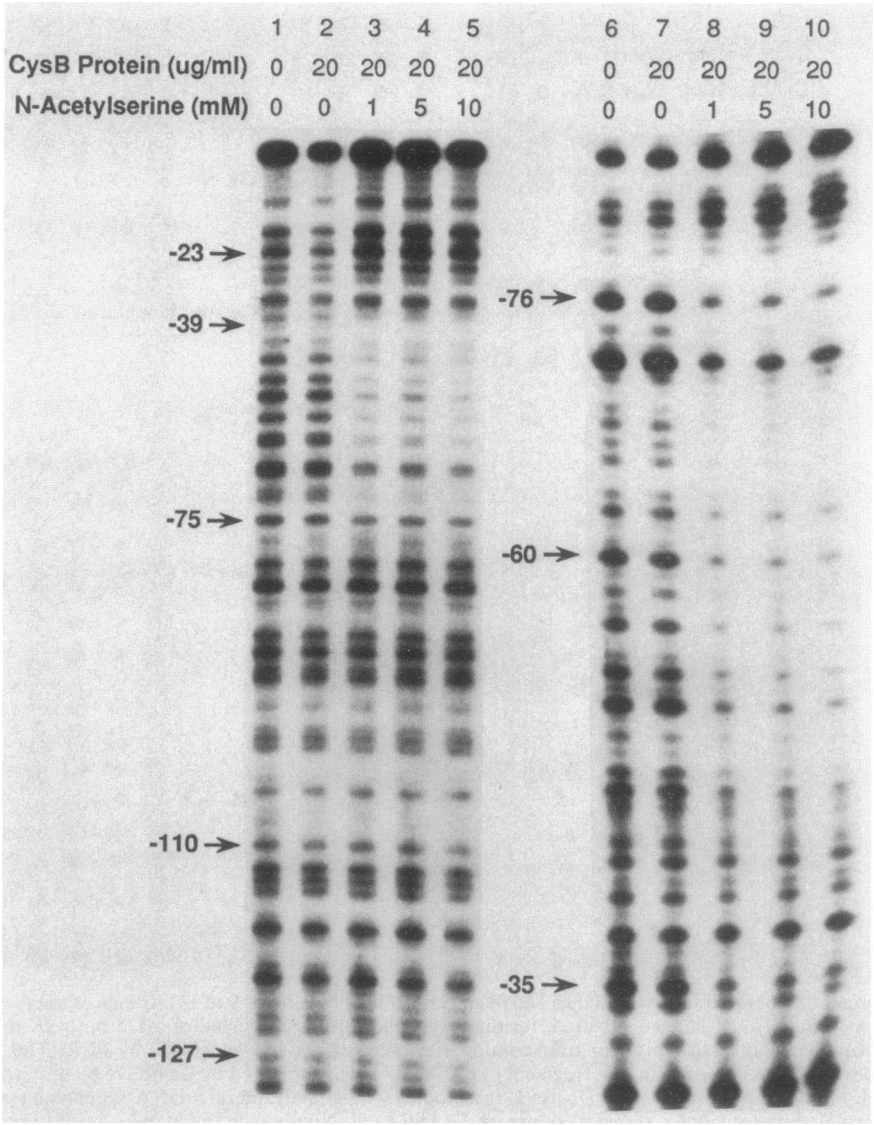

FIG. 5. DNase I protection of the nontranscribed (lanes 1 to 5) and transcribed (lanes 6 to 10) strands of the *cysJIH* promoter region by CysB protein and *N*-acetyl-L-serine. The substrates consisted of 221-bp *cysJIH* promoter fragments extending from positions −218 to +3 relative to the major transcription start site, which were generated from pGBK5 by PCR with primers that were 5′ labeled for one or the other strand. Following digestion with DNase I, the products were analyzed on a DNA sequencing gel as described in Materials and Methods. CysB protein and *N*-acetyl-L-serine were present as indicated. Numbers in the margins refer to positions relative to the major transcription start site.

two-thirds of this site, is necessary for formation of the slow complex but is not required for promoter activity.

As expected for a gene with a *cysK* promoter, chloramphenicol acetyltransferase levels were repressed in the L-cystine-grown wild-type host to the base-line value found with pRSM110 and were also very low in *cysB403* carrying pRSM112, pRSM120, or pRSM121 grown on either L-djenkolate or L-cystine (Table 1). In the *cysB1352* strain DW48, *cat* expression from pRSM112, pRSM119, and pRSM120 ranged from about 16 to 80% of that found in the wild-type host and was not repressed by growth on L-cystine (Table 1). This result provides additional evidence for control of *cat* expression by the *cysK* promoter, because *cysB1352* is known to direct high-level constitutive expression of the entire cysteine regulon during growth on L-cystine (15). The marked difference in *cat* expression between pRSM119 (3.4 U/mg) and pRSM120 (0.8 U/mg) in DW48 was not observed in the wild-type host and suggests that positions −77 and −76 of the *cysK* promoter region may be more important for transcription activation by the CysB protein encoded by *cysB1352* than for activation by the wild-type protein. *cat* expression from pRSM112 and pRSM120 in L-djenkolate-grown DW46 (*cysB484*) was approximately 20% of that observed in the wild-type host and was only partially lowered by growth on L-cystine (Table 1). These results are also consistent with the known behavior of *cysB484*, which causes low-level constitutive expression of *cysK* but is *cysB* mutant for the rest of the cysteine regulon (15).

DNase I protection studies were performed on the transcribed strand of several *cysK* promoter fragments (not shown). *O*-Acetyl-L-serine-stimulated protection by CysB protein was observed at the largely intact CBS-K1 site of

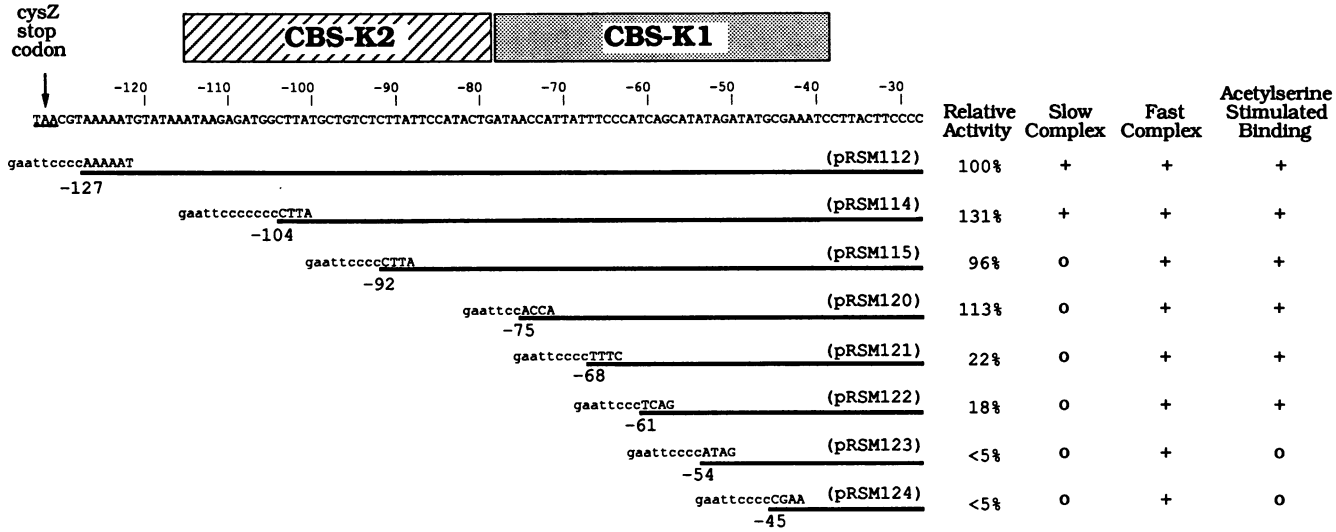

FIG. 6. Effects of deletions on in vivo *cysK* promoter activity and in vitro DNA binding. A series of deletions was created in the upstream portion of the *cysK* promoter region and inserted into pRSM106-A immediately upstream of a promoterless *cat* gene as described in Materials and Methods and depicted in Fig. 1. The uppercase letters above each solid bar represent the upstream boundary of intact *cysK* promoter sequence, and the lowercase letters represent the GAATT$(C)_n$ sequence generated in the construction of these deletions. In vivo promoter activity was measured as chloramphenicol acetyltransferase activity in sulfur-limited cultures of wild-type *S. typhimurium* LT2 carrying these plasmids and is expressed relative to the value for the wild-type promoter in pRSM112. DNA binding was measured by the gel mobility shift method with PCR-generated fragments extending 176 to 258 bp upstream of position +31 relative to the major transcription start site. Slow-complex formation was determined by incubation with 2 μg of CysB protein alone. Fast-complex formation and acetylserine-stimulated binding were evaluated with the further addition of 1 and 10 mM *O*-acetyl-L-serine. The positions of CBS-K1 and CBS-K2 are shown above the sequence.

pRSM120, but CysB protein alone at 25 μg/ml did not protect upstream of position −75, where CBS-K2 was replaced by sequence from pUC19. These results correlate with those of gel mobility shift experiments, in which total binding to the pRSM120 fragment was stimulated by *O*-acetyl-L-serine but slow-complex formation did not occur. The pRSM121 fragment, in which the deletion extends to position −68, was protected between positions −69 and −43, but only in the presence of 10 mM *O*-acetyl-L-serine. Protection could not be demonstrated with fragments containing deletions extending to positions −61, −54, and −45, even though all formed fast complexes in binding studies. These results indicate that upstream CBS-K1 sequences are required for tight binding to this site and illustrate the greater sensitivity of the gel mobility shift method.

Binding of CysB protein to CBS-K2 alone. A CBS-K2 fragment lacking CBS-K1 was generated by PCR from a pUC19-derived plasmid template similar to pRSM42 (Fig. 2B). The 127-bp product contained *cysK* promoter sequences between −144 and −79 and included 61 bp of pUC19 sequence at its upstream end, which was 5′ labeled. PCR was also used with a pRMS120 template to generate a 245-bp unlabeled CBS-K1 fragment containing the sequence between positions −75 and +31 (Fig. 6) plus 139 bp of

TABLE 1. Effects of upstream deletions on *cysK* promoter strength[a]

Plasmid	End of deletion[b]	Chloramphenicol acetyltransferase activity (U/mg)							
		Wild-type LT2		*cysB403*		DW48 (*cysB1352*)		DW46 (*cysB484*)	
		Djenkolate	Cystine	Djenkolate	Cystine	Djenkolate	Cystine	Djenkolate	Cystine
pRSM110[c]		0.2	0.2	0.6	0.1	0.2	0.1	0.4	0.2
pRSM112	−127	4.5	0.2	0.2	0.1	2.5	2.5	1.0	0.5
pRSM114	−104	5.9	0.1	—[d]	—	—	—	—	—
pRSM115	−92	4.3	0.1	—	—	—	—	—	—
pRSM119	−77	4.3	0.2	—	—	3.4	3.2	—	—
pRSM120	−75	5.1	0.1	0.2	0.1	0.8	0.9	0.9	0.7
pRSM121	−68	1.0	0.2	0.3	0.1	0.3	0.4	0.2	0.1
pRSM122	−61	0.8	0.1	0.3	0.2	0.3	0.3	—	—
pRSM123	−54	0.2	0.2	0.1	—	0.1	0.1	0.2	0.1
pRSM124	−45	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.1

[a] Cells were sulfur limited by growth on minimal medium containing L-djenkolic acid as the sole sulfur source. *cysB403* is *cysB* mutant, *cysB1352* is *cysB*(Con) for the entire cysteine regulon, and *cysB484* is *cysB* mutant for expression of sulfite reductase and other activities of sulfate reduction but *cysB*(Con) for expression of *O*-acetylserine (thiol)-lyase A, the product of *cysK*.

[b] Deletions extend downstream to but do not include this position, which is relative to the major transcription start site.

[c] Contains the promoterless *cat* gene.

[d] —, Not determined.

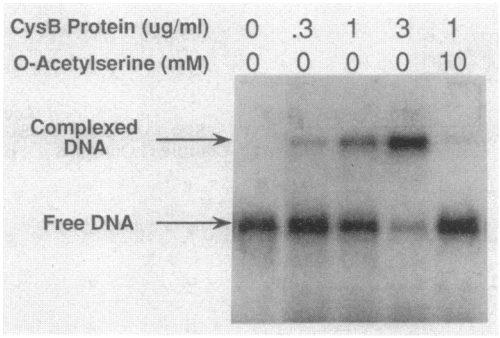

FIG. 7. Binding of CysB protein to a DNA fragment containing CBS-K2 and lacking CBS-K1. The gel mobility shift assay was done as described for Fig. 3 with a 127-bp PCR-generated fragment, which included 61 bp of pUC19 sequence at the upstream end and *cysK* promoter sequence extending from positions −144 to −79 relative to major transcription start site.

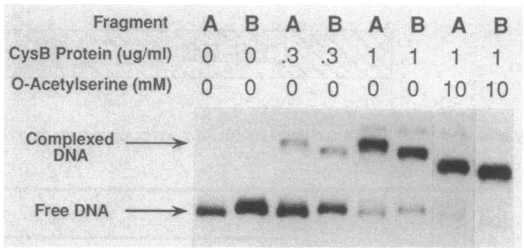

FIG. 8. Binding of CysB protein to DNA fragments containing CBS-K1 and CBS-K2 positioned either centrally or at one end. The gel mobility shift assay was done as described for Fig. 3. The boundary between CBS-K1 and CBS-K2 (position −79 relative to the major transcription start site) was located at 47% of the length of the 236-bp fragment A and at 26% of the length of the 247-bp fragment B.

pUC19 sequence. A gel mobility shift assay with the labeled CBS-K2 fragment showed binding of 10 to 90% of input DNA at 0.3 to 3 μg of CysB protein per ml (Fig. 7). *O*-Acetyl-L-serine markedly inhibited complex formation but did not alter complex mobility as it did with fragments containing both CBS-K1 and CBS-K2. Identical results were obtained when *O*-acetyl-L-serine was added either together with CysB protein or after a 2-min preincubation of DNA and CysB protein, and *N*-acetyl-L-serine was as effective as *O*-acetyl-L-serine (not shown).

Digestion of the CBS-K2 fragment with *Kpn*I gave a 59-bp 5′-labeled portion containing pUC19 sequence, to which CysB protein did not bind at concentrations as high as 10 μg/ml (not shown). Some degree of nonspecific binding was demonstrated, however, in competition studies; at a CBS-K2 concentration of 0.5 μg/ml (6 nM) and 2 μg of CysB protein per ml (14 nM tetramer), the addition of 2.5 μg of a *Pvu*II digest of unlabeled pUC19 per ml reduced binding to CBS-K2 from 90 to 50% of input. In contrast, addition of the unlabeled CBS-K1 fragment at 2.5 μg/ml (15 nM) reduced binding to CBS-K2 from 90 to 10% of input (not shown), indicating that these two specific sites compete for the same protein.

Evidence for induced bending of the *cysK* promoter by CysB protein. The demonstration of two binding sites for CysB protein in the *cysK* promoter region suggested a model in which the binding of two CysB protein molecules forms the slow complex observed in gel mobility shift binding studies and binding of a single protein forms the fast complex. Acetyl-L-serine would favor formation of the fast complex by inhibiting binding of one protein molecule to CBS-K2 while stimulating binding of the other to CBS-K1. An alternative explanation is that the two complexes contain the same quantity of CysB protein and differ because of DNA bending. The slow complex would then represent bent DNA, which is known to migrate more slowly during gel electrophoresis (35), and the fast complex would represent DNA that was less bent or not bent at all. To assess this possibility, we used PCR to prepare two different 5′-labeled DNA fragments containing the CysB protein-binding region either in the middle or at one end, since the effects of bending in gel mobility shift experiments are position dependent and the greatest when the bend point is in the middle of the fragment (35). Fragment A contained 236 bp and consisted of 61 bp of pUC19 sequence followed by positions −144 to +31 of the *cysK* promoter. Fragment B was slightly larger (247 bp) and consisted of 118 bp of pUC19 sequence followed by the *cysK* promoter sequence between −144 and −16. The boundary between CBS-K1 and CBS-K2 (position −79) was located at 47% of the total length for fragment A and at 26% for fragment B.

The gel mobility shift assay showed formation of a slow complex with both fragments, but the fragment A complex migrated more slowly than did the fragment B complex, even though the latter was longer by 9 bp (Fig. 8). This finding is consistent with bending of the DNA of the slow complex at a point more centrally located in fragment A than in fragment B, i.e., in or near the CysB protein-binding sites. *O*-Acetyl-L-serine increased complex mobility for both fragments and at 10 mM caused the fragment A complex to migrate almost as rapidly as the fragment B complex. The small difference between the two was even less at 30 mM *O*-acetyl-L-serine (not shown) and could have been due to either incomplete disruption of the slow complex or a small amount of bending in the fast complex itself.

DISCUSSION

Positive regulation of procaryotic gene expression frequently involves binding of an activator protein immediately upstream of the −35 promoter region, where it enables formation of a transcription initiation complex, either through an interaction with RNA polymerase or by an effect on the DNA itself or both (10, 27). The results presented here demonstrate that CysB protein binds at two different sites upstream of the *cysK* promoter and at one upstream site with the *cysJIH* promoter. Our deletion analysis of the *cysK* promoter shows that the CBS-K1 site is required for in vivo expression, and we assume that CBS-J is required for expression of the *cysJIH* promoter, since its position relative to the −35 region is the same as that of CBS-K1. Although CysB protein binds to CBS-K1 and CBS-J in the absence of inducer, in vitro transcription from the *cysJIH* promoter has been shown to require the additional presence of either *O*-acetyl-L-serine or *N*-acetyl-L-serine, even at CysB protein concentrations that ensure total occupancy of CBS-J (25, 26). We have obtained similar results with the *cysK* promoter (R. S. Monroe, Ph.D. thesis, Duke University, Durham, N.C., 1988, and unpublished data). Thus, increased binding in the presence of acetyl-L-serine does not cause formation of a transcription initiation complex and must be a consequence of changes in CysB protein that lead to such an event (26). It is interesting in this regard that the loss of stimulated binding in our deletion analysis of the *cysK* promoter corresponded with the complete loss of in vivo promoter activity.

```
St  CBS-K1    (-78) ATAACCATTATTTCCCATCAGCATATAGATATGCGAAATCCTTACTTCCCCAT (-26)
Ec "CBS-K1"   (-80) CATGTCATTATTTCCCTTCTGTATATAGATATGCTAAATCCTTACTTCCGCAT (-28)
St  CBS-J     (-79) AAACAGGTTAGTTCATTTGGTTATTT#GTTATTTCCAACCCTTCTTTAATTGT (-28)
Ec "CBS-J"    (-78) AAACAGGTTAGTCGATTTGGTTATTA#GTTATCGCTATCCCGTCTTTAATCCA (-27)
Consensus                 TTA.T.....T....AT...G.TAT....A..CC.T..TT

St  CBS-K2   (-117) AAATAAGAGATGGCTTATGCTGTCTCTTATTCCATACTGATA (-76)
Ec "CBS-K2"  (-119) GAAACAGGGGTGGCTTATGCCGCCCCTTATTCCATCTTGCAT (-80)
```

FIG. 9. Comparison of the sequences of CBS-K1, CBS-J, and CBS-K2 of *S. typhimurium* (St) and the corresponding regions in *E. coli* (Ec). The *E. coli* "CBS-K1" and "CBS-K2" sequences are from strain K-12 (4), and the *E. coli* "CBS-J" sequence is from strain B (25). A single gap (#) was introduced into CBS-J to increase the number of identities. The consensus sequence is only for CBS-K1 and CBS-J. Inverted repeats are shown with a double underline, and direct repeats are indicated with a single underline. The inverted repeats in the CBS-K2 sequences include GT pairs.

Not unexpectedly, segments of sequence identity are evident in a comparison of the CBS-K1 and CBS-J regions of *S. typhimurium* and the corresponding portions of the *cysK* promoter from *E. coli* K12 (4) and the *cysJIH* promoter from *E. coli* B (25). Insertion of a single gap in the *cysJIH* promoter region gives a consensus of $TTANTN_5TN_4ATN_{2-3}$ $GNTATN_4ANNCCNTNNTT$, with 17 of 40 identities for all four sequences (Fig. 9). Although most of the sequence ANNCCNTNNTT extends beyond the downstream boundary of DNase I protection for CBS-K1, it may confer binding specificity since it is retained in a deletion (pRSM124) that lacks promoter activity but still binds to CysB protein (Fig. 6). A 6-bp inverted repeat occurs in CBS-K1, and an almost identical sequence is found in *E. coli*, with matches at five positions (Fig. 9). CBS-J and its corresponding *E. coli* sequence, however, contain direct repeats of GTTATY in this region and an almost identical repeat at a third upstream position. Slight variations of the sequence GTTATTT appear at least twice in all four binding sites. The specific role of these sequences is unknown.

The CBS-K2 sequence differs considerably from the consensus derived from CBS-K1 and CBS-J. This is not unexpected given the fact that acetyl-L-serine inhibits binding to the first and stimulates binding to the latter two. Both the *S. typhimurium* CBS-K2 sequence and the corresponding region in *E. coli* contain 8-bp G+C-rich inverted repeats followed by the T-rich sequence TTATT (Fig. 9). The replacement of two A-T matches and one G-T match in the *S. typhimurium* inverted repeat by G-C matches in *E. coli* argues for a functional significance of this structure, which may be a Rho-independent terminator (28) for the upstream *cysZ* gene (4). Binding to CBS-K2, as evidenced by slow-complex formation, was not affected by deletion of the upstream half of this symmetric structure in pRSM114 but was eliminated with loss of the entire inverted repeat in pRSM115 (Fig. 6). The 12-bp difference between these two deletions includes the sequence TTAT at position −103, which may contribute to binding specificity since it contains the TTA and TAT conserved sequences of CBS-K1 and CBS-J. The sequence TTATT, which occurs in CBS-K1 and CBS-J, is also present in CBS-K2 at position −91 in *S. typhimurium* and at position −95 in *E. coli*.

Our deletion analysis indicates that CBS-K2 is not required for normal *cysK* promoter strength. One role for this binding region might be in regulating expression from a minor *cysK* promoter, designated P2, which has a start site located 70 bp upstream of the major start site (4). In theory, binding of CysB protein to either CBS-K2 or CBS-K1 could inhibit transcription from P2 by interfering with binding of RNA polymerase, as it does in *cysB* autoregulation (12; J. Ostrowski and N. M. Kredich, Abstr. Annu. Meet. Am. Soc. Microbiol. 1989, H182). In this way, CysB protein could provide dual control of *cysK* by activating the major promoter through an interaction at CBS-K1 when sulfur is limiting and by repressing the minor promoter by steric effects at CBS-K1 and CBS-K2 when sulfur is replete. In vivo observations, however, do not support such a model, because levels of mRNA originating from P2 do not vary significantly between sulfur-limited and sulfur-replete cells (4) or between Δ*cysB* and wild-type strains (R. S. Monroe and N. M. Kredich, unpublished data). Moreover, we have found no consistent differences in *O*-acetylserine sulfhydrylase levels between Δ*cysB* and wild-type strains during growth on L-cystine. At this time, the physiologic function of CBS-K2, if any, is unknown.

Our data indicate that the slow complex represents binding of CysB protein to both CBS-K1 and CBS-K2 and that the fast complex found in the presence of acetyl-L-serine is due to binding to CBS-K1 alone. The continuum of discrete bands migrating partway between the slow and fast complexes at nonsaturating acetyl-L-serine concentrations is presumed to represent an equilibrium between the two complexes during electrophoresis. With the wild-type *cysK* promoter, fast complexes were never detected in the absence of acetyl-L-serine, even at CysB protein concentrations low enough to bind only a few percent of input DNA. This finding indicates that binding to the two sites under these conditions is highly cooperative, which is relevant to our gel shift data suggesting that the slow complex contains bent DNA. We currently favor a model in which a single molecule of CysB protein bends *cysK* promoter DNA by binding simultaneously to both CBS-K1 and CBS-K2 in the absence of acetyl-L-serine. As a result of the effects of bending on electrophoretic mobility, this product would migrate more slowly during gel electrophoresis. Inhibition of binding to CBS-K2 by acetyl-L-serine would diminish or completely eliminate DNA bending and result in formation of the fast complex, in which CysB protein is bound only to CBS-K1. This model is consistent with our failure to observe mixtures of slow and fast complexes, since isomerization of a single complex between forms containing bent versus unbent DNA would be expected to give a single band during

electrophoresis, provided the rate of isomerization is rapid compared with the rate of migration. In contrast, a mixture containing a two-protein complex in equilibrium with a one-protein complex would be expected to lose protein during electrophoresis and result in either a discrete band of the fast complex (the one-protein complex) or a smear extending to the position of the fast complex. A one-protein model is more plausible with DNA bending, since the predicted diameter of a spherical, tetrameric CysB protein is only 6.9 nm and the 55 to 67 bp protected against DNase I in the absence of acetyl-L-serine represents a distance along a B-DNA axis of 18.2 to 22.1 nm. Without DNA bending, protection of such an expanse would require a highly elongated CysB protein. Stoichiometric studies will be required to distinguish between this model and others involving more than one CysB protein per complex.

If our one-protein model involving bent DNA is correct, it is not clear whether such bending would be functionally significant or simply an incidental consequence of binding of CysB protein to two separate sites. Bending cannot be required for *cysK* promoter activity because it would occur under conditions in which this promoter is almost totally inactive. It is also unlikely to serve a repressor function, since deletion of the entire CBS-K2 site in pRSM120 did not affect the ability of L-cystine to completely repress promoter activity in vivo (Table 1). DNA bending does allow an increase in the number of DNA-protein contact points and would facilitate binding to the long expanse of DNA encompassed by CBS-K1 and CBS-K2. The increased stability of such a complex would help sequester CysB protein at the *cysK* promoter while sulfur is replete and *cysK* expression is not required. Such a mechanism would ensure a rapid response of the *cysK* promoter in the event of a sudden decrease in sulfur availability.

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