JOURNAL OF BACTERIOLOGY, Jan. 1989, p. 130–140
0021-9193/89/010130-11$02.00/0

Vol. 171, No. 1

Molecular Characterization of the *cysJIH* Promoters of *Salmonella typhimurium* and *Escherichia coli*: Regulation by *cysB* Protein and *N*-Acetyl-L-Serine

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Received 6 July 1988/Accepted 30 September 1988

The *cysJIH* promoter regions from *Salmonella typhimurium* LT7 and *Escherichia coli* B were cloned and sequenced. Primer extension analyses showed that the major in vivo transcription initiation site in *S. typhimurium* is located 171 nucleotides upstream of the *cysJ* start codon. Minor start sites were found 8 and 9 nucleotides downstream of the major site. In vivo transcription initiation in *E. coli* was found to occur at a single site 66 nucleotides upstream of the *cysJ* start codon. Primer extension studies also indicated that chromosomal *cysJIH* transcription is stimulated by sulfur limitation and repressed by growth on L-cystine. Paradoxically, in strains carrying plasmids containing the *S. typhimurium cysJIH* region, the highest levels of primer extension products were found with RNA from cells grown on L-cystine, even though levels of the proteins encoded by *cysJ* and *cysI* were normally repressed. In vitro transcription runoff studies with DNA template from the *S. typhimurium cysJIH* promoter region showed synthesis of a product originating at the major in vivo start site, which was dependent on the presence of purified *cysB* protein and either *O*-acetyl-L-serine or *N*-acetyl-L-serine. *N*-Acetyl-L-serine was 10- to 30-fold more active than *O*-acetyl-L-serine as an in vitro inducer of *cysJIH* transcription.

Synthesis of L-cysteine by *Salmonella typhimurium* and *Escherichia coli* involves cellular uptake and reduction of sulfate to sulfide, the synthesis of *O*-acetyl-L-serine from L-serine and acetyl coenzyme A by serine transacetylase, and the reaction of *O*-acetyl-L-serine with sulfide in a reaction catalyzed by *O*-acetylserine (thiol)-lyase (reviewed in reference 20). Except for serine transacetylase, these biosynthetic activities are regulated at the gene level as a system of positive control termed the cysteine regulon, wherein gene expression requires sulfur limitation and the *cysB* regulatory protein (17, 19), a tetramer of identical 36-kilodalton subunits (27, 35). Serine transacetylase is controlled through feedback inhibition by L-cysteine (21, 22). In addition to acting as an L-cysteine precursor, *O*-acetyl-L-serine is considered an internal inducer of the cysteine regulon because *cysE* strains (lacking serine transacetylase) cannot be derepressed by sulfur limitation unless provided with an external source of *O*-acetyl-L-serine (18, 19).

Known genes of the cysteine regulon are widely scattered on the chromosome in five different clusters (2, 40). The *cysJIH* region consists of contiguous genes specifying the flavoprotein (*cysJ*) and hemoprotein (*cysI*) components of NADPH-sulfite reductase (42, 44) and for 3′-phosphoadenosine 5′-phosphosulfate sulfotransferase (*cysH*) (11). Regulation of *cysJIH* is at the level of transcription (12), and genetic studies indicate that these three genes constitute a single transcriptional unit with the order promoter-*cysJ-cysI-cysH* (9, 24, 34). We have cloned and sequenced the *cysJIH* regions from *S. typhimurium* (J. Ostrowski and N. M. Kredich, Abstr. Annu. Meet. Am. Soc. Microbiol. 1987, H183, p. 170) and *E. coli* (J. Y. Wu, J. Ostrowski and N. M. Kredich, manuscript in preparation) and have identified the open reading frames coding for all three genes. We report here in vivo and in vitro studies that characterize the *cysJIH* promoter and show that its activity is dependent on *cysB* protein and either *O*-acetyl-L-serine or its derivative *N*-acetyl-L-serine.

MATERIALS AND METHODS

Bacterial strains and media. The *S. typhimurium* and *E. coli* strains used in these studies are described in Table 1. Medium E with 0.5% glucose (45) was used as minimal medium and was supplemented with amino acids at 40 mg/liter and with uracil or thiamine hydrochloride at 4 mg/liter when appropriate for the growth of auxotrophs. An equimolar amount of $MgCl_2$ was substituted for $MgSO_4$, and either 0.5 mM reduced glutathione, 1.0 mM Na_2SO_4, 0.5 mM L-cysteine sulfinic acid, or 0.5 mM L-cystine was added as the sulfur source. Medium was adjusted to pH 6.8 with HCl before autoclaving in experiments testing in vivo effects of *O*-acetyl-L-serine and *N*-acetyl-L-serine (19). Rich medium consisted of LB for plasmid transformations and YT for phage M13 transformations (28). Solid medium contained 1.5% agar. When required, ampicillin was used at 25 mg/liter.

Plasmids. Plasmids are shown in Fig. 1. pGBK5 and pJYW2 were isolated by selecting for Cys^+ transformants of the *E. coli cysI* EC1124. pGBK5 was isolated by G. Jagura-Burdzy and contains *cysI* from *S. typhimurium* LT7 *hisG70* on a 13.0-kilobase (kb) *Sal*I fragment inserted in pBR322. pGBK5 was transferred by bacteriophage P22HT-mediated transduction (30) into the *S. typhimurium cysJ266*, *cysI68*, and *cysJIH383* and gave a Cys^+ phenotype with each, indicating that this plasmid contains the entire *cysJIH* region from *S. typhimurium* LT7. pJYW2 was isolated by Jer Yuarn Wu and contains *cysI* from *E. coli* B on a 9.5-kb fragment obtained from a partial *Sau*3A digest and inserted in the *Bam*HI site of pBR322. Comparison of the DNA sequences of pGBK5 and pJYW2 indicated that pJYW2 also carries the

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TABLE 1. Bacterial strains

Species and strain	Genotype	Origin or reference
E. coli[a]		
B	Wild type	ATCC 11303
JA199	*ΔtrpE5 leu-6 thi hsdR hsdM*$^+$	J. Carbon
NK1	*ΔtrpE5 leu-6 thi hsdR hsdM*$^+$ *cysB*	16
EC1124	*ΔtrpE5 leu-6 thi hsdR hsdM*$^+$ *cysI*	From JA199 by A. Wiater
S. typhimurium[b]		
LT2	Wild type	
cysB403	*cysB403*	29
cysJ266	*cysJ266*	9
cysI68	*Δ(cysI68)*	9
cysJIH383	*Δ(cysJIH383)*	9
hisG70	*hisG70*	14
DW18	*cysE2*	19
DW363	*leu-500 pyrF146 Δ(topA cysB1763)*	7
DW365	*leu-500 pyrF146 Δ(topA cysB1765)*	7
TK2167	*leu-500 pyrF146 Δ(topA cysB1763) cys2332*	34[c]
TK2192	*leu-500 pyrF146 Δ(topA cysB1765) cys2335*	Spontaneous from DW365[c]

[a] *E. coli* strains are all K-12 derivatives except wild-type *E. coli* B.
[b] *S. typhimurium* strains are all LT2 derivatives except *hisG70*, which is an LT7 strain.
[c] Selected for growth on L-cysteine sulfinic acid as a sulfur source.

entire *cysJIH* region from *E. coli* B (Wu et al., in preparation; also, see below). pRSM15 is a pBR322 derivative that contains *cysJIH* on a 7.8-kb fragment from wild-type *S. typhimurium* LT2 (30), which also was the product of a partial *Sau*3A digest.

TK2167 is a Δ*cysB* strain of *S. typhimurium* LT2 known to carry a spontaneous mutation in or near the *cysJIH* promoter (*cys2332*) that causes constitutive expression of NADPH-sulfite reductase in the absence of *cysB* protein (34). It was derived from DW363 by selection for the ability to grow on L-cysteine sulfinic acid, a convenient precursor of sulfite, as a sulfur source. TK2192 was obtained in the same way from DW365, another Δ*cysB* strain of *S. typhimurium* LT2, and also carries a mutation in or near the *cysJIH* promoter (*cys2335*). The *cysJIH* regions from these strains were cloned in pBR322 by a shotgun approach by transforming *E. coli cysB* NK1 and selecting for growth on L-cysteine sulfinic acid. Recombinant plasmids carrying *cysB*$^+$ were not obtained, because both TK2167 and TK2192 are Δ*cysB*. The *cysJIH* region from TK2167 was obtained as a 7.9-kb *Eco*RI-*Sal*I insert in a plasmid designated pJOK7 (Fig. 1). pJOH10 contains *cysJIH* from TK2192 as a 7.8-kb fragment obtained from a *Sau*3A partial digest, which was inserted into the *Bam*HI site of pBR322 (Fig. 1).

DNA sequencing. DNA sequencing was performed by the method of Sanger et al. (41) by using the Klenow fragment of *E. coli* DNA polymerase (Pharmacia, Inc.), [α-^{35}S]dATP (4) purchased from the Du Pont Co., and field-strength gradient, 6% polyacrylamide electrophoresis gels containing 7 M urea (33) and maintained at 55°C with thermostatic plates. Overlapping fragments of single-stranded DNA templates were generated from M13 phage derivatives (46) by the method of Dale et al. (8). Both DNA strands were completely sequenced for the *cysJIH* promoter regions from pGBK5 and pJYW2 (strategies not shown). The *cysJIH* promoter regions in pJOK7 and pJOH10 were sequenced in only one direction. pRSM15 was not sequenced at all.

Analyses of in vivo transcripts. Total cellular RNA was prepared by the method of Aiba et al. (1) from bacteria grown on minimal medium containing either reduced glutathione, sulfate, or L-cystine as a sulfur source. For primer extension experiments, 25 μg of total RNA and 0.1 pmol of 5′-[^{32}P]-labeled synthetic oligodeoxynucleotide were dissolved in 30 μl of 40 mM sodium PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)], pH 6.7, containing 1 mM Na_2-EDTA–0.2% sodium dodecyl sulfate–0.4 M NaCl and hybridized by incubating at 37°C for 16 h (23). After the addition of 0.5 ml of water, 0.2 ml of 4 M ammonium acetate, and 0.5 ml of isopropanol, the mixture was incubated for 10 min at 23°C. The precipitated RNA-oligodeoxynucleotide hybrid was collected by centrifugation and dissolved in 50 μl of 50 mM Tris hydrochloride, pH 8.3, containing 75 mM KCl, 6 mM $MgCl_2$, 10 mM dithiothreitol, and each of the four deoxynucleoside triphosphates at 0.5 mM. Primer extension was initiated by the addition of 6 U of avian myeloblastosis virus reverse transcriptase (U. S. Biochemical Corp.) in a 1-μl volume and carried out for 2 h at 41°C. Phenol-extracted and ethanol-precipitated radiolabeled DNA was then analyzed in sequencing gels. S1 nuclease protection experiments were performed as described earlier (30).

In vitro runoff transcription assays. The templates used in transcription runoff assays were derived from M13 phage derivatives, in which sequences downstream from the *S. typhimurium* LT7 *cysJIH* promoter were deleted and replaced by a $(C)_n$ GAATTC sequence at the *Eco*RI cloning site of M13mp19 (8). Fragments were subcloned in pUC19 and then purified from that vector by standard procedures (25).

The 20-μl preincubation mixture contained 40 mM Tris hydrochloride at a pH ranging between 7.4 and 8.0, 0.1 M KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM ATP, 100 μg of nuclease-free bovine serum albumin per ml, 2 nM purified template DNA, 50 μg (approximately 110 nM) of nuclease-free *E. coli* RNA polymerase holoenzyme per ml (Pharmacia), and various amounts of purified *cysB* protein, *O*-acetyl-L-serine, and *N*-acetyl-L-serine. After incubation for 2 to 8 min at 37°C to allow formation of initiation complexes, transcription was initiated by the addition of 2 μl of a solution containing 0.5 mg sodium heparin per ml, 10 μM [α-^{32}P]CTP (800 Ci/mmol; Du Pont Co.), and 2 mM each of ATP, GTP, and UTP (37). After another 5 min at 37°C, the reaction was terminated by adding 0.2 ml of 10 mM Na_2-EDTA containing 50 μg of yeast tRNA per ml. Phenol-

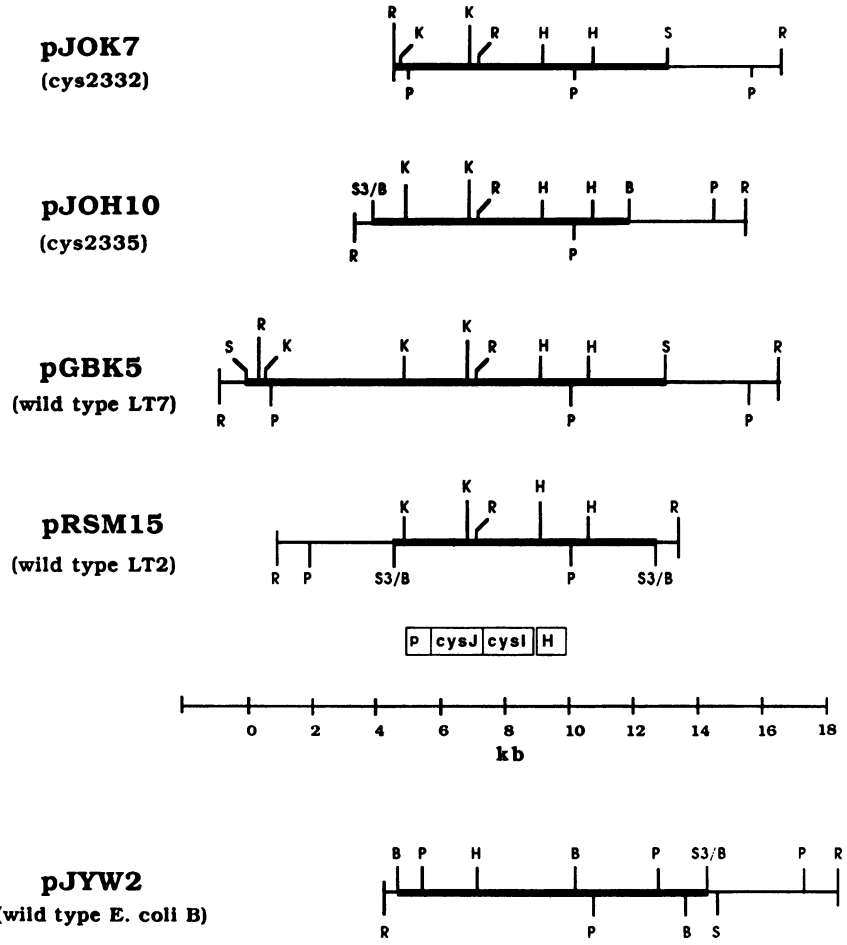

FIG. 1. Restriction endonuclease maps of plasmids used in this study. All are pBR322 derivatives, and cloned fragments of chromosomal DNA are shown as boldface lines. pGBK5 contains the wild-type *cysJIH* region from *S. typhimurium* LT7 *hisG70* on a 13.0-kb *Sal*I fragment. pRSM15 contains the *cysJIH* region from wild-type *S. typhimurium* LT2 on a 7.8-kb fragment obtained from a partial *Sau*3A digest. The *cysJIH* regions in pJOH10 and pJOK7 are from *S. typhimurium* LT2 derivatives: pJOH10 contains the *cys2335* allele from TK2192 on a 7.8-kb fragment obtained from a partial *Sau*3A digest, and pJOK7 contains the *cys2332* allele from TK2167 on a 7.9-kb *Eco*RI-*Sal*I fragment. pJYW2 contains wild-type *cysJIH* from *E. coli* B on a 9.5-kb fragment obtained from a partial *Sau*3A digest. The plasmids are aligned with respect to the positions of the promoter and individual genes of the *cysJIH* cluster, which are shown above the scale. Abbreviations: B, *Bam*HI; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; R, *Eco*RI; S, *Sal*I. Fragments obtained from partial *Sau*3A digests were inserted into the *Bam*HI site of pBR322. Resultant sites that are resistant to *Bam*HI are designated S3/B.

extracted and ethanol-precipitated radiolabel was then analyzed in sequencing gels.

Recombinant DNA methods. General methods were those described by Maniatis et al. (25). DNA ligase (Boehringer Mannheim Biochemicals) and restriction enzymes (Bethesda Research Laboratories, Inc.) were used according to the instructions of the suppliers. Oligodeoxynucleotides were prepared on an Applied Biosystems model 380A automated DNA synthesizer and purified on cartridges from the same company (26). They were radiolabeled at the 5′ terminus by dissolving 200 pmol in 30 μl of 0.1 M Tris hydrochloride, pH 9.0, containing an equimolar amount of [γ-^{32}P]ATP (3,000 Ci/mmol; Du Pont Co.), 5 mM $MgCl_2$, 5 mM dithiothreitol, and 7 U of polynucleotide kinase (Promega Biotec). After incubation at 37°C for 30 min, DNA was recovered by phenol extraction and ethanol precipitation and dissolved in sterile water at a concentration of 1 pmol/μl.

Preparation and analysis of *O*-acetyl-L-serine and *N*-acetyl-L-serine. *O*-Acetyl-L-serine (39) and *N*-acetyl-L-serine (31) were prepared as described earlier and analyzed by high-performance liquid chromatography with isocratic elution of a Partisil-10 SCX column (0.46 by 25 cm; Whatman, Inc.) with 0.02 M ammonium phosphate, pH 2.3, at a flow rate of 1.5 ml/min. The eluate was monitored with a model 450 variable wavelength detector (Waters Associates, Inc.) at 216 nm for *N*-acetyl-L-serine (ε_{216} = 755 M^{-1} cm^{-1}) and *O*-acetyl-L-serine (ε_{216} = 65 M^{-1} cm^{-1}) which were eluted at 3.1 and 7.7 ml, respectively. This method is not very sensitive for *O*-acetyl-L-serine, but 1 nmol of *N*-acetyl-L-serine in a volume of 0.1 ml or less was easily detected. Conversion of *O*-acetyl-L-serine to *N*-acetyl-L-serine was measured in a continuous spectrophotometric assay by monitoring the increase in A_{216} in a Shimadzu UV-260 recording spectrophotometer equipped with electronic temperature control.

Other methods. Our methods for the purification of *S. typhimurium cysB* protein (27), preparation of bacterial extracts (19), and assays for *O*-acetylserine (thiol)-lyase (3), NADPH-sulfite reductase and NADPH-cytochrome *c* reductase (43) have been described. Protein was determined by

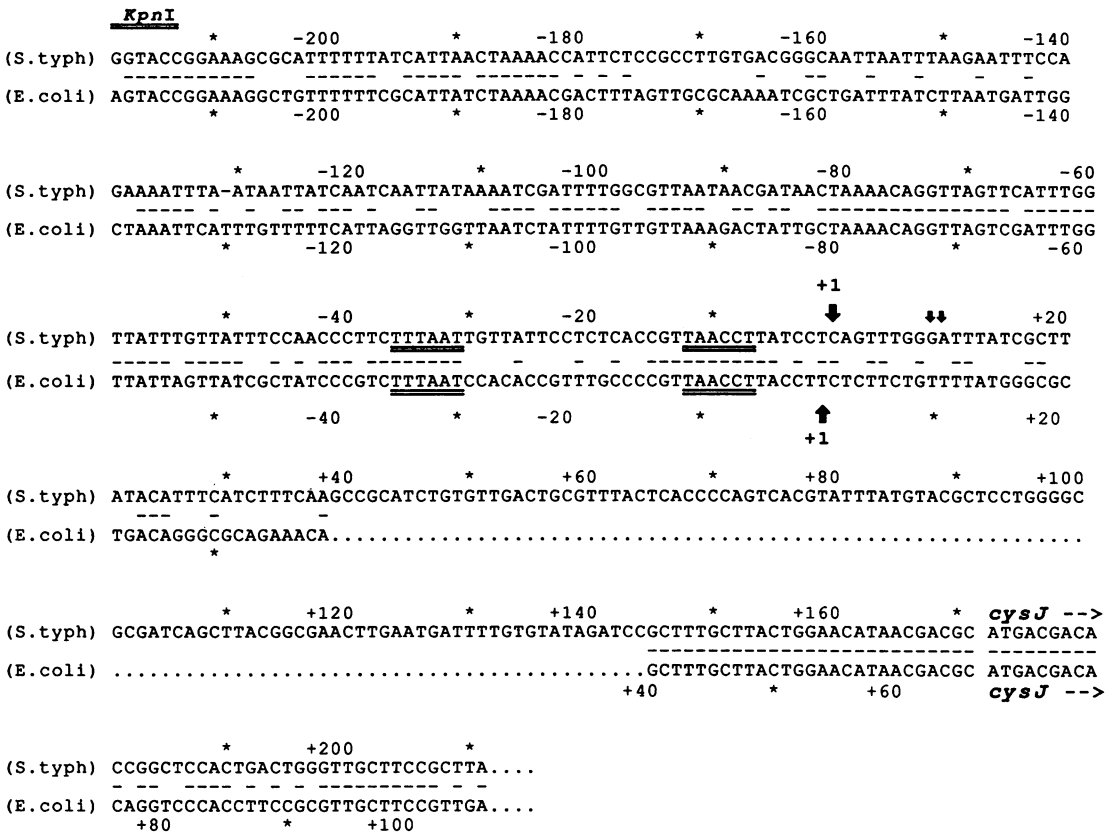

FIG. 2. DNA sequences of the wild-type *cysJIH* promoter regions of *S. typhimurium* LT7 and *E. coli* B. Identical nucleotides are indicated by a hyphen between the two sequences. The sequences were aligned for maximum identity by including a single-nucleotide gap in the *S. typhimurium* sequence and a 106-nucleotide gap in the *E. coli* sequence. An additional 316 nucleotides upstream of those shown were sequenced for *E. coli* B but are not included here. Nucleotide positions are numbered relative to in vivo major transcription start sites (⬆ and ⬇) that were determined by primer extension studies. Two minor transcription start sites at positions +9 and +10 were found for *S. typhimurium* (⬇⬇). The *cysJ* coding region begins at position +172 for *S. typhimurium* and at position +67 for *E. coli*. The *Kpn*I site at the beginning of the *S. typhimurium* sequence is labeled as a point of reference for comparison with Fig. 1. Regions corresponding to −10 and −35 promoter elements are double underlined.

the dye ligand method (5) by using bovine serum albumin as a standard. DNA sequence comparisons used the programs Compare and DotPlot from the Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin (10).

RESULTS

Comparison of *cysJIH* promoter sequences from wild-type *S. typhimurium* LT7 and *E. coli* B. The *S. typhimurium* LT7 sequence obtained from pGBK5 included 389 nucleotides upstream of the *cysJ* start codon, which was identified by amino-terminal analysis of purified NADPH-sulfite reductase flavoprotein (Ostrowski and Kredich, Abstr. Annu. Meet. Am. Soc. Microbiol. 1987). From pJYW2 we sequenced 600 nucleotides upstream of the *E. coli* B *cysJ* start codon. The entire 389 nucleotides from *S. typhimurium* and the corresponding region from *E. coli* were aligned for maximum identity by including a 1-nucleotide gap in the *S. typhimurium* sequence and a 106-nucleotide gap in that of *E. coli*. Each is numbered in Fig. 2 with respect to its major transcription initiation start site (see below). The overall identity between the two is 57% for the first 257 nucleotides of the *S. typhimurium* sequence and 100% for the last 27 nucleotides.

In vivo transcription start sites. Sites of in vivo *cysJ* transcription initiation were determined by primer extension analyses with three different 5′-^{32}P-labeled oligodeoxynucleotides. Primer A is a 30-mer complementary to the *S. typhimurium* sequence extending from +43 to +72 (Fig. 2). Primer B is a 29-mer complementary to the *S. typhimurium* sequence extending from position +134 to +162. Primer C is a 30-mer complementary to an *E. coli* sequence within the *cysJ* coding region extending from position +76 to +105 (Fig. 2). These primers were selected to be specific for either the *S. typhimurium* or *E. coli* sequence.

Extension of primer A with RNA template from wild-type *S. typhimurium* LT2 gave a major product of 72 nucleotides and minor products of 63 and 64 nucleotides (Fig. 3, lane 1). These size estimates were confirmed with the use of primer B, which gave a 162-nucleotide major product and a product of about 153 nucleotides, which was not resolved into two components (Fig. 3). These findings suggest the presence of multiple in vivo transcription initiation sites in the *cysJIH* promoter region of *S. typhimurium*. The major site is at the C residue located 171 nucleotides upstream of the *cysJ* start codon and is designated position +1 in Fig. 2. Two minor sites may exist at the G and A residues located 8 and 9 nucleotides downstream from the major site. S1 nuclease

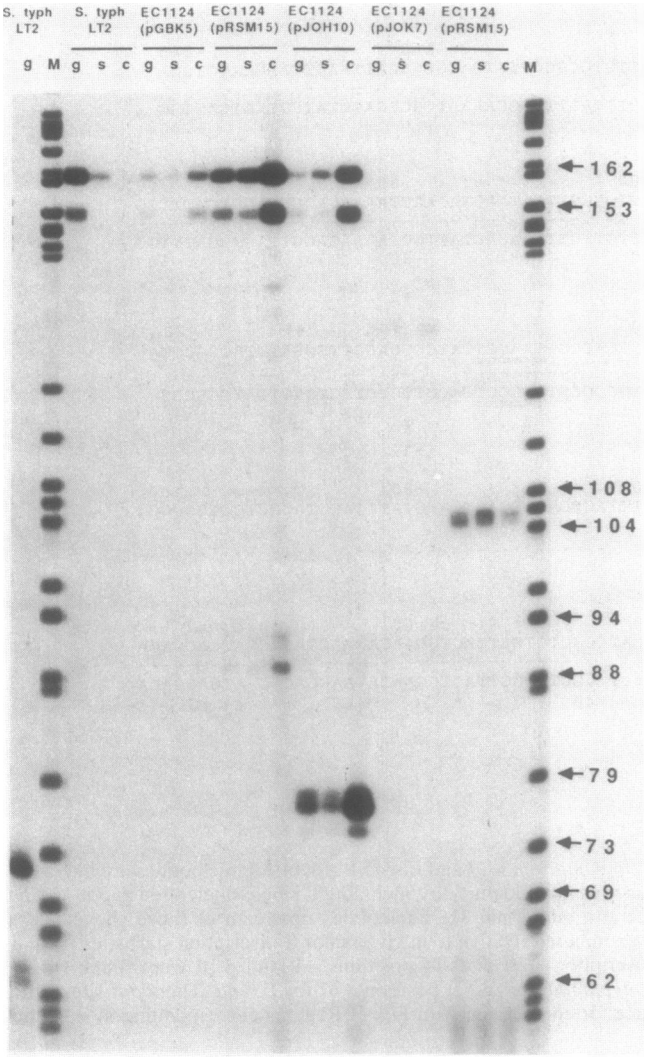

FIG. 3. Primer extension analyses of in vivo transcription initiation for *cysJIH*. RNA was isolated from each of the five strains grown on either reduced glutathione (g), sulfate (s), or L-cystine (c) as a sulfur source and used as template for primer extension. Identical amounts of primer and total RNA were used for each reaction. Product sizes were estimated by comparison with G-reaction lanes (M) from a DNA sequencing reaction by using M13mp18 as a template. The values given are corrected for the assumption that the 5′-^{32}P-phosphorylated primer extension products migrate further by 1 nucleotide than the nonphosphorylated DNA products. The first lane shows extension products of 72, 64, and 63 nucleotides, which were obtained with RNA from glutathione-grown *S. typhimurium* LT2 annealed to primer A, a 30-mer complementary to the *S. typhimurium* sequence between positions +43 and +72 (Fig. 2). For the 15 lanes after lane M, RNA was annealed to primer B, a 29-mer complementary to the *S. typhimurium* sequence between positions +134 and +162. Extension products of 162 and 153 (presumably a doublet) nucleotides were found with each strain except EC1124(pJOK7). RNA from EC1124 (pJOH10) also gave products of 74, 76, and 77 nucleotides. Minor products of 89 and 92 nucleotides were found with RNA from EC1124(pRSM15) and were faintly visible with RNA from EC1124(pGBK5) and wild-type *S. typhimurium* LT2 grown on glutathione. The three lanes preceding the final lane M used RNA from EC1124(pRSM15) annealed to the *E. coli*-specific primer C, a 30-mer complementary to the *E. coli* sequence between positions +76 and +105 (Fig. 2). The 105-nucleotide product represents a transcript originating from the *E. coli cysJIH* promoter of EC1124(pRSM15).

protection experiments (results not shown) confirmed these results by showing two different protected RNA species, one of the length expected from the major initiation site defined by primer extension analyses and the other 7 to 10 nucleotides shorter. An alternative explanation of these data is that the two shorter transcripts may have been derived from the longer one by RNA processing. S1 nuclease protection experiments failed to detect transcripts that might have originated from positions +134 to +171, which would not have been detected by our primer extension experiments.

With RNA template from the *E. coli* K-12 derivative EC1124(pRSM15), extension of the *E. coli* B-specific primer C gave a 105-nucleotide product (Fig. 3), which we assume was directed by mRNA from the *cysJIH* region of *E. coli* K-12. If the *cysJIH* promoter region of *E. coli* K-12 is identical to that of *E. coli* B, these results indicate the presence of an initiation start site at the T residue located 66 nucleotides upstream of the *cysJ* start codon and designated position +1 in Fig. 3. There was no evidence of a second transcription initiation start site in *E. coli*.

The −10 element of the major *cysJIH* promoter in both sequences is represented by TAACCT and begins at −12 for *S. typhimurium* and at −11 for *E. coli*. These two promoters also share the sequence TTTAAT in the −35 region, which is separated from the −10 element by 18 nucleotides, only 6 of which are identical (Fig. 2). The sequence TATCCT beginning at position −6 may serve as the −10 element of the postulated minor *cysJIH* promoter for *S. typhimurium*.

In vivo regulation of *cysJIH* promoter activity. In wild-type *S. typhimurium* LT2 carrying no plasmid, the highest levels of major and minor primer extension products were found with RNA from bacteria that were sulfur-limited by growth on glutathione as a sole sulfur source (Fig. 3). RNA from sulfate-grown cells gave lesser amounts of these products, and RNA from cells repressed for the cysteine regulon by growth on L-cystine gave no detectable products. These findings confirm those of Fimmel and Loughlin (12) for *E. coli* and are consistent with the notion that regulation of the cysteine regulon occurs at the level of transcription. The results obtained with EC1124 carrying wild-type *S. typhimurium cysJIH* on either pGBK5 (from strain LT7) or pRSM15 (from strain LT2) were quite different and showed the highest primer extension product levels with RNA from cells grown on L-cystine (these results are discussed below). Additional minor extension products of 89 and 92 nucleotides were noted with EC1124(pRSM15) and were found in highest amounts in L-cystine-grown cells (Fig. 3). Very small amounts of products of the same length were also detected with template RNA from sulfur-limited cultures of plasmid-free wild-type *S. typhimurium* LT2 and with EC1124 (pGBK5). We do not know whether these products are artifacts of the primer extension reaction, mRNA degradation products, or products of another minor in vivo promoter with start sites at positions +73 and +76. Levels of the *E. coli*-specific primer extension product in sulfur-limited and sulfur-replete EC1124(pRSM15) did not vary as dramatically as in *S. typhimurium* LT2 but were still significantly lower in cells grown on L-cystine (Fig. 3).

Characterization of the *cysJIH* promoter regions of *cys2335* and *cys2332*. Extension of primer B with RNA template from EC1124 carrying *cys2335* on pJOH10 gave not only the products expected from expression of the wild-type *S. typhimurium* promoters but also a new major product of 76 nucleotides and two additional minor products of 74 and 77 nucleotides (Fig. 3). The DNA sequence of the *cysJIH* promoter region from pJOH10 was found to be identical to

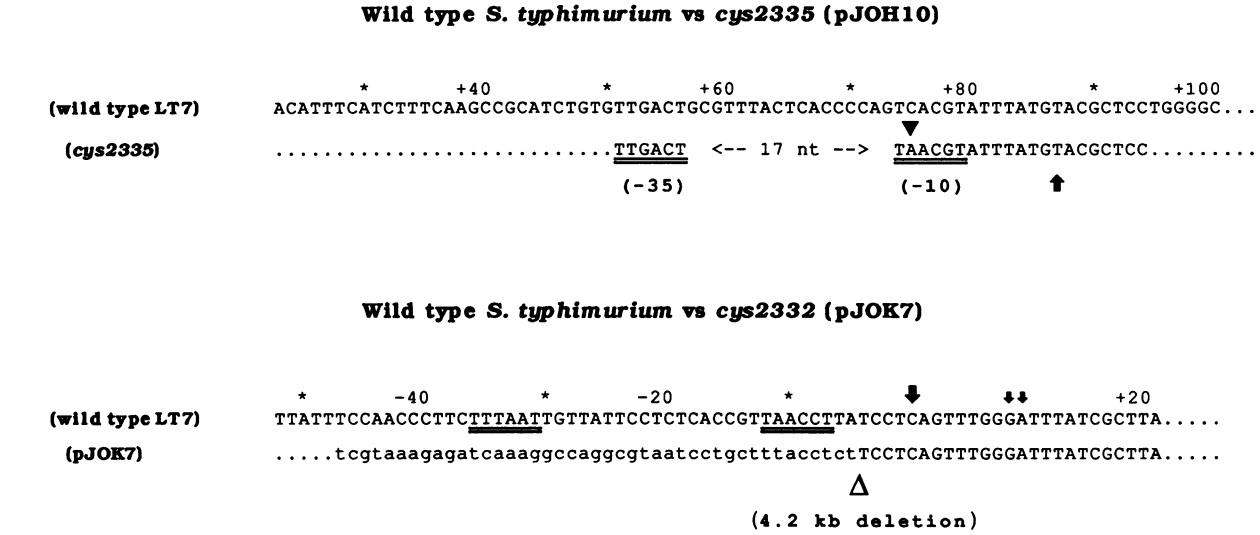

FIG. 4. Comparison of promoter regions from wild-type *S. typhimurium* LT7 and the LT2 derivatives *cys2335* and *cys2332*. Only the relevant portions of sequences are shown. The 389 nucleotides upstream of the *cysJ* start codon in *cys2335* were identical to the wild-type LT7 sequence except for a C-to-A transversion shown at position +75 (▼), which creates a −10 promoter element 17 nucleotides downstream from a nearly consensus −35 sequence. The major initiation start site of this mutant promoter was determined by primer extension studies and is marked at position +87 (⬆). Minor initiation sites were noted at +86 and +89 (not shown). A total of 680 nucleotides upstream to the *cysJ* start codon were sequenced for the *cys2332* allele of pJOK7. Similarity to the wild-type LT7 promoter region was not observed until position –4, after which the next 175 nucleotides were identical. Lowercase letters indicate nonidentical sequences of *cys2332* (except for random matches). Major (⬇) and minor (⬇⬇) transcription start sites of the wild-type sequence are marked as in Fig. 2.

that of wild-type LT7 between positions −218 and +171 except for a C→A substitution at position +74 (Fig. 4). This change cannot be due to a difference between strains LT2 and LT7, because it was not present in the other LT2 derivative, pJOK7 (see below). This mutation changes a TCACGT sequence to TAACGT, which has the highly conserved TA - - - T residues of the −10 element of a promoter. Upstream and separated from this sequence by 17 nucleotides is the nearly consensus −35 promoter sequence TTGACT (15). The results of our primer extension experiments indicate that the C→A mutation in *cys2335* has created a new promoter, which initiates transcription primarily at the T residue at position +87 and with much less efficiency from sites 1 nucleotide upstream and 2 nucleotides downstream. It is of interest that, as was seen with EC1124(pGBK5) and EC1124(pRSM15), the highest levels of extension products from both the wild-type promoters and the mutant promoter were obtained with RNA from cells grown on L-cystine.

Primer extension experiments gave no product with RNA template from EC1124 carrying *cys2332* on pJOK7 except for a small amount of material of about 133 nucleotides observed with primer B (Fig. 3). Of the 680 nucleotides that were sequenced upstream of the *cysJ* start codon of *cys2332*, the first 505 were found to differ completely from any portion of the promoter regions of wild-type LT7 and the *cys2335* allele of pJOH10 (Fig. 4). The last 175 nucleotides were identical to those of wild-type LT7 and differed from *cys2335* only at the position of the *cys2335* mutation. These findings indicate that *cys2332* is either a large deletion or some other type of rearrangement extending upstream from wild-type position −5. The restriction map of the pGBK5 insert (wild type) shows a cluster of *Eco*RI, *Kpn*I, and *Pst*I sites at about 0.5 kb that are found 4.3 kb closer to *cysJIH* in the pJOK7 insert (Fig. 1). Therefore, we believe that *cys2332* is a deletion, which has eliminated most of the wild-type promoter sequences and fused *cysJIH* to an upstream promoter that does not require *cysB* protein for activity. Our inability to detect a primer extension product with RNA from EC1124(pJOK7) suggests this promoter is a kilobase or more upstream of our primer. S1 nuclease protection studies with RNA from EC1124(pJOK7) showed the presence of a small amount of protected fragment, which was approximately the size of that obtained with RNA from EC1124(pGBK5) (data not shown). This is the result expected from a *cys2332* transcript originating upstream of the wild-type initiation site, because such an mRNA would anneal to a DNA probe as far upstream as position −4 and give a protected fragment only 4 nucleotides longer than that obtained with a wild-type transcript.

In vitro transcription. In vitro transcription initiation at the *S. typhimurium cysJIH* promoter(s) was characterized in a transcription runoff assay, with two different duplex DNA fragments used as templates. Each began at the *Kpn*I site at position −218 and extended downstream into *cysJ* to either position +215 or position +271 relative to the major in vivo transcription initiation site (Fig. 2). As a result of the method used to obtain these templates (see Materials and Methods), the shorter ended in a $(C)_{12}$ GAATT-5′ segment not present in the *cysJ* sequence, and the longer had a $(C)_{16}$ GAATT-5′ segment, giving totals of 232 and 292 nucleotides, respectively, expected for runoff products originating from the major in vivo initiation site.

These two templates gave the expected runoff products of either 232 or 292 nucleotides, which were dependent on the addition of both purified *cysB* protein and acetyl-L-serine. With the shorter template and 3 mM *O*-acetyl-L-serine, synthesis of the 232 nucleotide product was directly proportional to *cysB* protein concentration over a range of 0.5 to 10 μg/ml (Fig. 5, lanes 3 through 8). Higher concentrations of *cysB* protein gave no further increase in runoff product, as determined from visual inspection of gel autoradiographs (data not shown). Since *O*-acetyl-L-serine is considered the coinducer of the cysteine regulon, we were surprised to find

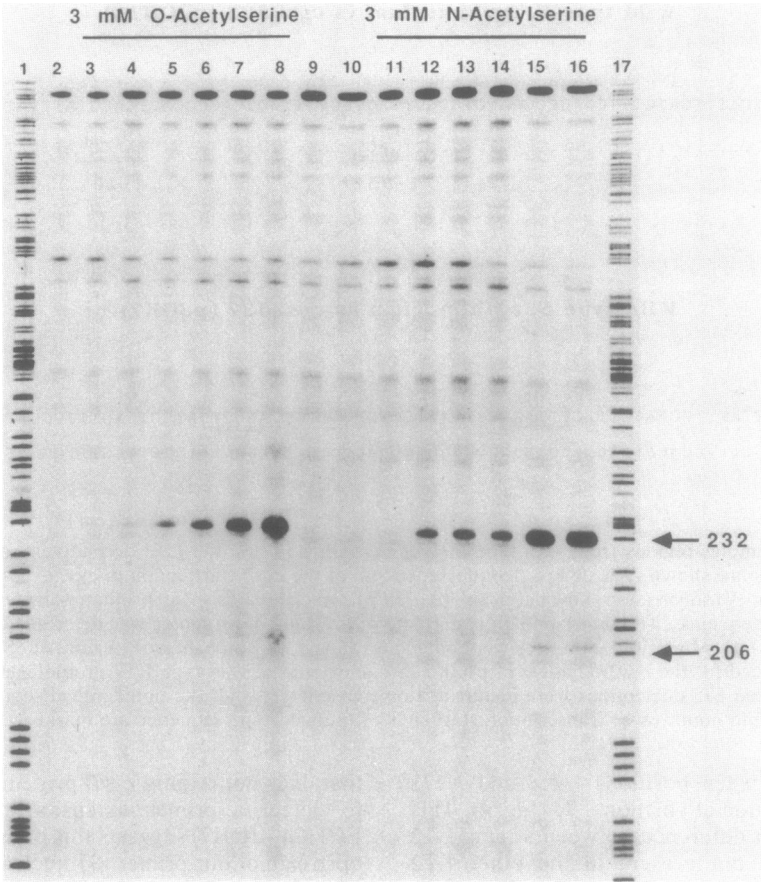

FIG. 5. Dependence of *cysJIH* promoter activity on purified *cysB* protein in an in vitro transcription runoff assay. Initiation complexes between DNA template and RNA polymerase holoenzyme were allowed to form for 2 min at pH 7.4 before addition of sodium heparin and nucleoside triphosphates. Transcription runoff products were then analyzed on polyacrylamide sequencing gels, and product sizes were estimated by comparison with a G-reaction lane from a DNA sequencing reaction with M13mp18 used as a template (lanes 1 and 17). Values are corrected for the assumption that the transcription products migrate faster by 1 nucleotide than the nonphosphorylated DNA products in the standard. The DNA template used in this experiment contained 232 nucleotides downstream of (and including) the major transcription start site for the *S. typhimurium cysJIH* promoter. Reaction mixtures contained no *cysB* protein and no acetyl-L-serine (lane 2); 3.0 mM *O*-acetyl-L-serine and *cysB* protein at 0, 0.5, 1, 2, 5, and 10 μg/ml (lanes 3 to 8, respectively); no acetyl-L-serine and 10 μg of *cysB* protein per ml (lane 9); 3 mM L-serine and 10 μg of *cysB* protein per ml (lane 10); and 3.0 mM *N*-acetyl-L-serine and *cysB* protein at 0, 0.5, 1, 2, 5, and 10 μg/ml (lanes 11 to 16). Production of a 232-nucleotide transcript was clearly dependent on the concentration of *cysB* protein and on the presence of either *O*-acetyl-L-serine or *N*-acetyl-L-serine.

that *N*-acetyl-L-serine was an effective substitute for *O*-acetyl-L-serine in the generation of this in vitro transcription fragment (Fig. 5, lanes 11 through 16). No appreciable product was noted with *cysB* protein at 10 μg/ml in the absence of acetyl-L-serine or with 3 mM L-serine (Fig. 5, lanes 9 and 10). Small amounts of several shorter transcripts that were also dependent on *cysB* protein and acetyl-L-serine were noted, but comparison of gels from experiments using the two templates indicated that these products were due to premature transcription termination (data not shown). There was no evidence in these in vitro experiments for transcription initiation at positions +9 and +10, suggesting that the products found in primer extension analyses (Fig. 3) and in S1 nuclease protection experiments may represent processed derivatives of the transcript originating at position +1.

Our finding that *N*-acetyl-L-serine was active in stimulating in vitro *cysJIH* transcription raised the possibility that conversion of *O*-acetyl-L-serine to *N*-acetyl-L-serine might be responsible for the observed effects of *O*-acetyl-L-serine. This reaction is known to occur spontaneously by means of an intramolecular *O*-to-*N* acetyl shift with a first-order rate constant of 0.98% per min at pH 7.5 and 29°C (13). This constant is directly proportional to the concentration of nonprotonated amino group, viz., NH_2, and therefore is larger at higher pHs. The reverse reaction, viz., formation of *O*-acetyl-L-serine from *N*-acetyl-L-serine, does not occur to any significant extent except in strong acid (32). To minimize conversion of *O*-acetyl-L-serine to *N*-acetyl-L-serine, we used a preincubation time of 2 min at pH 7.4 in all experiments reported here. These conditions gave approximately the same amount of *cysB* protein-dependent transcription as the 8 min at pH 8.0 called for in our model protocol (37). As a further measure, freshly prepared stock solutions of 0.1 M *O*-acetyl-L-serine were kept on ice for no more than 2 h before use. At this low temperature and pH of 5.1, conversion to *N*-acetyl-L-serine was found to be negligible (<0.001%/min at 1°C).

At a constant *cysB* protein concentration of 10 μg/ml, transcription initiation at the *cysJIH* promoter occurred with *N*-acetyl-L-serine concentrations as low as 0.05 mM but was only slight until 0.3 mM (Fig. 6). *O*-Acetyl-L-serine was far less effective and required concentrations of 1 to 3 mM for

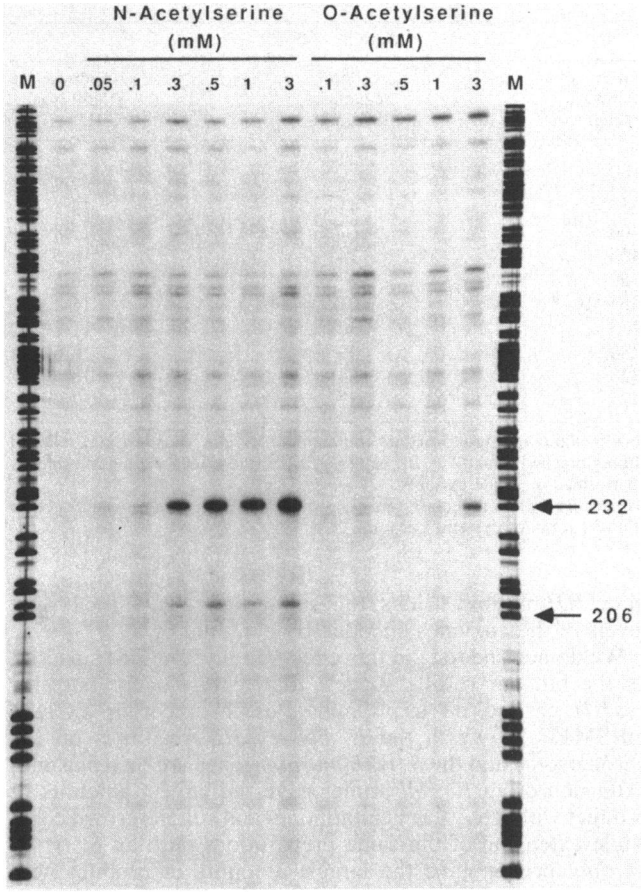

FIG. 6. Relative effects of *N*-acetyl-L-serine and *O*-acetyl-L-serine in stimulating *cysB* protein-dependent in vitro transcription from the *S. typhimurium cysJIH* promoter. The DNA template and general conditions were the same as those described in Materials and Methods and in the legend to Fig. 5. Initiation complexes were allowed to form for 2 min at pH 7.4 to minimize conversion of *O*-acetyl-L-serine to *N*-acetyl-L-serine. The G-reaction lane from a DNA sequencing reaction with M13mp18 used as a template was used as a standard (M). All reaction mixtures contained 10 μg of *cysB* protein per ml and either *N*-acetyl-L-serine or *O*-acetyl-L-serine at the concentrations indicated. With 3 mM *O*-acetyl-L-serine, the amount of specific product at 232 nucleotides was slightly less than that obtained with 0.3 mM *N*-acetyl-L-serine.

significant stimulation. The amount of product found at 3 mM *O*-acetyl-L-serine was equivalent to that formed at between 0.1 and 0.3 mM *N*-acetyl-L-serine in the same experiment. If *O*-acetyl-L-serine itself were totally inactive, this effect would require an *N*-acetyl-L-serine contaminant of 3 to 10% and more likely between 5 and 8%. Analysis of our *O*-acetyl-L-serine preparation by high-performance liquid chromatography showed an *N*-acetyl-L-serine content of <0.2% at zero time. When incubated at 37°C in the runoff assay preincubation buffer (40 mM Tris hydrochloride, 0.1 M KCl, 10 mM $MgCl_2$) at pH 7.4, *O*-acetyl-L-serine was converted to *N*-acetyl-L-serine at a rate of 0.31%/min. *cysB* protein at 10 μg/ml had no appreciable effect on this rate. From these values we estimate that the *N*-acetyl-L-serine concentration of reaction mixtures preincubated with *O*-acetyl-L-serine at 37°C for 2 min at pH 7.4 would be less than 1% of the initial *O*-acetyl-L-serine concentration. Since an *N*-acetyl-L-serine contaminant of 5 to 8% would be required to account for the observed effects, it follows that *O*-acetyl-L-serine itself has some activity as an in vitro activator of *cysJIH* transcription. Conclusive evidence on this point will require a more quantitative type of transcription initiation assay.

In vivo induction of the cysteine regulon by *N*-acetyl-L-serine. Previous studies had shown that *cysE* strains of *E. coli* (18) and *S. typhimurium* (19) could not be derepressed for activities of the cysteine regulon unless provided with an exogenous source of *O*-acetyl-L-serine. Because of our finding that *N*-acetyl-L-serine was more effective than *O*-acetyl-L-serine in stimulating in vitro transcription initiation at the *S. typhimurium cysJIH* promoter, we decided to measure the in vivo effects of *N*-acetyl-L-serine. Sulfur-limited cultures of *S. typhimurium* DW18(*cysE2*) were treated with various concentrations of either *O*-acetyl-L-serine or *N*-acetyl-L-serine, and extracts were assayed for NADPH-cytochrome *c* reductase, a sensitive indicator of *cysJ* flavoprotein activity; for NADPH-sulfite reductase holoenzyme activity, which depends on both *cysJ* and *cysI* expression; and for *O*-acetylserine (thiol)-lyase, a measure of *cysK* expression. The results showed that the cysteine regulon can be induced in vivo by *N*-acetyl-L-serine but required much higher concentrations than are necessary with *O*-acetyl-L-serine (Table 2). With *O*-acetyl-L-serine, enzyme activities reached half-maximal values at concentrations of 0.01 to 0.03 mM and peaked at about 0.1 mM. In contrast to our in vitro comparisons of these two compounds, 10-fold higher concentrations of *N*-acetyl-L-serine were required to achieve similar levels of induction in vivo. Since we did not measure transport rates or steady-state cellular levels of these two compounds, the significance of these in vivo differences cannot be assessed.

DISCUSSION

Our primer extension analyses have defined the in vivo transcriptional start sites and promoter regions for the *cysJIH* operons of *S. typhimurium* and *E. coli*. The deviation of the −35 region of the *cysJIH* promoter from the consensus sequence (15) has also been observed for the *cysK* promoters of *S. typhimurium* and *E. coli*, which are also dependent on *cysB* for expression (6), and appears to be a general characteristic of positively regulated promoters (38). Presumably, *cysB* protein interacts with nearby DNA sequences or with RNA polymerase or both to facilitate transcription initiation at what is otherwise an inefficient promoter. The DNA sequences of the *cys2332* and *cys2335* alleles indicate that the ability of Δ*cysB* strains carrying these mutations to express *cysJIH* is due to the creation of new *cysB*-independent promoters either through a point mutation in *cys2335* or by fusion to another promoter in *cys2332*.

Comparison of the promoter regions of *cysJIH* from *S. typhimurium* and *E. coli* with those of *cysK* showed a number of identities for the four sequences including TA - - CT in the −10 region, CTT in the −35 region, and 12 of 39 nucleotides immediately upstream of the −35 regions (Fig. 7). The last are of particular interest because preliminary studies in this laboratory indicate that the 39 to 41 nucleotides immediately upstream of the −35 region of the *S. typhimurium cysK* promoter are required for positive control by *cysB* protein (R. Monroe and N. M. Kredich, Abstr. Annu. Meet. Am. Soc. Microbiol. 1988, H156, p. 171). Sequences separating the −10 and −35 regions are quite different from one another and consist of 18 nucleotides for the *cysJIH* promoters and only 16 nucleotides in the *cysK* promoters.

TABLE 2. Induction of the cysteine regulon in DW18(*cysE2*) by *O*-acetyl-L-serine and *N*-acetyl-L-serine[a]

Concn (mM) of added:		Activity[b] of:		
O-Acetyl-L-serine	*N*-Acetyl-L-serine	Cytochrome *c* reductase (mU/mg)	Sulfite reductase (mU/mg)	*O*-Acetylserine (thiol)-lyase (U/mg)
0	0	55	<1	3.1
0.01	0	75	5	7.2
0.03	0	255	41	15.1
0.1	0	400	67	17.8
0.3	0	405	74	16.5
1	0	360	47	16.5
3	0	355	61	16.2
0	0.1	155	30	11.2
0	0.3	280	49	16.6
0	1	325	53	18.5
0	3	475	82	22.9

[a] *S. typhimurium* DW18(*cysE2*) was grown with vigorous shaking at 37°C in minimal medium adjusted to pH 6.8 containing 0.5% glucose and 1 mM reduced glutathione (19). Either *O*-acetyl-L-serine or *N*-acetyl-L-serine was added when cultures reached about 2×10^8 cells per ml, and bacteria were harvested by centrifugation at densities of 4×10^9 to 6×10^9 cells per ml. Enzyme assays were performed on crude extracts.

[b] For both reductases, 1 U of activity catalyzes the oxidation of 1 μmol of NADPH per min. Sulfite reductase was assayed with hydroxylamine as an electron acceptor (43). One unit of *O*-acetylserine (thiol)-lyase activity catalyzes the formation of 1 μmol of cysteine per min.

The semiquantitative results of our primer extension analyses with RNA from wild-type *S. typhimurium* carrying no plasmid confirmed previous studies on the effects of different sulfur sources on *cysJIH* expression (11, 19, 36). The very high levels of extension product with RNA from plasmid-carrying strains grown on L-cystine were unexpected, however, and prompted us to measure enzyme levels in plasmid-carrying strains. EC1124 carrying either pRSM15, pJOH10, or pJOK7 was grown on minimal medium with different sulfur sources under conditions identical to those used to prepare RNA for primer extension studies. Extracts were assayed for NADPH-cytochrome *c* reductase and NADPH-sulfite reductase to evaluate *cysJ* and *cysJI* expression and for *O*-acetylserine (thiol)-lyase to estimate *cysK* expression as a control. As might be expected from a copy-number effect, the absolute level of cytochrome *c* reductase was approximately 30-fold higher in L-cystine-grown EC1124 (pRSM15) than in a plasmid-free *cysJIH*$^+$ strain. However, in contrast to the results expected from primer extension experiments, this high level of enzyme activity increased another 10- to 20-fold during growth on glutathione or sulfate. As expected, *O*-acetylserine (thiol)-lyase levels were the lowest in L-cystine-grown cells. Similar results were obtained with EC1124(pJOH10) and EC1124(pJOK7), in which the lowest levels of all three enzymes were also found in L-cystine-grown cells (data not shown). Thus, even though growth on L-cystine gave the highest transcript levels in *cysJIH* plasmid strains, this sulfur source gave the lowest levels of the enzymes encoded by *cysJ* and *cysI*.

We do not understand this effect of L-cystine on transcript levels, but our results suggest that it may occur only for *cysJIH* carried on a plasmid. For instance, in EC1124 (pRSM15), in which the *E. coli cysJIH* region is on the chromosome and the *S. typhimurium* genes are on a plasmid, extension of an *E. coli* primer gave the largest amounts of product with RNA from glutathione- and sulfate-grown cells, while extension of the same preparations with an *S. typhimurium* primer gave the largest amounts of product with RNA from L-cystine-grown cells (Fig. 3). Therefore, in EC1124(pRSM15), levels of *cysJ* transcript were "appropriate" for the sulfur source for the chromosomal gene and inappropriate for the plasmid gene. Furthermore, in EC1124(pJOH10), growth on L-cystine gave the highest levels of transcript originating from the *cysB*-independent promoter created by the point mutation in *cys2335*, which was also carried on a plasmid (Fig. 3). It has yet to be determined why the very high transcript levels found in L-cystine-grown plasmid strains are not accompanied by correspondingly high levels of enzyme activity.

Our in vitro transcription studies using templates containing the *S. typhimurium cysJIH* promoter establish a role for *cysB* in regulating transcription from this promoter and provide a direct demonstration of biochemical activity for purified *cysB* protein. Although these experiments did not

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                                          -35                  -10
S.typh cysJIH   AACAGGTTAGTTCATTTGGTTATTTGTTATTTCCAACCCTTCTTTAATTGTTATTCCTCTCACCGTTAACCTTATCCTC
E.coli cysJIH   AACAGGTTAGTCGATTTGGTTATTAGTTATCGCTATCCCGTCTTTAATCCACACCGTTTGCCCCGTTAACCTTACCTT
S.typh cysK     ACCATTATTTCCCATCAGCATATAGATATGCGAAATCCTTACTTCCCCATATCTGGCTGGAAGG--TATGCTGGGAAG
E.coli cysK     GTCATTATTTCCCTTCTGTATATAGATATGCTAAATCCTTACTTCCGCATATTCTCTGAGCGGG--TATGCTACCTGTTG
(conserved)     ..CA...T......T..G..TAT...T.......A.CC...CTT.....................TA..CT........
```

FIG. 7. Comparison of the *cysJIH* and *cysK* promoter regions of *S. typhimurium* and *E. coli*. Sequences are aligned with respect to −35 and −10 regions (double underline) and have been adjusted by including two blank spaces (hyphens) just before the −10 regions of the *cysK* promoters. The last nucleotide of each sequence is the major transcription start site. The *cysK* data are from the work of Byrne et al. (6). Residues that are identical in all four sequences are shown on the bottom line.

define an exact mechanism of action for *cysB* protein, they are consistent with the notion that this regulatory element acts at the level of transcription initiation. As predicted from in vivo studies, in vitro transcription from the *cysJIH* promoter requires not only *cysB* protein but also a coinducer, which has been thought to be *O*-acetyl-L-serine (17, 19). Our transcription runoff assays, however, show that *N*-acetyl-L-serine is far more effective than *O*-acetyl-L-serine as a coinducer. It is not clear whether the major regulatory effect of *O*-acetyl-L-serine is due to a low level of intrinsic activity as a coinducer or is mediated through its conversion to *N*-acetyl-L-serine. The greater in vivo activity of externally supplied *O*-acetyl-L-serine as a coinducer may be due to more rapid cellular uptake, followed perhaps by conversion to *N*-acetyl-L-serine.

By analogy with other regulatory systems, the most likely role for coinducer would be to bind to *cysB* protein, thereby effecting a change in conformation necessary for stimulating transcription initiation at *cys* promoters. In support of this model is the fact that certain *cysB* point mutations causing single-amino-acid changes (T. E. Colyer, J. Ostrowski, R. S. Monroe, and N. M. Kredich, Abstr. Annu. Meet. Am. Soc. Microbiol. 1988, H154, p. 170) obviate the requirement for coinducer and result in the constitutive expression of *cys* genes, even in a *cysE* background and regardless of the sulfur source used for growth (19). A previously unsuccessful effort to demonstrate binding of *O*-acetyl-L-serine to purified *cysB* protein (27) is now understandable in light of our finding that this compound has only weak activity as a coinducer in transcription runoff assays with the *cysJIH* promoter. If these in vitro experiments are any indication, it may also be difficult to demonstrate binding of *N*-acetyl-L-serine to *cysB* protein by direct means. The limited solubility of *cysB* protein limits the sensitivity of binding studies to a K_d of 1×10^{-4} M or less, and only a very weak effect on in vitro transcription initiation was noted at that concentration of *N*-acetyl-L-serine. Preliminary studies from this laboratory suggest that it may be possible to quantify binding of *N*-acetyl-L-serine to *cysB* protein indirectly by measuring effects on binding of *cysB* protein to DNA.

ACKNOWLEDGMENTS

This work was supported by Public Health Service research grant DK-12828 from the National Institutes of Health.

We thank Humphrey Kendall for his excellent technical assistance.

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