JOURNAL OF BACTERIOLOGY, Jan. 1992, p. 390–397
0021-9193/92/020390-08$02.00/0

Vol. 174, No. 2

Regulation of the *Salmonella typhimurium metA* Gene by the MetR Protein and Homocysteine

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Received 19 July 1991/Accepted 5 November 1991

The DNA sequence of the *Salmonella typhimurium metA* control region is presented. S1 nuclease mapping was used to determine the transcription initiation site. By measuring β-galactosidase levels in *Escherichia coli* strains lysogenized with λ phage carrying a *metA-lacZ* gene fusion, the MetR protein was shown to activate the *metA* gene. Homocysteine, an intermediate in methionine biosynthesis, plays a negative role in the MetR-mediated activation mechanism. Gel mobility shift assays and DNase I protection experiments showed that the MetR protein binds to a DNA fragment carrying the *metA* control region and protects a 26-bp region beginning 9 bp upstream of the −35 promoter sequence.

In *Salmonella typhimurium* and *Escherichia coli*, the first step in the biosynthesis of methionine is the conversion of homoserine to *O*-succinylhomoserine by homoserine succinyltransferase, the *metA* gene product (Fig. 1) (for a review, see reference 21). The *metA* gene, as well as all of the other methionine biosynthetic genes except *metH*, is negatively regulated by the MetJ repressor, with *S*-adenosylmethionine acting as a corepressor. In addition to MetJ-mediated repression, at least two genes, the *metE* and *metH* genes, are activated by MetR, a DNA-binding protein (4, 28). Although homocysteine is required for the MetR-mediated activation of the *metE* gene, homocysteine plays an inhibitory role in the MetR-mediated activation of the *metH* gene (5, 29).

In *S. typhmurium metE* mutants, the *metA* gene is not expressed during methionine-limited growth (22). Since homocysteine is expected to accumulate in *metE* mutants (Fig. 1) and plays a negative role in the MetR-mediated activation of the *metH* gene, we tested whether homocysteine also plays a negative role in regulation of the *S. typhimurium metA* gene. We show here that the *metA* gene is positively regulated by the MetR protein and that homocysteine plays an inhibitory role in this MetR-mediated activation.

MATERIALS AND METHODS

Bacterial strains, bacteriophages, and plasmids. All bacterial strains used in this study are derivatives of *E. coli* K-12 and are described in Table 1. Phage λgt2 (18) was obtained from R. Davis, and λ*c*I90*c*17 (24) was obtained from M. Feiss. Plasmids pBR322 (2), pMC1403 (6), and pMF7 (10) were described previously. Other plasmids and phages were isolated during this investigation.

Media. Luria agar and glucose minimal medium (GM) have been described previously (25). GM was supplemented with vitamin B_1 (1 μg/ml) and phenylalanine (50 μg/ml), since most strains carry the *pheA905* and *thi* mutations. Other supplements were added at the following concentrations: ampicillin, 100 μg/ml; tetracycline, 10 μg/ml; amino acids, 50 μg/ml; purines, 10 μg/ml; 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal), 40 μg/ml; and DL-homocysteine, 100 μg/ml.

DNA manipulations. Procedures for the isolation of DNA, restriction enzyme digestion, ligation, and bacterial transformation have been described previously (14).

Construction of plasmids containing the *metA* gene. *S. typhimurium* chromosomal DNA (25 μg) was cut with the restriction enzyme *Eco*RI under partial digestion conditions, the DNA was electrophoresed on a 0.8% low-melting-temperature agarose gel, and fragments of about 35 to 45 kb were isolated (14). The DNA fragments were ligated into the *Eco*RI site of the cosmid vector pMF7 (1 μg). The ligated DNA was ethanol precipitated and dissolved in 12 μl of TE buffer (10 mM Tris-HCl [pH 8], 1 mM EDTA), and a 4-μl aliquot was packaged with a Gigapack Gold λ DNA packaging kit (Stratagene, La Jolla, Calif.). The packaged DNA was used to infect strain GS765 (*hsdR4 hsdM*$^+$ *metA28*), and the cells were plated on a GM plate supplemented with ampicillin. Plasmid DNA was isolated from one Met$^+$ Ap^r (ampicillin-resistant) transformant and designated pGS233. An *Eco*RI digest of pGS233 showed the presence of several *Eco*RI fragments. To reduce the size of the original insert, plasmid pGS233 was digested with *Eco*RI, and the DNA fragments were ligated into the *Eco*RI site of plasmid pBR322. The ligation mixture was used to transform the *metA* mutant strain AB1927, and the transformed cells were plated on a GM plate supplemented with purines, arginine, and tetracycline. Plasmid DNA was isolated from one Met$^+$ Tc^r (tetracycline-resistant) transformant and designated pGS234. An *Eco*RI digest showed that plasmid pGS234 carries a single *Eco*RI insert fragment of about 28 kb. To further reduce the size of the cloned DNA, a restriction map of this plasmid was determined (data not shown). Three *Ase*I sites were located within the cloned fragment. Plasmid pGS234 was digested with *Eco*RI and *Ase*I, and the fragments were ligated into the *Ase*I and *Eco*RI sites of plasmid pBR322. The ligated mixture was used to transform the *metA* mutant strain AB1927, and the transformed cells were plated on a GM plate supplemented with arginine, purines, and tetracycline. Plasmid DNA was prepared from one Met$^+$ Tc^r colony and designated pGS235. Restriction enzyme analysis showed the presence of a single 3.4-kb *Ase*I-*Eco*RI insert fragment.

DNA sequence analysis. DNA sequencing was done by the method of Maxam and Gilbert (15).

Construction of a *metA-lacZ* gene fusion. A 527-bp *Hpa*I-*Xmn*I DNA fragment that carries the *metA* control region and the first 19 amino acid codons of the *metA* structural

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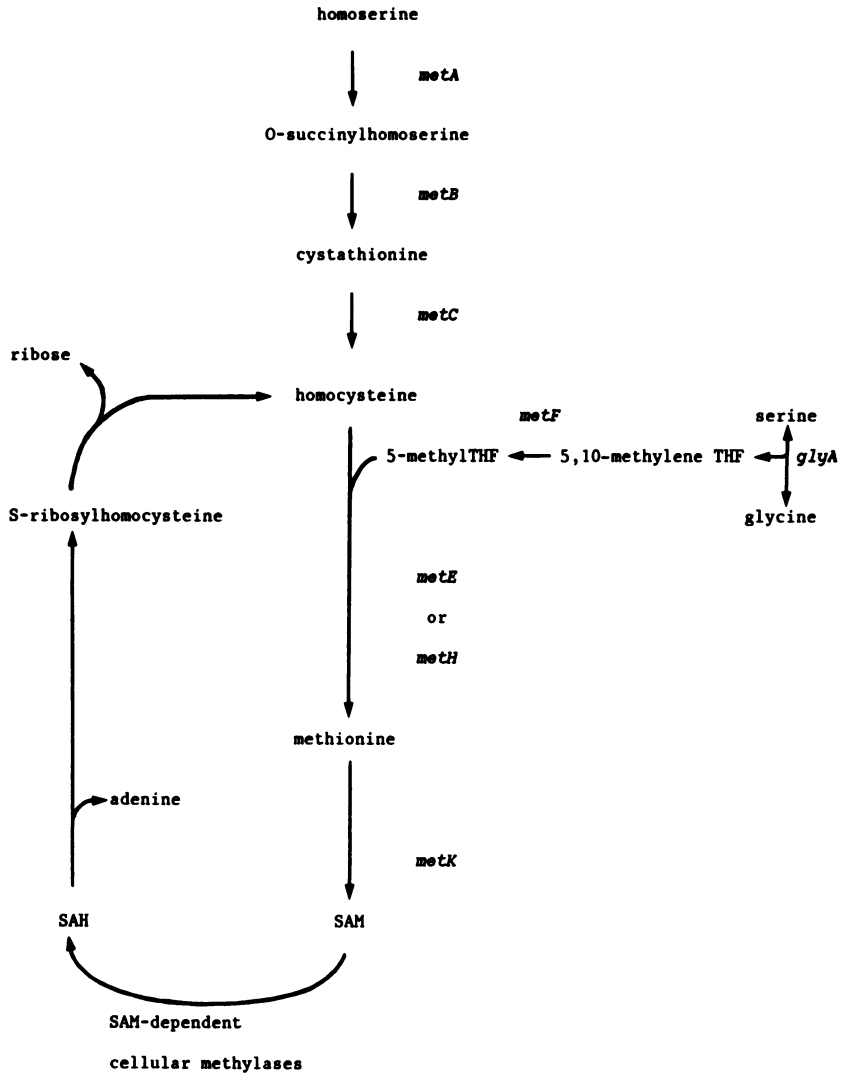

FIG. 1. The methionine biosynthetic pathway in *E. coli* and *S. typhimurium* (7, 21). The nonfolate branch includes the *metA*, *metB*, and *metC* gene products and produces homocysteine. The folate branch includes the *metF* and *glyA* gene products and produces the methyl group added to homocysteine to form methionine. The enzymes encoded by the *metE* and *metH* genes catalyze the transmethylation reaction. The methionine made is used in protein synthesis or converted to *S*-adenosylmethionine (SAM), a principal methyl-group donor. *S*-Adenosylhomocysteine (SAH), a product of SAM-dependent methylation reactions, is converted to adenine and homocysteine by the cyclical regenerative branch of the pathway (9), which has not been characterized genetically. THF, tetrahydrofolate.

gene was isolated from plasmid pGS235. This fragment was ligated into the *lac* fusion vector pMC1403, which had been cut with *Xma*I and whose ends had been filled in with deoxynucleoside triphosphates and the large fragment of DNA polymerase I. The reading frame of the *metA* gene and the *lacZ* gene are maintained in this fusion. The ligation mixture was used to transform the *lac* deletion strain GS245, and the transformed cells were plated on Luria agar supplemented with ampicillin and X-Gal. Plasmid DNA was isolated from one blue Ap^r transformant, and the *metA-lacZ* fusion site was confirmed by DNA sequencing. The fusion plasmid was designated pAlac.

Construction of λ lysogens carrying the *metA-lacZ* gene fusion. The *Eco*RI-*Sal*I fragment in plasmid pAlac, which carries the *metA-lacZ* gene fusion and the *lacY* and *lacA* genes, was cloned into the *Eco*RI site of the temperature-sensitive phage λgt2 by a procedure described previously

TABLE 1. *E. coli* strain descriptions

Strain[a]	Genotype
GS162	Wild type
GS245	Δ*glyA*::Mu
GS597	*metJ97*
GS718	*metB1*
GS719	*metB1 metJ97*
GS722	*metB1 metJ97* Δ*metF*::Mu
GS747	*metB1 metJ97 metE163*::Tn*10*
GS748	*metJ97* Δ*metR*::Mu
GS761	*metB1 metJ97* Δ*metR*::Mu
GS765	*metA28 gal* $T1^r$ *endA sbc-15 hsdR4 hsdM*$^+$ *malE52*::Tn*10 thi*
AB1927	*metA28 argH1 purF1 xyl-7*

[a] Strain AB1927 was obtained from the *E. coli* Genetic Stock Center. All other strains were constructed in our laboratory. In addition to the genotypes shown, all strains except AB1927 and GS765 carry the *pheA905*, *thi*, Δ*lacU169*, *araD129*, and *rpsL* mutations.

(26). This phage, designated λAlac, was then used to lysogenize appropriate bacterial strains as described previously (26). Lysogens were tested for a single copy of λAlac phage by infection with phage λ*c*I90*c*17 (24).

β-Galactosidase assay. β-Galactosidase activity was measured by the chloroform-sodium dodecyl sulfate lysis procedure described by Miller (17). The results are averages of two or more assays in which the values were determined in triplicate for each assay.

Gel mobility shift assay. The gel mobility shift assay was based on the methods of Fried and Crothers (11) and Garner and Revzin (12). Plasmid pAlac was digested with *Eco*RI and *Bam*HI, and approximately 0.25 μg of the digested DNA was added to 20-μl reaction mixtures containing DNA-binding buffer (DBB; 10 mM Tris-HCl [pH 7.5], 50 mM KCl, 1 mM EDTA, 5% glycerol, 1 mM dithiothreitol) plus 125 μg of bovine serum albumin (BSA) per ml and, where indicated, 1 mM L-homocysteine. The reaction mixtures were incubated for 5 min at 37°C. Next, 2 μl of purified *S. typhimurium* MetR protein (3) in 2× DBB plus 125 μg of BSA per ml in a twofold dilution series ranging from 0.8 to 0.1 μg of protein was added, and incubation was continued for 15 min at 37°C. One microliter of dye mix (0.1% xylene cyanol and 50% glycerol in H_2O) was added to each reaction mixture, and the samples were loaded immediately onto a 5% polyacrylamide gel (bisacrylamide-acrylamide [1/30] buffered with 10 mM Tris-HCl [pH 7.4], 0.38 M glycine, and 1 mM EDTA). The gel was prerun at 9 V/cm for 1 h prior to loading.

DNase I protection assay. The DNase I protection assay is based on the method of Schmitz and Galas (23) with modifications. Plasmid pAlac, which carries the *metA* control region, was digested with *Bam*HI and labeled at the 3′ ends with [α-^{32}P]dGTP and T7 DNA polymerase. The labeled DNA was digested with *Eco*RI, and a 527-bp fragment carrying the *metA* control region was isolated by gel electrophoresis (14). A 114-μl reaction mixture containing the labeled fragment in DBB plus 125 μg of BSA per ml was incubated for 5 min at 37°C. Twelve microliters of MetR protein (9.6 μg) in 2× DBB plus 125 μg of BSA per ml or 2× DBB alone was added, and the mixtures were incubated for an additional 15 min at 37°C. Six microliters of DNase I (1.25 μg/ml dissolved in 20 mM sodium acetate [pH 7]–32 mM $CaCl_2$) was added to the reaction mixtures, and incubation was continued for 30 s. The digestions were stopped with 25 μl of DNase I stop mix (3 M ammonium acetate, 0.25 M EDTA, 15 μg of sonicated calf thymus DNA per ml), and the mixtures were precipitated with ethanol. The DNase I digestion products were resuspended in sequencing dye mix and run alongside the Maxam-Gilbert sequencing reaction mixtures of the DNA probe.

S1 nuclease mapping. The S1 nuclease mapping procedure of Weaver and Weissmann (31) was used with minor modifications. Plasmid pAlac was digested with *Eco*RI and *Bam*HI, and the 527-bp *Eco*RI-*Bam*HI DNA fragment containing the *metA* control region was isolated and labeled at the 5′ ends with [γ-^{32}P]ATP and T4 polynucleotide kinase (14). The two strands were then separated on an 8% polyacrylamide gel, and the strand complementary to the *metA-lacZ* transcript was isolated and used as a probe (15). Total cellular RNA was isolated as described previously (1). Twenty micrograms of total cellular RNA was precipitated along with an aliquot of the ^{32}P-labeled single-stranded DNA probe. The DNA pellets were resuspended in 10 μl of hybridization buffer {(0.4 M NaCl, 0.04 M PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); pH 6.4], 1 mM EDTA}, heated to 90°C for 2 min, and then allowed to hybridize at 65°C for 1 h. A 100-μl portion of cold S1 nuclease buffer (0.25 M NaCl, 0.06 M sodium acetate [pH 4.6], 1 mM $ZnSO_4$, 5% glycerol) and 300 U of S1 nuclease were added, and digestion was carried out at 20°C for 1 h. The S1 nuclease-resistant products were then precipitated and electrophoresed on a DNA sequencing gel adjacent to the Maxam and Gilbert G sequencing reaction ladder of the DNA probe.

Scanning densitometry. The optical density of each band from the S1 nuclease mapping autoradiogram was measured with a scanning densitometer. The results from the scanning densitometer were quantified by cutting out and weighing peaks representing each band in photocopies of the tracings. The percentages were calculated by comparing the ratio of the weight of the band from each lane to the weight of the band from 719λAlac without homocysteine.

Nucleotide sequence accession number. The nucleotide sequence data reported have been deposited with the GenBank nucleotide sequence data base under accession number M74188.

RESULTS

Sequence of the *metA* control region. The *metA* gene was initially cloned on an *Eco*RI DNA fragment in cosmid vector pMF7 and subsequently subcloned on a 3.4-kb *Ase*I-*Eco*RI DNA fragment into plasmid pBR322 (see Materials and Methods). The location and orientation of the *metA* gene in this fragment was determined as follows. A physical map of the fragment was prepared and compared with the physical map deduced from the sequence of the *E. coli metA* gene (16). Several restriction enzyme sites appeared to be common to the *E. coli metA* gene and the 3.4-kb *Ase*I-*Eco*RI fragment carrying the *S. typhimurium metA* gene. A 207-bp sequence near an *Alw*NI site, deduced to be near the *metA* promoter, was determined and compared with the *E. coli metA* sequence. The sequence showed 90% homology with the *E. coli metA* structural gene sequence (16). The DNA sequence was then extended to include the *metA* control region and the beginning of the *metA* structural gene (Fig. 2).

Regulation of a *metA-lacZ* gene fusion. To facilitate regulatory studies, we constructed a λAlac phage which carries a *metA-lacZ* gene fusion (see Materials and Methods). This fusion encodes a chimeric β-galactosidase whose synthesis is directed by the transcriptional and translational signals of the *metA* control region. The λAlac phage was used to lysogenize strains GS162 (wild type), GS718 (*metB*), and GS597 (*metJ*). The *metB* mutation results in methionine auxotrophy and should cause overexpression of *metA* during methionine-limited growth. The lysogens 162λAlac, 718λAlac, and 597λAlac were grown in GM and, where indicated, supplemented with either L-methionine (repressing condition) or D-methionine, and β-galactosidase levels were measured (D-methionine is converted to L-methionine by the cells and serves as a limiting source of methionine [13]). Expression of the *metA-lacZ* fusion was derepressed about eight- and sevenfold in the wild-type lysogen 162λAlac grown in GM or GM supplemented with D-methionine, respectively, compared with L-methionine (Table 2). Expression of the *metA-lacZ* fusion was derepressed 44-fold in the *metB* lysogen 718λAlac grown in the presence of D-methionine in comparison with L-methionine. Expression of the *metA-lacZ* fusion was further elevated in the *metJ* lysogen 597λAlac, and L-methionine did not significantly repress expression of the fusion. These results are consistent with the MetJ-mediated repression reported previously for the

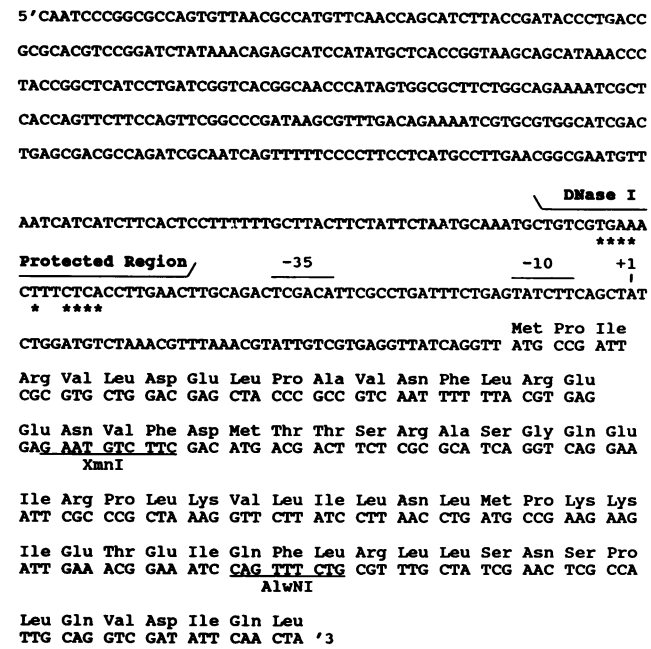

FIG. 2. DNA sequence of the *S. typhimurium metA* control region. The transcriptional start point (+1) was determined by S1 nuclease mapping (Fig. 5). The most likely −35 and −10 promoter regions are overlined and were assigned on the basis of their locations relative to the transcription start site. The 26-bp region of DNA protected from DNase I digestion by the MetR protein (Fig. 4) is indicated by the bracket. The interrupted palindrome 5'-TGAANNTNNCTCA-3' (N = any nucleotide) that may constitute the MetR recognition sequence is indicated by asterisks. The *Alw*NI site that was used to determine the location and orientation of the *metA* gene in the cloned fragment and the *Xmn*I site that was used to construct the *metA-lacZ* gene fusion are indicated.

intact *S. typhimurium metA* gene (22), indicating that the λAlac fusion is regulated normally.

Effect of homocysteine on *metA-lacZ* expression. Homocysteine has been shown to play a positive role in MetR-mediated activation of the *metE* gene but a negative role in activation of the *metH* gene (29). To determine whether homocysteine is involved in regulation of *metA* gene expression, the λAlac phage was used to lysogenize strain GS719 (*metJ metB*). This strain does not accumulate high levels of homocysteine because of the *metB* mutation (Fig. 1), and it can be used to test the effect of homocysteine supplementation on the expression of the *metA-lacZ* gene fusion. However, homocysteine cannot be completely eliminated because of the cyclical branch of the methionine pathway (Fig. 1). The *metJ* mutation eliminates changes in β-galactosidase levels being mediated by the MetJ repressor. Lysogen 719λAlac was grown in GM supplemented with D-methionine or D-methionine plus homocysteine, and β-galactosidase levels were determined. In lysogen 719λAlac, homocysteine supplementation resulted in about a 2.4-fold decrease in β-galactosidase activity (Table 3).

TABLE 2. Reguation of the *metA-lacZ* gene fusion

Lysogen	Relevant genotype	β-Galactosidase activity with indicated supplement[a]		
		No addition	L-Methionine	D-Methionine
162λAlac	Wild type	81	10	74
712λAlac	*metB*	ND[b]	10	443
597λAlac	*metJ*	1,490	1,480	1,357

[a] Units of activity are Miller units (17). Cells were grown in GM and, where indicated, supplemented with either L-methionine or D-methionine.
[b] ND, not determined (since GM alone does not support growth).

TABLE 3. Effects of homocysteine on expression of the *metA-lacZ* gene fusion

Lysogen	Relevant genotype	β-Galactosidase activity with indicated supplement[a]	
		D-Methionine	D-Methionine plus homocysteine
719λAlac	*metJ metB*	1,302	551
722λAlac	*metJ metB metF*	487	368
747λAlac	*metJ metB metE*	694	538

[a] Units of specific activity are Miller units (17). Cells were grown in GM supplemented with D-methionine, either with or without DL-homocysteine.

The λAlac phage was also used to lysogenize strains GS722 (*metJ metB metF*) and GS747 (*metJ metB metE*). The *metE* and *metF* mutations should result in the accumulation of intracellular levels of homocysteine (8) through the cyclical regenerative branch of the methionine pathway (Fig. 1). When lysogens 722λAlac and 747λAlac were grown in GM plus D-methionine, β-galactosidase levels were significantly reduced compared with lysogen 719λAlac (Table 3). The addition of homocysteine to the growth medium resulted in a small but reproducible decrease in β-galactosidase levels. These results suggest that homocysteine has an inhibitory role in the regulation of the *metA-lacZ* gene fusion.

Effect of the MetR protein on *metA-lacZ* expression. To determine whether the MetR protein mediates the inhibitory effect of homocysteine on *metA-lacZ* expression, strains GS719 (*metJ metB*), GS748 (*metJ metR*), and GS761 (*metJ metB metR*) were lysogenized with the λAlac phage. These lysogens were grown in GM supplemented with D-methionine or D-methionine plus homocysteine, and β-galactosidase levels were determined. In the *metR* lysogens 748λAlac and 761λAlac grown in GM supplemented with D-methionine, β-galactosidase levels were about 2.9- and 3.1-fold lower than in the *metR*$^+$ lysogen 719λAlac (Table 4). Homocysteine supplementation still resulted in a small but reproducible decrease in β-galactosidase levels in these lysogens.

Expression of the *metE* gene requires a functional MetR protein (30). Thus, *metR* mutants are phenotypically like *metE* mutants. Since homocysteine would be expected to accumulate to high levels in *metR* mutants, it is possible that the reduced β-galactosidase levels in the *metR* lysogens

TABLE 4. Effect of MetR protein on expression of the *metA-lacZ* gene fusion

Lysogen	Relevant genotype	β-Galactosidase activity with indicated supplement[a]	
		D-Methionine	D-Methionine plus homocysteine
719λAlac	*metJ metB*	1,302	551
748λAlac	*metJ metR*	444	369
761λAlac	*metJ metB metR*	420	342
761λAlac(pGS69)	*metJ metB metR/metE*$^+$	343	322

[a] Units of specific activity are Miller units (17). Cells were grown in GM supplemented with D-methionine, either with or without DL-homocysteine.

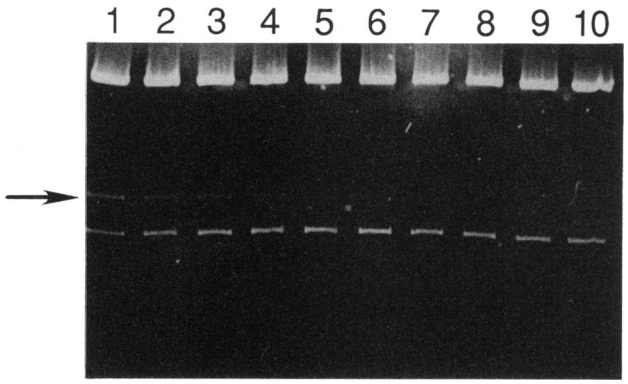

FIG. 3. Gel mobility shift assay for binding of MetR protein to the *metA* control region. Plasmid pAlac, which contains the *metA* control region, was digested with *Eco*RI and *Bam*HI and then incubated with various dilutions of MetR protein to allow specific protein-DNA complexes to form. The samples were then electrophoresed on a nondenaturing 5% polyacrylamide gel. The effect of homocysteine on DNA-MetR complexes was determined by adding 1 mM L-homocysteine to the binding reaction mixtures. Lanes 1, 3, 5, and 7, twofold dilutions of purified MetR protein from 0.8 to 0.1 μg; lanes 2, 4, 6, and 8, twofold dilutions of purified MetR protein from 0.8 to 0.1 μg and 1 mM L-homocysteine; lane 9, no MetR protein; lane 10, 1 mM L-homocysteine and no MetR protein. The arrow indicates the position of the DNA-MetR complex.

748λAlac and 761λAlac are due to high intracellular levels of homocysteine rather than to the absence of a functional MetR protein. To determine which of these possibilities is most likely, lysogen 761λAlac was transformed with plasmid pGS69, a multicopy plasmid containing the *S. typhimurium metE* gene (30). Although the *metE* gene is expressed at only about 5% of the wild-type level in *metR* mutants, the multiple copies of the *metE* gene in the transformed cells provide sufficient homocysteine transmethylase levels to utilize homocysteine produced by the cyclical pathway. Thus, if high intracellular homocysteine were responsible for decreased *metA-lacZ* expression in the *metR* mutants, β-galactosidase levels would be expected to increase in lysogen 761λAlac (pGS69). As shown in Table 4, β-galactosidase levels in lysogen 761λAlac (pGS69) were not changed significantly from those in the untransformed cells, suggesting that the MetR protein is required for full *metA-lacZ* gene expression.

Binding of the MetR protein to the *metA* control region. The MetR protein has been shown to activate *metE* gene expression by binding to *metE* promoter region DNA (4, 28). A gel mobility shift assay was used to determine whether the MetR protein binds to the *metA* control region and whether homocysteine contributes to the binding of this protein. Plasmid pAlac was digested with *Eco*RI and *Bam*HI to generate a 527-bp *Eco*RI-*Bam*HI fragment containing the *metA* control region. The DNA was incubated with or without MetR protein and with or without 1 mM L-homocysteine (see Materials and Methods). The protein-DNA complexes were then analyzed on a nondenaturing 5% polyacrylamide gel. The addition of the MetR protein shifted the normal mobility of the DNA fragment to a more slowly migrating form (Fig. 3, lanes 1 to 8). As the amount of MetR protein decreased from 0.8 to 0.1 μg, a decreasing amount of the DNA fragment was shifted. The addition of 1 mM L-homocysteine did not significantly alter the affinity of binding of the MetR protein to the DNA.

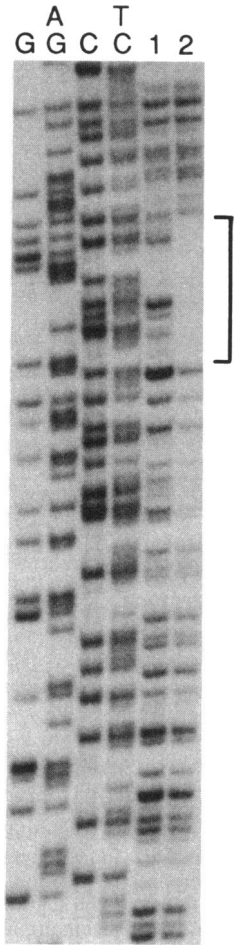

FIG. 4. Protection from DNase I digestion of the *metA* control region by the purified MetR protein. The ^{32}P-labeled 527-bp *metA* DNA probe was incubated with (lane 2) or without (lane 1) 9.6 μg of purified MetR protein and then subjected to partial DNase I digestion. The reaction mixtures were run alongside Maxam and Gilbert sequencing reaction mixtures of the same DNA fragment. The region of the probe protected from DNase I digestion is indicated by the bracket and is shown in Fig. 2.

Location of the MetR-binding site. A DNase I protection assay was used to locate the binding site for the MetR protein on the 527-bp *Eco*RI-*Bam*HI DNA fragment carrying the *metA* control region (see Materials and Methods). As shown in Fig. 4, the MetR protein protected a 26-bp region from DNase I attack (lane 2). This region was not protected in the absence of MetR protein (lane 1). The sequence protected by the bound MetR protein is indicated by the bracket in Fig. 2.

Transcriptional regulation of the *metA* gene. To determine whether regulation of the *metA-lacZ* gene fusion by the MetR protein and homocysteine occurs at the transcriptional level, an S1 nuclease assay was used to measure relative levels of *metA-lacZ* mRNA (see Materials and Methods). The cellular RNA used was prepared from lysogens 719λAlac and 761λAlac grown in GM supplemented either with D-methionine or with D-methionine plus homocysteine. The results are shown in Fig. 5. The relative amounts of *metA-lacZ* mRNA were quantitated from the autoradiogram by densitometry (see Materials and Methods). For lysogen

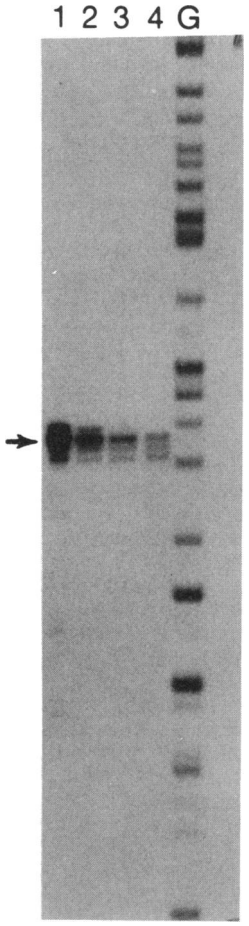

FIG. 5. Transcriptional regulation of the *metA-lacZ* gene fusion by the MetR protein and homocysteine. Lysogens 719λAlac and 761λAlac were grown in GM supplemented with either D-methionine or D-methionine plus homocysteine and were used to prepare total cellular RNA. Equal amounts of RNA from each culture were hybridized to a ^{32}P-labeled DNA probe containing the *S. typhimurium metA* control region and then digested with S1 nuclease. The S1 nuclease-resistant products (arrow) were run on a DNA sequencing gel adjacent to the G sequencing reaction mixture of Maxam and Gilbert (15). Lane 1, 719λAlac grown with D-methionine; lane 2, 719λAlac grown with D-methionine plus DL-homocysteine; lane 3, 761λAlac grown with D-methionine; lane 4, 761λAlac grown with D-methionine plus DL-homocysteine.

719λAlac, the addition of homocysteine resulted in about a fourfold decrease in *metA-lacZ* mRNA (Fig. 5; compare lanes 1 and 2). For lysogen 761λAlac, grown either with or without homocysteine, there was a 9- to 10-fold decrease in *metA-lacZ* mRNA compared with 719λAlac grown with D-methionine (Fig. 5; compare lane 1 with lanes 3 and 4). These results suggest that homocysteine and the MetR protein control *metA-lacZ* gene expression at the level of transcription.

DISCUSSION

The *S. typhimurium metA* gene was cloned, and the nucleotide sequence of the control region and the beginning of the structural gene was determined (Fig. 2). The sequence determined is similar to that of the *metA* control region from *E. coli* (16) and allowed us to predict the promoter region and translation start site. The −10 and −35 promoter sequence elements are identical in *E. coli* (16) and *S. typhimurium* and show matches of 4 and 5 out of 6 bp with the promoter consensus sequences (20), respectively. From S1 nuclease mapping (Fig. 5), it was deduced that transcription initiates 6 bases downstream from the proposed −10 sequence. This is the same transcription start site found in *E. coli*. A second transcriptional start site reported for the *E. coli metA* gene (16) at position −76 was not found in *S. typhimurium* by S1 nuclease mapping. The translation start codon is preceded by a 48-base leader transcript.

A λAlac phage carrying a *metA-lacZ* gene fusion (λAlac) was constructed in order to study regulation of the *metA* gene. In the wild-type lysogen 162λAlac, methionine repressed *metA-lacZ* expression about 8-fold (Table 2), whereas in the *metB* lysogen 718Alac, methionine repressed expression 44-fold. In the *metJ* lysogen 597λAlac, methionine-mediated repression was lost (Table 2). These results indicate that the *metA-lacZ* gene fusion is expressed from the normal *metA* control region.

Homocysteine has been shown to play a positive role in MetR-mediated activation of the *metE* gene and a negative role in activation of the *metH* gene (29). In the *metJ metB* lysogen 719λAlac, which does not accumulate high levels of homocysteine because of the *metB* mutation, homocysteine supplementation of the medium resulted in a 2.4-fold decrease in β-galactosidase levels (Table 3). In lysogens 722λAlac and 747λAlac, the *metF* and *metE* mutations result in the accumulation of intracellular homocysteine produced endogenously from *S*-adenosylmethionine by the cyclical branch of the methionine pathway (Fig. 1). These strains have low β-galactosidase levels without homocysteine supplementation. The addition of homocysteine, however, still results in a small but reproducible decrease in β-galactosidase levels. We have no explanation for why this additional decrease occurs. The results indicate that homocysteine plays an inhibitory role in *metA-lacZ* expression.

In the *metJ metR* lysogen 748λAlac and the *metJ metB metR* lysogen 761λAlac, where the MetR protein is inactivated, *metA* expression is decreased 2.9- to 3.1-fold (Table 4). Since *metR* mutants behave phenotypically like *metE* mutants and are expected to accumulate high intracellular levels of homocysteine, the decrease in β-galactosidase synthesis in the *metR* lysogens could be due to an absence of the MetR protein, high homocysteine levels, or both. However, when the *metJ metB metR* lysogen 761λAlac was transformed with the multicopy plasmid pGS69, which carries the *S. typhimurium metE* gene, β-galactosidase levels were essentially the same as in the untransformed cells grown in GM plus D-methionine (Table 4). The multiple copies of *metE* provide enough homocysteine transmethylase to keep intracellular homocysteine concentrations low. These results suggest that the MetR protein is a positive activator of the *metA* gene. It should be noted that in the *metR* lysogens, homocysteine addition to the growth medium still results in a small but reproducible decrease in β-galactosidase levels. It is possible that part of the homocysteine effect occurs via a second mechanism independent of the MetR protein.

Although the MetR protein appears to serve as a positive regulator for the *metA* gene, its absence does not completely eliminate *metA-lacZ* expression (Table 4). Similar results are also observed for MetR-mediated activation of the *E. coli glyA* gene (19) and the *S. typhimurium metH* gene (27). This is consistent with previous reports that although *metR* mutants reduce *metE* gene expression sufficiently to cause

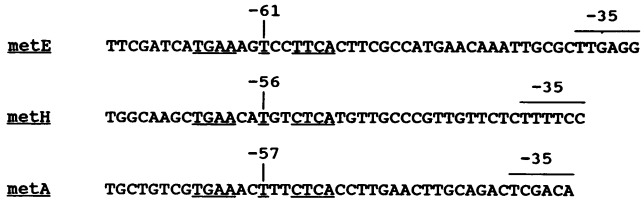

FIG. 6. Comparison of the 26-bp MetR-binding sites from the *S. typhimurium metE* (28), *metH* (27), and *metA* genes. The interrupted palindromes are underlined, and the −35 promoter regions are overlined. The number at the center of each interrupted palindrome is the distance from the respective transcription start site.

methionine auxotrophy, vitamin B_{12} supplementation allows slow growth via the alternate MetH transmethylase, implying that homocysteine is produced by the nonfolate branch at levels high enough to sustain slow growth (30).

Although the S1 nuclease mapping results suggest that homocysteine and the MetR protein control *metA-lacZ* gene expression at the level of transcription, we are not certain why there is not a better correlation between the levels of mRNA and the levels of β-galactosidase. For lysogen 719λAlac, the addition of homocysteine resulted in about a 4-fold decrease in *metA-lacZ* mRNA (Fig. 5; compare lanes 1 and 2) and about a 2.4-fold decrease in β-galactosidase levels (Table 3). For lysogen 761λAlac, grown either with or without homocysteine, there was a 9- to 10-fold decrease in *metA-lacZ* mRNA (Fig. 5; compare lane 1 with lanes 3 and 4), and about a 3.1-fold decrease in β-galactosidase levels (Table 4). It is possible that *metA* is also regulated posttranscriptionally. We are constructing appropriate *metA-lacZ* transcriptional fusions to test this possibility.

A gel mobility shift assay showed that the MetR protein binds to a DNA fragment carrying the *metA* control region (Fig. 3). Homocysteine did not significantly alter the DNA-binding affinity of MetR protein. Similar results have been found for the *S. typhimurium metE* and *metH* genes (3, 28).

By using a DNase I footprinting assay with purified MetR protein, a 26-bp segment beginning 9 bp upstream from the −35 promoter sequence that is protected from DNase I attack was identified (Fig. 4). This 26-bp region was compared with the DNA sequences of the control regions of the *S. typhimurium metE* (28) and *metH* (27) genes, which are also activated by MetR. Within the protected region is an interrupted 8-bp palindrome showing a match of 7 out of 8 bp with the palindrome found in the *metE* promoter region and a match of 8 out of 8 bp with the palindrome found in the *metH* promoter region (Fig. 6). The interrupted palindrome is conserved in the *E. coli metA* sequence (16), suggesting that MetR plays a role in regulating the *metA* gene in this organism as well. The center of the palindrome for the MetR binding site is located 23 bp upstream from the −35 promoter region of the *metA* gene. The analogous distances are 24 bp for the *metH* gene (27) and 29 bp for the *metE* gene (28). It is interesting that homocysteine plays a positive role in *metE* expression (29) but an inhibitory role in both *metH* (29) and *metA* expression. The results are consistent with the hypothesis that the 5- or 6-bp difference in spacing between the MetR-binding site and the −35 promoter elements plays a role in the opposite effects of homocysteine on expression of the *metE* gene and on expression of the *metA* and *metH* genes.

ACKNOWLEDGMENT

This investigation was supported by Public Health Service grant GM38912 from the National Institute of General Medical Sciences.

REFERENCES

1. **Baker, R. F., and C. Yanofsky.** 1968. The periodicity of RNA polymerase initiations: a new regulatory feature of transcription. Proc. Natl. Acad. Sci. USA **60**:313–320.
2. **Bolivar, F., R. L. Rodriguez, P. J. Greene, M. C. Betlach, H. L. Heyneker, H. W. Boyer, J. H. Crosa, and S. Falkow.** 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene **2**:95–113.
3. **Byerly, K. A., M. L. Urbanowski, and G. V. Stauffer.** 1991. The MetR binding site in the *Salmonella typhimurium metH* gene: DNA sequence constraints on activation. J. Bacteriol. **173**: 3547–3553.
4. **Cai, X.-Y., M. E. Maxon, B. Redfield, R. Glass, N. Brot, and H. Weissbach.** 1989. Methionine synthesis in *Escherichia coli*: effect of the MetR protein on *metE* and *metH* expression. Proc. Natl. Acad. Sci. USA **86**:4407–4411.
5. **Cai, X.-Y., B. Redfield, M. Maxon, H. Weissbach, and N. Brot.** 1989. The effect of homocysteine on MetR regulation of *metE*, *metR* and *metH* expression *in vitro*. Biochem. Biophys. Res. Commun. **163**:79–83.
6. **Casadaban, M. J., J. Chou, and S. N. Cohen.** 1980. In vitro gene fusions that join an enzymatically active β-galactosidase segment to amino-terminal fragments of exogenous proteins: *Escherichia coli* plasmid vectors for the detection and cloning of translational initiation signals. J. Bacteriol. **143**:971–980.
7. **Dev, I. K., and R. J. Harvey.** 1984. Regulation of synthesis of serine hydroxymethyltransferase in chemostat cultures of *Escherichia coli*. J. Biol. Chem. **259**:8394–8401.
8. **Dev, I. K., and R. J. Harvey.** 1984. Role of methionine in the regulation of the synthesis of serine hydroxymethyltransferase in *Escherichia coli*. J. Biol. Chem. **259**:8402–8406.
9. **Duerre, J. A., and R. D. Walker.** 1977. Metabolism of adenosylhomocysteine, p. 43–57. *In* F. Salvatore, E. Borek, V. Zappia, H. G. Williams-Ashman, and F. Schlenk (ed.), The biochemistry of adenosylmethionine. Columbia University Press, New York.
10. **Feiss, M., D. A. Siegele, C. F. Rudolph, and S. Frackman.** 1982. Cosmid DNA packaging *in vivo*. Gene **17**:123–130.
11. **Fried, M., and D. M. Crothers.** 1981. Equilibria and kinetics of lac repressor-operator interactions by polyacrylamide gel electrophoresis. Nucleic Acids Res. **9**:6505–6525.
12. **Garner, M. M., and A. Revzin.** 1981. A gel electrophoresis method for quantifying the binding of proteins to specific DNA regions: application to components of the *Escherichia coli* lactose operon regulatory system. Nucleic Acids Res. **9**:3047–3060.
13. **Greene, R. C.** 1973. Methionine limitation in *Escherichia coli* K-12 by growth on the sulfoxides of D-methionine. J. Bacteriol. **116**:230–234.
14. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
15. **Maxam, A. M., and W. Gilbert.** 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. Methods Enzymol. **65**:499–560.
16. **Michaeli, S., M. Mevarech, and E. Z. Ron.** 1984. Regulatory region of the *metA* gene of *Escherichia coli* K-12. J. Bacteriol. **160**:1158–1162.
17. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
18. **Panasenko, S. M., J. R. Cameron, R. W. Davis, and I. R. Lehman.** 1977. Five hundred-fold overproduction of DNA ligase after induction of a hybrid lambda lysogen constructed *in vitro*. Science **196**:188–189.
19. **Plamann, M. D., and G. V. Stauffer.** 1989. Regulation of the *Escherichia coli glyA* gene by the *metR* gene product and homocysteine. J. Bacteriol. **171**:4958–4962.
20. **Rosenberg, M., and D. Court.** 1979. Regulatory sequences involved in the promotion and termination of RNA transcrip-

tion. Annu. Rev. Genet. **13**:319–353.
21. **Saint-Girons, I., C. Parsot, M. M. Zakin, O. Barzu, and G. N. Cohen.** 1988. Methionine biosynthesis in Enterobacteriaceae: biochemical, regulatory, and evolutionary aspects. Crit. Rev. Biochem. **23**:S1–S42.
22. **Savin, M. A., M. Flavin, and C. Slaughter.** 1972. Regulation of homocysteine biosynthesis in *Salmonella typhimurium*. J. Bacteriol. **111**:547–556.
23. **Schmitz, A., and D. J. Galas.** 1979. The interaction of RNA polymerase and lac repressor with the lac control region. Nucleic Acids Res. **6**:111–137.
24. **Shimada, K., R. A. Weisberg, and M. E. Gottesman.** 1972. Prophage lambda at unusual chromosomal locations. I. Location of the secondary attachment sites and the properties of the lysogens. J. Mol. Biol. **63**:483–503.
25. **Stauffer, G. V., M. D. Plamann, and L. T. Stauffer.** 1981. Construction and expression of hybrid plasmids containing the *Escherichia coli glyA* gene. Gene **14**:63–72.
26. **Urbanowski, M. L., and G. V. Stauffer.** 1986. Autoregulation by tandem promoters of the *Salmonella typhimurium* LT2 *metJ* gene. J. Bacteriol. **165**:740–745.
27. **Urbanowski, M. L., and G. V. Stauffer.** 1988. The control region of the *metH* gene of *Salmonella typhimurium* LT2: an atypical *met* promoter. Gene **73**:193–200.
28. **Urbanowski, M. L., and G. V. Stauffer.** 1989. Genetic and biochemical analysis of the MetR activator-binding site in the *metE metR* control region of *Salmonella typhimurium*. J. Bacteriol. **171**:5620–5629.
29. **Urbanowski, M. L., and G. V. Stauffer.** 1989. Role of homocysteine in the *metR*-mediated activation of the *metE* and *metH* genes in *Salmonella typhimurium* and *Escherichia coli*. J. Bacteriol. **171**:3277–3281.
30. **Urbanowski, M. L., L. T. Stauffer, L. S. Plamann, and G. V. Stauffer.** 1987. A new methionine locus, *metR*, that encodes a *trans*-acting protein required for activation of *metE* and *metH* in *Escherichia coli* and *Salmonella typhimurium*. J. Bacteriol. **169**:1391–1397.
31. **Weaver, R. F., and C. Weissmann.** 1979. Mapping of RNA by a modification of the Berk-Sharp procedure: the 5′ termini of 15S β-globin mRNA precursor and mature 10S β-globin mRNA have identical map coordinates. Nucleic Acids Res. **7**:1175–1193.