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Operator-Constitutive Mutations of the *Escherichia coli metF* Gene

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The *Escherichia coli metF* gene codes for 5,10-methylene-tetrahydrofolate reductase, the enzyme that leads to the formation of *N*-methyltetrahydrofolate, supplying the methyl group of methionine. Transcription of *metF*, as well as of most of the methionine genes, is repressed by the *metJ* gene product complexed with *S*-adenosylmethionine. A *metF'-'lacZ* gene fusion was used to isolate mutants that have altered expression from the *metF* promoter. The nucleotide sequences of the *metF* regulatory region from five such mutants were determined. The mutations were located in the region previously defined as the potential target of the methionine repressor by its similarity to other binding sites. The mutationally defined *metF* operator thus consists of a 40-base-pair-long region, with five 8-base-pair imperfect palindromes spanning the *metF* transcription start. The altered operators do not recognize the purified repressor in an in vitro transcription-translation system, although the repressor binds efficiently to the *metF* wild-type operator.

The control of gene expression depends mainly on the specific recognition of short stretches of DNA sequences by proteins. The recognition of the promoter sequence by RNA polymerase constitutes the first level of control over transcription. In addition, initiation of transcription may be controlled by repressor and activator proteins.

All the *Escherichia coli* methionine biosynthetic genes (except *metH*, coding for the B_{12}-methyltransferase) are subject to negative control by methionine via the methionine repressor, the product of the *metJ* gene (4, 6). Since mutants in the *metK* gene which are deficient in *S*-adenosylmethionine synthetase are also regulatory mutants, *S*-adenosylmethionine is implicated in the regulation of the system (7, 8). It has been shown that MetJ protein inhibits in vitro expression of the *metF* gene and that *S*-adenosylmethionine functions as a corepressor (16, 20, 21). In addition, a second mechanism exists that specifically affects the synthesis of the *metF* and *metE* gene products (5,10-methylene-tetrahydrofolate reductase and non-B_{12}-methyltransferase, respectively) when *E. coli* is grown in the presence of vitamin B_{12}. This control, which is independent of the *metJ* gene product, is mediated by the holoenzyme composed of the *metH* gene product and cobalamin (13).

A better understanding of the mechanisms underlying regulation by repression requires precise identification of the repressor target. In this paper, we report the first isolation of operator-constitutive mutations for a *met* gene, namely, the *metF* gene, the determination of their nucleotide sequences, and their in vivo and in vitro characterization.

MATERIALS AND METHODS

Enzymes and chemicals. L-[^{35}S]methionine (800 Ci/mmol), [α-^{35}S]dATP, and the M13 sequencing kit were from Amersham Corp. (Arlington Heights, Ill.). The *para*-toluenesulfonate salt of *S*-adenosylmethionine was obtained from Sigma Chemical Co. (St. Louis, Mo.). Restriction enzymes were purchased from Boehringer Mannheim Biochemicals (Indianapolis, Ind.) and used as recommended by the manufacturer. The methionine aporepressor protein was purified as previously described (16).

Bacterial strains, plasmids, and growth media. *E. coli* strains used throughout this work were the following. TG1[*Δlac-proAB thi rpsL supE endA sbcB15 hsdR4*/F' *traD36 proAB lacI lacZΔM15*)] was the host used for M13 derivatives. The mutator strain, GT942, was constructed from TP2100 [*lacΔX74 argH ilvA*] (14) by transduction with P1 bacteriophage which was previously grown on RP526 *zac-13*::Tn*10 mutD5* (9). DNAs of plasmids pMC1403 (3), pDIA13 (4a), and their derivatives were purified in dye-CsCl gradient (10) or by the rapid alkaline lysis method (2). Cells were grown aerobically at 37°C in LB medium or minimal medium 63 supplemented with 5 μg of thiamine per ml, 0.4% glucose, and 0.01% of the required amino acid except when specified (12). Ampicillin and kanamycin were used at 50 and 25 μg/ml, respectively, as required.

In vitro fusion of *metF* transcriptional and translational signals to *lacZ* gene. A 320-base-pair DNA fragment from pMAD4 (23) carrying the regulatory region and the first 33 codons of the *metF* gene (18) was inserted into the *SmaI* site of pMC1403 (3). The transcriptional and translational signals of the *metF* gene were thereby fused to the *lacZ* gene devoid of its promoter and its first eight codons (Fig. 1, step 1). The recombinant plasmid thus obtained (pIP36) was digested with *Eco*RI and *Sal*I restriction enzymes. The *Eco*RI-*Sal*I fragment carrying the whole of the hybrid gene was then inserted into the corresponding sites of pDIA13, a derivative of a low-copy-number plasmid, giving rise to pIP51 (Fig. 1, step 2).

Mutagenesis and nucleotide sequence. Mutagenesis was performed with a mutator strain or chemical mutagens. Mutator strain GT942 was transformed with pIP51 with selection for kanamycin resistance on 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) indicator plates. The mutagenesis was performed in vivo with bacteria from two single light blue colonies of the Kan^r strain inoculated into LB medium containing kanamycin (25 μg/ml) followed by overnight incubation with aeration at 37°C. Other mutants were derived from TP2100 transformed with pIP51. They were obtained by mutagenizing cells with ethyl methanesulfonate or *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine

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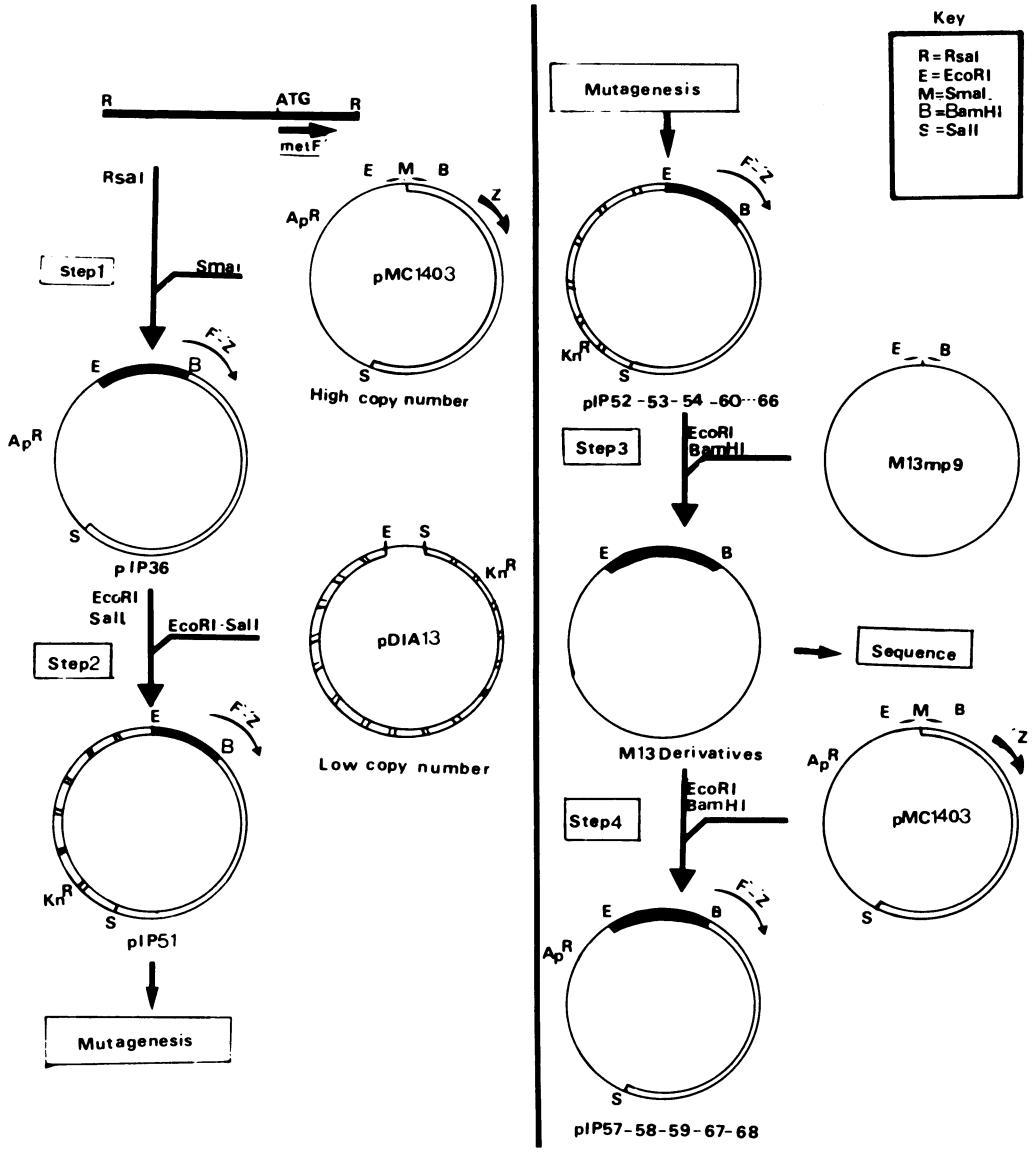

FIG. 1. Construction of a *metF'-lac'Z* hybrid gene in the multiple-copy-number plasmid pMC1403 and in the low-copy-number plasmid pDIA13. Enzymes used are indicated by a code (see key). Step 1: An *Rsa*I-*Rsa*I fragment of pMAD4 plasmid (23) carrying the whole of the regulatory region of the *metF* gene and its first 33 codons was cloned into the *Sma*I site of pMC1403 carrying the *lacZ* gene devoid of its promoter and its first 8 codons. In the recombinant plasmid pIP36, transcription and translation of the *lacZ* gene is under the control of the *metF* regulatory region. Step 2: Cloning of the *Eco*RI-*Sal*I fragment of pIP36 carrying the whole *metF'-lac'Z* hybrid gene between the *Eco*RI and *Sal*I sites of pDIA13 yielded pIP51, a low-copy-number plasmid. Mutations were then obtained in the regulatory region of the *metF* gene to give pIP52, -53, -54, -60, -62, -63, -64, -65, and -66. Step 3: The *Eco*RI-*Bam*HI fragments of plasmids pIP52, -53, -54, -60, -62, -63, -64, and -66 were cloned into M13mp8 and M13mp9 for sequencing. Step 4: The *Eco*RI-*Bam*HI fragments from pIP52, -53, -54, -60, and -63 were subsequently cloned into pMC1403 to give pIP57, -58, -59, -67, and -68, respectively.

at concentrations permitting 5 and 50% survival, respectively (12). The mutagens were eliminated by washing, and the cells were transferred to broth and grown overnight.

Plasmids extracted from overnight cultures of GT942 (pIP51) or mutagen-treated TP2100(pIP51) were transformed into strain TP2100. Transformants from independent experiments showing higher levels of *lacZ* expression (deep blue colonies on X-gal indicator plates) were selected for further study. The *Eco*RI-*Bam*HI restriction fragments of plasmids pIP52, -53, -54, -60, -62, -63, -64, and -66 carrying the regulatory region of the *metF* gene with a presumed mutation were cloned into M13mp8 and M13mp9 for sequencing by the dideoxy nucleotide chain termination method (19) (Fig. 1, step 3).

Cell-free protein synthesis. The system described by Zubay (12) was used with the following modifications. The bacteria were lysed by passing them through a French pressure cell at 2,000 lb/in^2 (13.8 $\times$ 10^6 P), polyethylene glycol 6000 was added (1.5% wt/vol) to the reaction mixture, folinic acid was omitted, and the $CaCl_2$ concentration was reduced to 3.7 mM. The S-30 extract was prepared from strain TP2339 (15). The template DNA with a wild-type methionine operator was from plasmid pIP36. After identification of the nucleotide change, the replicative forms of M13 carrying the

TABLE 1. β-Galactosidase assays as an index of *metF* expression in crude extracts of strain TP2100 harboring pIP51 or its mutagenized derivatives[a]

Bacterial strain	Plasmid	*metFo* allele no.	Mutagen[b]	β-Galactosidase activity		Repression factor
				Minimal medium	Minimal medium + methionine	
GT950	pIP51			478	79	6
GT944	pIP52	*1000*	*mutD5*	1,285	592	2
GT946	pIp53	*1001*	*mutD5*	1,411	607	2.3
GT965	pIP60	*1003*	*mutD5*	1,083	416	2.6
GT945	pIP54	*1002*	*mutD5*	1,447	466	3.1
GT973	pIP64	*1002*	EMS	1,932	627	3.0
GT971	pIP62	*1004*	EMS	1,174	881	1.3
GT970	pIP63	*1004*	EMS	1,153	766	1.5
GT976	pIP66	*1004*	NG	1,504	990	1.5

[a] Units of β-galactosidase activity are nanomoles of product formed per minute per milligram of protein at 28°C. The strains were grown for three generations in minimal medium in the presence or absence of 5 mM L-methionine. The repression factor is defined as the ratio between the values of β-galactosidase obtained in the absence and presence of methionine in the growth medium. Allele numbers were given to the mutations in the *metF* regulatory region after determination of the nucleotide sequences.

[b] *mutD5*, mutator gene (9); EMS, ethyl methanesulfonate; NG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.

mutations were isolated, and the *Eco*RI-*Bam*HI fragment was cloned into pMC1403, yielding plasmids pIP57, pIP58, pIP59, pIP67, and pIP68. These plasmids are isogenic to pIP36 except for the mutation in the regulatory region of the *metF* gene (Fig. 1, step 4). β-Galactosidase assays were performed as described previously (12).

RESULTS

Isolation of mutations in the regulatory region of the *metF* gene. The aim of this work was to obtain mutations in the *metF* regulatory region. To obtain a scoreable phenotype, we constructed a derivative of pMC1403 (pIP36). In this plasmid a DNA fragment carrying the promoter and the first 33 codons of the *metF* gene was fused to the *lacZ* gene devoid of its promoter and its first 8 codons (Fig. 1). Unfortunately, β-galactosidase production by strains carrying pIP36 was so high that it prevented selection of a further increase in *lacZ* expression. Therefore, we subcloned the *metF'-'lacZ* hybrid gene into a low-copy-number plasmid to give pIP51. β-Galactosidase production in strains carrying pIP51 was about 8% of that in strains carrying pIP36. This low level of expression allowed us to use X-gal indicator plates to detect increases in β-galactosidase production.

To isolate mutations altering the regulatory region of the *metF* gene, we used either a mutator strain or a chemical mutagen as described in Materials and Methods. To avoid screening of unwanted chromosomal mutations, we transferred mutagenized plasmids to a nonmutagenized Δ*lac* strain. Clones producing dark blue colonies on X-gal plates were further analyzed. Three events could cause the increased *lacZ* expression in these clones: (i) a mutation in the regulatory region of the *metF* gene, (ii) an increase in translation of the *lacZ* gene, or (iii) a higher plasmid copy number. To distinguish among these three possibilities, we assayed the β-galactosidase activity in the mutant strains grown in the absence or presence of methionine at a repressing concentration (5 mM). The results indicated that all clones had a higher than normal level of β-galactosidase activity in agreement with their deep blue color on X-gal indicator plates. Strains GT944 through -946 and -965 through -976 produced two to four times more β-galactosidase activity than the wild-type strain GT950 when grown in minimal medium (Table 1). That regulation via the methionine holorepressor was altered in the mutant strains was indicated by the slight repression observed when methionine was added to the growth medium.

Nucleotide sequence of mutations. The eight mutants showing very low repression levels by methionine were chosen for DNA sequencing. We determined the nucleotide sequence of the whole regulatory region and the 33 first codons of the *metF* gene from the plasmids extracted from GT944 to -946 and -965 to -976. Nucleotide changes (all transitions) were found at five distinct positions of the *metF* regulatory region (Fig. 2). To show that the nucleotide changes found were indeed responsible for the altered repression of the mutants, we inserted the *Eco*RI-*Bam*HI fragment carrying

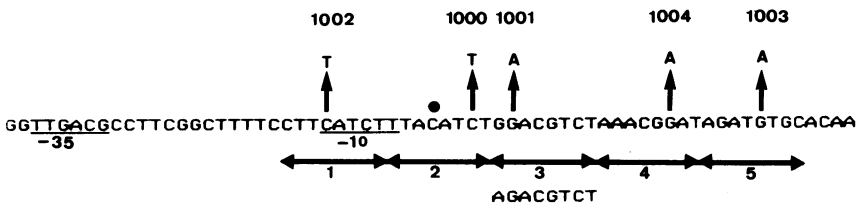

FIG. 2. Nucleotide sequence of the *metF* regulatory region and positions of the mutations. Nucleotide positions (−32 through +33) are relative to the transcription initiation site taken as +1 (●) and correspond to nucleotides 6 to 63 taken from reference 18. Vertical arrows indicate the base substitutions at position −8 for allele *1002*, at position +4 for allele *1000*, at position +7 for allele *1001*, at position +19 for allele *1004*, and at position +26 for allele *1003*. The double-faced arrows (1 to 5) refer to the repetitive units indicated below as the consensus AGACGTCT (1). The −35 and −10 regions of the *metF* promoter are underlined.

TABLE 2. β-Galactosidase assays as index of *metF* expression in a cell-free system[a]

Plasmid	*metFo* allele no.	β-Galactosidase activity[b]	
		No addition	+ MetJ protein, + *S*-adenosylmethionine
pIP36		812	2
pIP57	*1000*	501	444
pIP58	*1001*	635	484
pIP59	*1002*	2,878	885
pIp67	*1003*	573	141
pIp68	*1004*	981	945

[a] Protein synthesis was programmed with DNA from a high-copy-number plasmid, pIP36, with a wild-type operator or from its derivatives with a mutation in the *metF* regulatory region. MetJ protein was at 6.4 μg/ml, and *S*-adenosylmethionine was used at 0.1 mM.

[b] β-Galactosidase activity is nanomoles of product formed per minute per milligram of protein.

only the *met* DNA of each mutant between the *Eco*RI and *Bam*HI sites of pIP51 that had not been mutagenized. Strains carrying the recombinant plasmids produced elevated, nonrepressible levels of β-galactosidase activity, confirming that the nucleotide changes were indeed the cause of the altered phenotype of the mutants (data not shown). Plasmids pIP52, -53, -54, -60, and -62 were chosen as representative of the five mutant classes for further characterization.

Further characterization of regulatory mutants with a cell-free system. We had previously shown that the production of β-galactosidase under the control of the *metF* gene was subject to strong repression by the methionine repressor in a cell-free system (16). To analyze the consequences of the nucleotide changes described here, we performed an in vitro transcription-translation experiment using DNA from plasmids carrying the wild-type or the altered *metF* operators. Surprisingly, the derivatives of pDIA13 such as pIP51, -52, -53, -54, -60, and -62 directed such a low level of protein synthesis in the cell-free system that the results were difficult to interpret (data not shown). The altered operators were therefore cloned in pMC1403 to give pIP57, -58, -59, -67, and -68 (Fig. 1, step 4). The results shown in Table 2 indicate that in the absence of the methionine repressor, the production of β-galactosidase (an index of *metF* expression) was of the same order of magnitude for pIP57, -58, -67, and -68 as for the wild type (pIP36). For pIP59, a threefold increase was observed. In the presence of the methionine holorepressor (MetJ protein plus *S*-adenosylmethionine), repression was 400-fold with the wild-type *metF* operator (as expected from previous experiments), whereas no to fivefold repression was observed with the altered operators.

The concentration of the methionine holorepressor in vivo in a wild-type strain does not vary (600 dimeric molecules per cell) (16), whereas in vitro, it can be set to a much higher value, which may explain why the repression is much more efficient (400 in vitro compared with 6 in vivo). In a cell in which the *metJ* gene is on a plasmid under *tac* control, the concentration of the methionine repressor is increased 200-fold compared with the wild-type strain, and the strain becomes auxotrophic for methionine which indicates that the repression is extreme (16).

DISCUSSION

All *met* genes have been cloned, and the nucleotide sequences of the *metJ*, *metBL*, *metF*, *metC*, and part of the *metA* operons have been reported (1, 5, 11, 17, 18). Analysis of the regulatory regions of these transcriptional units showed that they do not contain attenuatorlike features (22). Since it was known that the expression of the *met* biosynthetic genes was repressed via the *metJ* gene product, it was important to find the target of the repressor protein. In this paper we describe the isolation of mutations in the regulatory region of the *metF* gene. Mutagenesis was performed in vivo by increasing the spontaneous rate of mutation through the use of a mutator gene or chemical mutagens, in strains carrying a plasmid bearing a *metF'-'lacZ* hybrid gene. The mutations carried by the recombinant plasmid were identified by increased β-galactosidase production, the *lacZ* gene being under the control of the *metF* promoter. The strain in which the mutations were isolated carried a chromosomic wild-type *metJ* allele. This indicated that β-galactosidase production was probably insensitive to repression by the *metJ* gene product, although other explanations such as an increase in plasmid copy number or an increase in β-galactosidase expression were also possible. The results of the assay of β-galactosidase activity in extracts of cultures grown in the presence or absence of methionine indicated that the mutations affected repression. Moreover, β-galactosidase activity in a cell-free system programmed with DNA from the *metF'-'lacZ* gene with a wild-type operator was repressed 400-fold in the presence of the *met* repressor, whereas little or no repression was obtained with the altered operators. Three of the eight mutated *metF* regulatory regions sequenced were identical; we thus identified five unique base pair changes (Fig. 2).

The C→T nucleotide change corresponding to allele *1002* is in the −10 box of the *metF* promoter (18); the CATCTT sequence is modified to TATCTT, which is closer to the consensus TATAAT. In agreement with these results, a threefold promoter up effect is observed in the cell-free system directed by pIP59 DNA carrying the *1002* allele. Since the target of the *met* repressor is also altered by this mutation, the promoter and operator of the *metF* gene must overlap.

The regulatory regions of the *metB*, *metF*, *metA*, and *metC* genes contain a region of homology (*met* box) which could be the methionine repressor target (1, 5, 11, 18). A palindromic repetitive unit of eight nucleotides, AGACG TCT, is present, in slightly altered forms, in all the *met* boxes, twice for *metC*, four times for *metA*, and five times for *metB* and *metF* (1). Derepression of *metF* expression resulted from single-site mutations in any of the five repetitive units. All these changes produced typical operator-constitutive mutations. It should be noted that four of five mutations reduce the homology of each affected octamer with that of the consensus sequence, AGACGTCT. All five repetitive units are thus required for fully effective binding of the *metJ* gene product to the *metF* operator. However, the mutant operators analyzed seem to retain some affinity for the methionine repressor since addition of methionine to the growth medium still caused some repression of *metF'-'lacZ* with mutated operators (Table 1). Although the *1004* allele constitutes an exception in that it changes a nonconsensus base to another nonconsensus base, the constitutive effect of this mutation is strong (Tables 1 and 2). When we established the consensus sequence of the *met* boxes, the T at the position occupied by an A in allele *1004* was deduced from 15T, 1C, and 1G (1), and it should be noted that no A appears at this position. Perhaps an A at this position may affect drastically the affinity for the methionine repressor. The role of the octamer for the suggested *metA*, *metB*, and *metC* binding sites can be inferred from analogy to the mutation-

ally defined *metF* operator. Although the size of the *metF* operator is 40 base pairs long, it seems that the methionine repressor target of the *metC* gene as defined by sequence comparison is only 16 base pairs long. This could be related to the different extents of repression elicited by the methionine repressor (6).

It should be emphasized that we report here the first operator-constitutive mutations in a methionine gene, an important aspect of the study of the interactions of the methionine repressor with DNA. Whether or not regulation via vitamin B_{12} relies on the same target is under investigation.

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