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Cloning and Characterization of the *Salmonella typhimurium ada* Gene, Which Encodes O^6-Methylguanine-DNA Methyltransferase

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The *ada* gene of *Escherichia coli* encodes O^6-methylguanine-DNA methyltransferase, which serves as a positive regulator of the adaptive response to alkylating agents and as a DNA repair enzyme. The gene which can make an *ada*-deficient strain of *E. coli* resistant to the cell-killing and mutagenic effects of *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine (MNNG) has been cloned from *Salmonella typhimurium* TA1538. DNA sequence analysis indicated that the gene potentially encoded a protein with a calculated molecular weight of 39,217. Since the nucleotide sequence of the cloned gene shows 70% similarity to the *ada* gene of *E. coli* and there is an *ada* box-like sequence (5′-GAATTAAAACGCA-3′) in the promoter region, we tentatively refer to this cloned DNA as the ada_{ST} gene. The gene encodes Cys-68 and Cys-320, which are potential acceptor sites for the methyl group from the damaged DNA. The multicopy plasmid carrying the ada_{ST} gene significantly reduced the frequency of mutation induced by MNNG both in *E. coli* and in *S. typhimurium*. The Ada_{ST} protein encoded by the plasmid increased expression of the *ada′-lacZ* chromosome fusion about 5-fold when an *E. coli* strain carrying both the fusion operon and the plasmid was exposed to a low concentration of MNNG, whereas the *E. coli* Ada protein encoded by a low-copy-number plasmid increased it about 40-fold under the same conditions. The low ability of Ada_{ST} to function as a positive regulator could account for the apparent lack of an adaptive response to alkylation damage in *S. typhimurium*.

Methylating agents produce various methylated purine and pyrimidine adducts as well as forming methylphosphotriesters (7). Among these adducts, O^6-methylguanine (O^6-MeG) is thought to be the most relevant adduct in terms of induction of mutations because this altered base directs the incorporation of either thymine or cytosine without blocking DNA replication, resulting in GC-to-AT transition mutation (3, 18, 54). This DNA adduct is mainly repaired by the O^6-MeG-DNA methyltransferase (MTase) in many species, which directly transfers the methyl group from O^6-MeG to one of its cysteine residues in an autoinactivating stoichiometric fashion (16, 17, 35).

Escherichia coli cells have the ability to acquire increased resistance to the cell-killing and mutagenic effects of alkylating agents during exposure of cells to sublethal concentrations of the methylating and ethylating agents (11, 44). This phenomenon has been termed the adaptive response to alkylating agents and requires a functional *ada* gene, which encodes a 39-kDa MTase (12, 17, 48, 59). The Ada protein transfers the methyl group from O^6-MeG and O^4-methylthymine (O^4-MeT) to its Cys-321 residue and also transfers the methyl group from one of the two stereoisomers of methylphosphotriester (the *S* configuration) to its Cys-69 residue (4, 21, 22, 23, 35, 55, 63). The methylation at the Cys-69 residue converts the Ada protein into an efficient transcriptional activator of its own gene and other genes (*alkB*, *alkA*, and *aidB*) (15, 30, 32, 52, 55, 60, 61). The *alkA* gene product, 3-methyladenine DNA glycosylase II, efficiently repairs the potentially lethal lesions of N^3-methylpurine and O^2-methylpyrimidine in DNA (29, 31). Thus, the Ada protein plays an important role both as a repair enzyme and as a positive regulator in the adaptive response. The Ada protein is composed of two functional domains, each possessing Cys-69 or Cys-321, and is cleaved into the two domains by endogenous protease in *E. coli* (49, 58). Interestingly, *E. coli* has a second MTase, which is a predominant MTase in unadapted *E. coli* (19, 39, 41, 42, 51). The second MTase, the Ogt protein, repairs the O^6-MeG and O^4-MeT but not the methylphosphotriesters and repairs the O^6-ethylguanine in DNA at a higher rate than does the Ada protein (64). The amino acid sequence of this 19-kDa protein shares homology with the *C*-terminal domain of the Ada protein (19, 39). The expression of the *ogt* gene is constitutive (19). The *ogt* gene is located at 29 min on the *E. coli* chromosome map, whereas the *ada* gene is located at 47 min (38, 47).

In contrast to *E. coli*, the adaptive response to alkylating agents does not seem to occur in *Salmonella typhimurium* (8, 9, 17). Guttenplan and Milstein reported that levels of O^6-MeG as well as mutagenesis induced by *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine (MNNG) are biphasic in *S. typhimurium* TA1535 and suggested that *S. typhimurium* has a saturable constitutive MTase (9). Although many derivatives of *S. typhimurium* have widely been used in the Ames test for the detection of environmental mutagens and carcinogens, including alkylating agents (20), the molecular nature of its MTases has not been characterized.

To clarify the molecular basis of the apparent lack of the adaptive response in *S. typhimurium*, we have searched for the *ada* gene of *S. typhimurium* by screening for the gene which makes a Δ*ada-25* strain of *E. coli* resistant to the cell-killing and mutagenic effects of MNNG. Consequently, we have cloned the gene encoding the 39-kDa MTase, which shows 75% similarity to the Ada protein of *E. coli* at the amino acid level. We have tentatively referred to the cloned gene as the ada_{ST} gene. The gene product, Ada_{ST}, was found to be a weak activator of expression from the *ada* promoter of *E. coli*. The accompanying report (60a) provides biochemical evidence that the ada_{ST} gene in *S. typhimurium* is weakly induced by MNNG. The low ability of Ada_{ST} to

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Description[a]	Source
Strains		
E. coli		
XL-1-Blue	*endA1 hsdR17*(r_K^- m_K^+) *supE44 thi-1 recA1 gyrA96 relA* Δ(*lac*) F′(*proAB*$^+$ *lacI*^{q} *lacZ* ΔM15 Tn*10*)	Stratagene
AB1157	F$^-$ *thr-1 leu-6 proA2 his-4 thi-1 argE3 lacY1 galK2 ara-14 xyl-5 mtl-1 tsx-33 rpsL31 supE37*	G. C. Walker
GW7101	As AB1157 but has *ada-25*	G. C. Walker
GW5354	As AB1157 but has *ada′-lacZ*	G. C. Walker
S. typhimurium		
TA1535[b]	As LT2 but has *hisG46*	B. N. Ames
TA1538[b]	As LT2 but has *hisD3052*	B. N. Ames
Plasmids		
pYG7001	As pBR322 but has a 5.5-kb fragment of TA1538 DNA carrying the ada_{ST} gene	This study
pYG7034	As pBluescript KS+ but has a 2.0-kb *Eco*RV (2.5 kb)-*Eco*RV (4.5 kb) fragment of pYG7001 carrying the ada_{ST} gene	This study
pYG7034′	As pYG7034, but the 2.0-kb fragment DNA carrying the ada_{ST} gene was directly cloned from the library DNA of TA1538	This study
pYG7070	As pHSG576, but its *Bam*HI-*Hin*dIII region is replaced by a 2.0-kb *Bam*HI-*Hin*dIII fragment of pYG7034 carrying the ada_{ST} gene	This study
pYN3059[c]	As pUC9 but has the *E. coli ada* gene	M. Sekiguchi
pYG7050	As pSE101 but has a 1.3-kb fragment of pYN3059 carrying the *E. coli ada* gene at its *Hpa*I site	This study
pYG7090	As pHSG576, but its *Hin*dIII-*Sma*I region is replaced by a 1.3-kb *Hin*dIII-*Sma*I fragment of pYN3059 carrying the *E. coli ada* gene	This study

[a] Numbers in parentheses indicate the map position of each restriction site in pYG7001 (Fig. 3). pSE101 is a derivative of pSC101 with the kanamycin resistance gene (6). pHSG576 is a derivative of pSC101 with the chloramphenicol resistance gene (56).
[b] TA strains have the genotype Δ(*gal bio chl uvr*B) *rfa*.
[c] Detailed information of pYN3059 is in reference 28.

function as a positive regulator could account for the apparent lack of the adaptive response in *S. typhimurium*. Possible factors which reduce the inducibility of the ada_{ST} gene in *S. typhimurium* are discussed.

MATERIALS AND METHODS

Bacterial strains and plasmids. All bacterial strains and plasmids used are described in Table 1.

Media and culture. LB broth and agar were used for routine bacterial culture and supplemented, when necessary, with 50 μg of ampicillin per ml for plasmid selection and maintenance. MNNG-containing agar plates contained the following: M9 salts (24) adjusted to pH 5.6; 0.2% glucose; 2 mM $MgCl_2$; 0.1 mM $CaCl_2$; 40 μg each of threonine, leucine, isoleucine, proline, valine, histidine, and arginine per ml; 5 μg of thiamine per ml; 1.5 μg of MNNG per ml; 50 μg of ampicillin per ml; and 1.5% Bacto-Agar (Difco). Semienriched-medium agar plates used for the reversion assay of *argE3* to Arg$^+$ of *E. coli* were prepared as described previously (34). Vogel-Bonner minimal agar plates and top agar used for the reversion assay of *hisG46* to His$^+$ of *S. typhimurium* TA1535 were prepared as described previously (20). Supplemented M9-glucose medium for induction of the adaptive response of *E. coli* was prepared as described previously (34) except that it contained an increased amount of arginine (40 μg/ml). Histidine-fortified Vogel-Bonner medium for induction of the adaptive response of *S. typhimurium* was prepared as described previously (20) except that it contained histidine (50 μg/ml) and biotin (3 nmol/ml).

Cloning of the ada_{ST} gene encoding the MTase of *S. typhimurium*. A gene library of *S. typhimurium* TA1538 was constructed by ligating partially *Sau*3AI-digested genomic DNA, whose size is about 10 kb, with *Bam*HI-digested pBR322 (62). The library DNA was modified by introducing it into an XL1-blue strain (r_K^- m_K^+) of *E. coli*. More than 30,000 transformants of XL1-blue were collected, and plasmid DNA was extracted. A Δ*ada-25* strain of *E. coli* (GW7101) was transformed with the modified library DNA, and the transformants were directly spread on MNNG-containing agar plates. After incubation for 3 days at 37°C, colonies that survived on the plates were selected. Plasmid DNA was reisolated from such colonies and introduced into a fresh Δ*ada-25* background. The plasmid which makes a Δ*ada-25* strain resistant to the mutagenic effect of MNNG was selected by checking the second transformants for their mutability to MNNG by a quantitative MNNG mutagenesis assay. The selected plasmid (pYG7001) was introduced into *S. typhimurium* TA1535. A Δ*ada-25* strain containing pYG7001 was subjected to an assay for MTase enzyme activity.

Recloning of the ada_{ST} gene. The genomic DNA of *S. typhimurium* TA1538 was partially digested with *Eco*RV and then subjected to 0.8% agarose gel electrophoresis. A band of about 2 kb was excised from the gel and ligated with an *Eco*RV-digested pBluescript KS+ vector. A Δ*ada-25* strain of *E. coli* was transformed with the DNA, and ampicillin-resistant colonies were selected. Each transformant was tested for resistance to the cell-killing effect of MNNG by streaking the cells on MNNG-containing agar plates. Plasmid DNA was isolated from the master colonies of apparent MNNG-resistant transformants and subjected to restriction enzyme analysis with *Eco*RV, *Cla*I, *Nru*I, and *Dra*I. The plasmid DNA (pYG7034′) which showed the same pattern of digestion as that of pYG7034 was selected and used for confirmation of the DNA sequence of the ada_{ST} gene.

Quantitative MNNG mutagenesis assay. An aliquot (1 ml) of the culture of log-phase cells was mixed with MNNG dissolved in dimethyl sulfoxide (10 μl) and incubated for 5 min at 37°C with shaking. The treated cells were washed twice with cold saline and resuspended. A portion (0.1 ml) of the cells was spread on semienriched-medium agar plates with a sterile glass rod (*E. coli*) or spread on Vogel-Bonner agar plates with 2 ml of molten top agar (*S. typhimurium*). For counting the surviving cells, the suspension was diluted

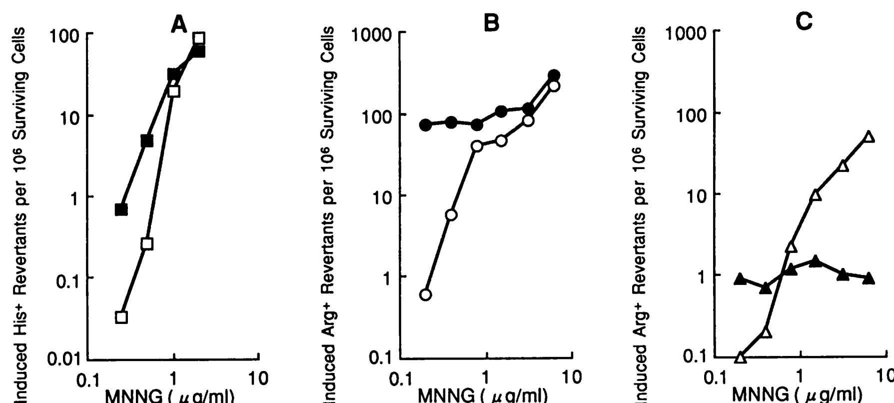

FIG. 1. Induced-mutation frequencies by MNNG in *S. typhimurium* TA1535 (A), *E. coli* AB1157 Δ*ada-25* (B), and *E. coli* AB1157 (C). Cells were adapted with 0.3 μg of MNNG per ml for 1.5 h and then challenged with different concentrations of MNNG. The reversion frequencies of *hisG46* to His$^+$ (*S. typhimurium*) or of *argE3* to Arg$^+$ (*E. coli*) were determined. Closed and open symbols represent the frequencies of the adapted and unadapted cells, respectively.

10^5-fold with cold saline and 0.1 ml of the diluted suspension was spread on semienriched-medium agar plates or on Vogel-Bonner agar plates with top agar. The induced-mutation frequency was calculated as described previously (34). The Ames test using *S. typhimurium* TA1535 and its derivatives was carried out as described previously (20) except that the cells treated with MNNG for 5 min at 37°C were washed twice with cold saline before addition of the top agar. All plates were incubated at 37°C for 2 days. The adaptive response was induced by exposing exponential-phase cultures (A_{600} of 0.3 to 0.4) to MNNG (1.5 μg/ml) for 1.5 h unless otherwise indicated.

MTase assay. Crude extracts were prepared by sonicating a culture as previously described (28). ^{3}H-labeled methylated DNA was prepared by the reaction of calf thymus DNA (Sigma Chemical Co., St. Louis, Mo.) with *N*-[^{3}H]methyl-*N*-nitrosourea (1.0 Ci/mmol; Amersham, Buckinghamshire, United Kingdom) (37). The extracts were assayed by incubation at 37°C for 15 min of a reaction mixture containing 35 mM *N*-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-KOH buffer (pH 7.8), 10 mM dithiothreitol, 500 μM spermidine hydrochloride, 20 μg of bovine serum albumin, 50 μg of ^{3}H-labeled methylated DNA containing 2.27 pmol of O^6-MeG adduct, and the crude extracts in a total volume of 0.4 ml (25). After acid hydrolysis of a pelleted DNA, the hydrolysate was separated by high-performance liquid chromatography on a column of Whatman Partisil 10SCX (4.6 by 250 mm). The sample was eluted with 75 mM ammonium formate (pH 4.0) at room temperature at a flow rate of 2 ml/min. Each fraction was collected, and the radioactivity was determined in a liquid scintillation counter. The retention times of 7-methylguanine, O^6-MeG, and 3-methyladenine were 3.8, 6.5, and 13.2 min, respectively.

Labeling of plasmid-coded proteins in maxicells. The maxicell method of Sancar et al. (45) was used to label the proteins encoded by plasmids pYG7034 and pYN3059 in strain CSR603 with [^{35}S]methionine. Samples were run on a 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel and visualized by fluorography.

DNA sequencing. A set of deletion derivatives of pYG7034 was constructed by using the exonuclease III-mung bean nuclease digestion protocol from Stratagene. Both strands of the 1.3-kb region containing the ada_{ST} gene were sequenced by the dideoxy-chain termination method (46), using the Sequenase sequencing kit, version II (U.S. Biochemical Corp., Cleveland, Ohio). DNA sequencing of pYG7034' was carried out as described above except that a set of synthetic oligonucleotide primers was used instead of creating the deletion derivatives. Sequencing data were analyzed by using SDC-Genetyx software (SDC Software Development Co., Tokyo, Japan).

β-Galactosidase assay. The β-galactosidase assay was performed as described by Miller (24). MNNG (1 μg/ml) was added to the cells (A_{600} of 0.2) of derivatives of GW5354 grown in supplemented M9-glucose medium and remained in the samples throughout the experiments (53). β-Galactosidase activity was assayed 2 h after the addition of MNNG.

Nucleotide sequence accession number. The nucleotide sequence data reported here will appear in the DDBJ, EMBL, and GenBank nucleotide sequence data bases under accession number D90221.

RESULTS

Apparent lack of the adaptive response to MNNG in *S. typhimurium* TA1535. To validate the notion that *S. typhimurium* lacks the adaptive response to alkylating agents, we compared the response of *S. typhimurium* TA1535 to sublethal MNNG treatment with those of an *E. coli* AB1157 Δ*ada-25* strain (GW7101) and of *E. coli* AB1157.

The MNNG-induced mutation frequencies of *S. typhimurium* TA1535 as well as of a Δ*ada-25* strain were not decreased but rather increased by pretreatment of the cells with 0.3 μg of MNNG per ml for 1.5 h (Fig. 1A and B). Pretreatment with lower (0.0375, 0.075, and 0.15 μg/ml) or higher (0.5 and 0.7 μg/ml) concentrations of MNNG did not induce the adaptive response in *S. typhimurium* TA1535 (data not shown). In contrast, the MNNG-induced mutation frequencies of strain AB1157 were significantly reduced by pretreatment of the cells with MNNG, as expected (Fig. 1C). The induced-mutation frequency was lowered about 50-fold by the pretreatment when the frequencies were compared at a challenging dose of 8 μg of MNNG per ml. From these results, we concluded that *S. typhimurium* TA1535 actually lacks the adaptive response to MNNG, and we investigated the molecular basis of this lack by cloning the *ada* gene of *S. typhimurium*.

Cloning of the gene encoding the MTase of *S. typhimurium*.

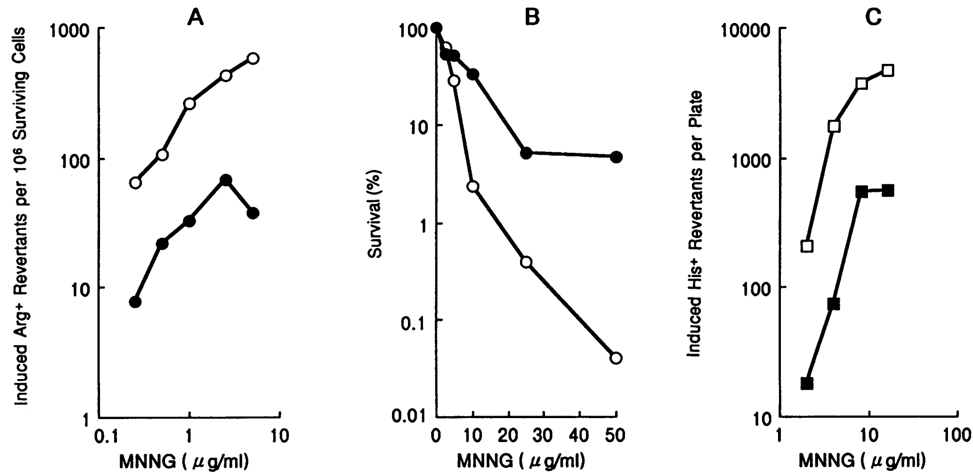

FIG. 2. Effects of pYG7001 on the mutagenesis and cell killing induced by MNNG in *E. coli* AB1157 Δ*ada-25* (A and B) and on the mutagenesis induced by MNNG in *S. typhimurium* TA1535 (C). Symbols: ●, *E. coli* AB1157 Δ*ada-25* containing pYG7001; ○, *E. coli* AB1157 Δ*ada-25* containing pBR322; ■, *S. typhimurium* TA1535 containing pYG7001; □, *S. typhimurium* TA1535 containing pBR322.

To clone the ada_{ST} gene, we have screened for the gene which makes a Δ*ada-25* strain resistant to the cell-killing and mutagenic effects of MNNG. We obtained 10 candidates which apparently showed resistance to the cell-killing effect of MNNG by selecting the transformants of a Δ*ada-25* strain with the library DNA of *S. typhimurium* TA1538 on MNNG-containing agar plates. About 10,000 ampicillin-resistant colonies appeared when the transformants of a Δ*ada-25* strain with the same amount of the library DNA were incubated on the same agar plates without MNNG. To confirm that this resistance is due to the presence of the plasmids carrying a part of the chromosome DNA of TA1538, we isolated the plasmids from the resistant colonies, introduced them into a fresh Δ*ada-25* background, and then performed a quantitative MNNG mutagenesis assay.

One plasmid, which we designated pYG7001, made the host strain resistant to the mutagenic and cell-killing effects

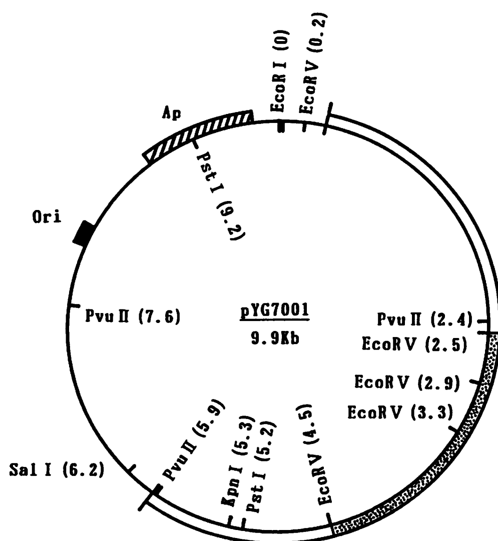

FIG. 3. Restriction map of pYG7001. The *Eco*RI restriction site derived from pBR322 was assigned map position 0 kb of the 9.9-kb pYG7001 map. A DNA fragment (5.5 kb) derived from *S. typhimurium* TA1538 was inserted into the *Bam*HI site of pBR322. ada_{ST} was located in a 2.0-kb *Eco*RV-*Eco*RV fragment (▨) which was used for subcloning into pBluescript KS+. Ori, DNA replication origin; Ap, ampicillin resistance gene.

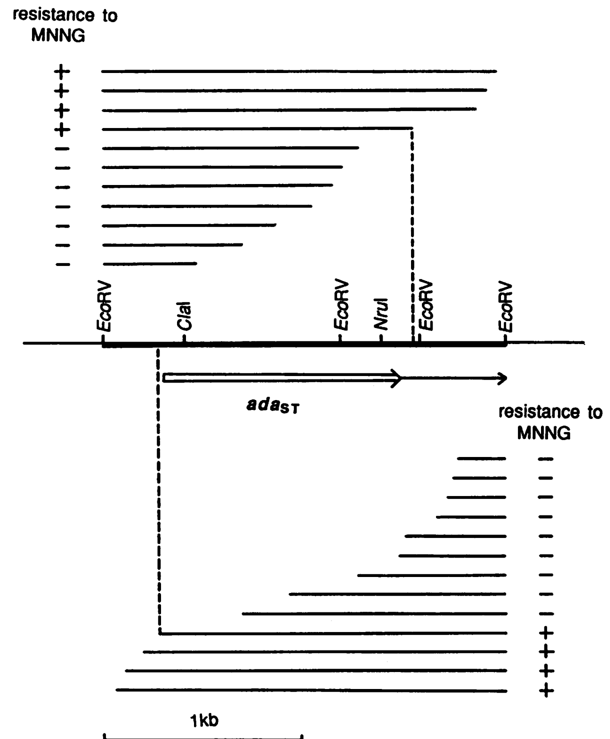

FIG. 4. Partial restriction map of the 2.0-kb *Eco*RV-*Eco*RV region of pYG7034 and predicted location of the ada_{ST} gene. Bars represent DNA of the 2.0-kb region remaining after exonuclease III-mung bean nuclease digestion. + and − indicate the ability and inability, respectively, of each subclone to make *E. coli* AB1157 Δ*ada-25* resistant to the cell-killing effect of MNNG. Resistance of each transformant was checked by using MNNG-containing agar plates.

1 2
97.4kD —
66.2kD —
42.7kD —
E. coli Ada
Ada$_{ST}$
31.0kD —
β-lactamase
21.5kD —
14.4kD —

FIG. 5. [^{35}S]methionine-labeled proteins synthesized in maxicells containing pYG7034 carrying the ada_{ST} gene (lane 1) and pYN3059 carrying the *E. coli ada* gene (lane 2).

of MNNG. A Δ*ada-25* strain containing pYG7001 showed about 10 times lower mutability to MNNG than did a Δ*ada-25* strain containing pBR322 (Fig. 2A). Since other plasmids did not make a Δ*ada-25* strain resistant to the mutagenic effect of MNNG, we did not study them further. A Δ*ada-25* strain containing pYG7001 showed higher resistance to the cell-killing effect of MNNG than did the control strain (Fig. 2B). Plasmid pYG7001 was transferred to *S. typhimurium* TA1535. The resulting transformant, *S. typhimurium* TA1535 containing pYG7001, also showed about 10 times lower mutability toward MNNG than did TA1535 containing pBR322 (Fig. 2C). To confirm that the cloned plasmid pYG7001 encodes the MTase, we assayed the MTase activity of the crude extract of a Δ*ada-25* strain containing pYG7001, using high-performance liquid chromatography. The extract showed activity of more than 1.1 pmol of O^6-MeG removed per mg of protein. From these results,

```
       10        20        30        40        50        60        70        80        90
GGATGTCGCCGCAGTTCAAAACGTTCCTGGTGAGCGATAAGAATTAAAACGCAAGATTACGGTTTTTGTGGCGGATTTACGGACACAC
                                                 ada box       -35                   -10
      100       110       120       130       140       150       160       170       180
TGCATGTAAGCAAGCACTATGCTTAATGAAGGAGTCTATGATGAAAAAAGCGTTACTTATCGATGATGAATGCTGGCTGCGGGTGCAGGC
                                  S.D.       M  K  K  A  L  L  I  D  D  E  C  W  L  R  V  Q  A

      190       200       210       220       230       240       250       260       270
GCGCGATGCCAGCGCGGATGGGCGTTTCGTTTTTGCGGTGCGAACCACCGGCGTTTTTGCCGTCCTTCTTGTCGCTCGAAGCGGGCGTT
 R  D  A  S  A  D  G  R  F  V  F  A  V  R  T  T  G  V  F  C  R  P  S  C  R  S  K  R  A  L

      280       290       300       310       320       330       340       350       360
ACGTAAAAATGTTCGCTTTTTTGCCAACGCGCAGCAGGCGCTGGACGCCGGTTTTCGCCCCTGCAAGCGCTGTCAGCCGGATAATGCGCG
 R  K  N  V  R  F  F  A  N  A  Q  Q  A  L  D  A  G  F  R  P  C  K  R  C  Q  P  D  N  A  R

      370       380       390       400       410       420       430       440       450
CGCGCAGCAACGGCGGTTGGATAAGATTGCCTGCGCCTGCCGTTTGCTTGAGCAGGAGACGCCGGTAACGCTGGCGTTTCTGGCGCAGGC
 A  Q  Q  R  R  L  D  K  I  A  C  A  C  R  L  L  E  Q  E  T  P  V  T  L  A  F  L  A  Q  A

      460       470       480       490       500       510       520       530       540
GGTGGCGATGAGCCCGTTTCATCTGCACCGTCTGTTTAAAGCGAGCACCGGAATGACGCCGAAAGGGTGGCAGCAGGCGTGGCGCGCCCG
 V  A  M  S  P  F  H  L  H  R  L  F  K  A  S  T  G  M  T  P  K  G  W  Q  Q  A  W  R  A  R

      550       560       570       580       590       600       610       620       630
GCGGCTGCGTGAGGCTTTGGCGAAAGGAGAGCCGATCACGGCGGCTATTTACCGCGCTGGCTTCCCGGATAGCAGTAGCTACTACCGTCA
 R  L  R  E  A  L  A  K  G  E  P  I  T  A  A  I  Y  R  A  G  F  P  D  S  S  S  Y  Y  R  H

      640       650       660       670       680       690       700       710       720
TGCCGACCAGACGCTGGGCATGACGGCAAAACAGTTTCGCAAAGGCGGCGATAATGTCTCCGTTCGCTATGCGCTGACGGACTGGGTTTA
 A  D  Q  T  L  G  M  T  A  K  Q  F  R  K  G  G  D  N  V  S  V  R  Y  A  L  T  D  W  V  Y

      730       740       750       760       770       780       790       800       810
CGGACGGTGCCTGGTGGCGGAGAGCGAGCGGGGGATTTGCGCGATTCTCCCCGGTGATAGCGACGACGCGCTACTGGCTGAATTACACAC
 G  R  C  L  V  A  E  S  E  R  G  I  C  A  I  L  P  G  D  S  D  D  A  L  L  A  E  L  H  T

      820       830       840       850       860       870       880       890       900
CCTTTTCCCGTCGGCCCGCCACGAACCTGCTGACGCGCTTTTTCAGCAACGGGTGCGGCAGGTTGTCGCGGCTATCAACACACGCGATGT
 L  F  P  S  A  R  H  E  P  A  D  A  L  F  Q  Q  R  V  R  Q  V  V  A  A  I  N  T  R  D  V

      910       920       930       940       950       960       970       980       990
GCTGCTCTCGTTGCCGCTGGATATCCAGGGAACCGCGTTTCAACAGCAGGTCTGGCAGGCGTTATGCGCAATTCCCTGCGGCGAAACCGT
 L  L  S  L  P  L  D  I  Q  G  T  A  F  Q  Q  Q  V  W  Q  A  L  C  A  I  P  C  G  E  T  V

     1000      1010      1020      1030      1040      1050      1060      1070      1080
AAGCTATCAACAGCTTGCCGCGACTATCGGCAAACCCACGGCGGTACGCGCGGTCGCCAGCGCGTGCGGCGCGAATAAACTGGCGATGGT
 S  Y  Q  Q  L  A  A  T  I  G  K  P  T  A  V  R  A  V  A  S  A  C  G  A  N  K  L  A  M  V

     1090      1100      1110      1120      1130      1140      1150      1160      1170
GATCCCGTGTCATCGGGTCGTGCGTCGCGATGGCGCGCTCTCCGGTTATCGTTGGGGCGTGCGTCGAAAAGCGCAGCTATTAAAGCGAGA
 I  P  C  H  R  V  V  R  R  D  G  A  L  S  G  Y  R  W  G  V  R  R  K  A  Q  L  L  K  R  E

     1180      1190      1200      1210      1220      1230      1240      1250      1260
AGCGCAAAAAGAGGAGTAGCAATGCTGGATCTATTTGCTGATGAAGCGCCCTGGCAAGAGCCCCTGGCGCCTGGCCGGGTGGTGTTGCGC
 A  Q  K  E  E           M  L  D  L  F  A  D  E  A  P  W  Q  E  P  L  A  P  G  R  V  V  L  R

CGCTTT
 R
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ada_{ST}

FIG. 6. Nucleotide and predicted amino acid sequences of the ada_{ST} gene. An ORF corresponding to the ada_{ST} gene extends from nucleotides +131 to +1186. The putative *ada* box, ribosome-binding site (Shine-Dalgarno [S.D.] sequence), and −35 and −10 regions are underlined. Nucleotide and predicted amino acid sequences of the 5′ region of the *alkB*-like gene extending from nucleotide +1192 are also presented.

```
                                                                GCATGTAAGC
ada_ST : AGCGATAAGAATTAAAACGCAAGATTACGGGTTTTTGTGGGCGGATTTACGGACACACTAAGCACTATGCTTAATGAAGGAGTCTATGATG
                  ada box          -35                          -10                    S.D.

ada    : AGCGAAAAAAATTAAAGCGCAAGATTGTTGGTTTTTGCGTGATGGTGACCGGGCAGCCTAAAGGCTATCCTTAACCAGGGAGCTGATTATG
                  ada box          -35                          -10                    S.D.

alkA   : ATGTTGCCGTCGCGACAACCGGAATATGAAAGCAAAGCGCAGCGTCTGAATAACGTTTATGCTGAAAGCGGATGAATAAGGAGATGCGATG
                                    ada box                       -10                   S.D.
                                         -35
```

FIG. 7. Promoter region of the ada_{ST}, *E. coli ada*, and *alkA* genes. The conserved *ada* box sequence, proposed −35 and −10 regions, and Shine-Dalgarno (S.D.) sequence are underlined. The transcription initiation site is represented by a dot. The initiation codon (ATG) is represented by capital letters.

we have concluded that plasmid pYG7001 encodes the MTase of *S. typhimurium*.

Determination of the minimum region of cloned DNA essential for MTase activity. A restriction map of pYG7001 was constructed by digesting the plasmid with several restriction enzymes (Fig. 3). The plasmid (9.9 kb) was composed of pBR322 (4.4 kb) and genomic DNA of TA1538 (5.5 kb). To determine the region necessary for encoding the MTase, we partially digested pYG7001 with *Eco*RV and constructed a set of deletion derivatives. Upon checking the sensitivities of Δ*ada-25* strains containing such deletion plasmids on MNNG-containing agar plates, we concluded that the MTase is encoded in the 2.0-kb region spanning from map position 2.5 kb (the second *Eco*RV site) to 4.5 kb (the fifth *Eco*RV site) of pYG7001. The restriction map within the 2.0-kb region was different from those of the *ada* and *ogt* genes of *E. coli*.

To further define the minimum DNA region necessary for encoding the MTase, we subcloned the 2.0-kb DNA fragment into an *Eco*RV site of the pBluescript KS+ vector. The resulting plasmid, pYG7034, efficiently made a Δ*ada-25* strain resistant to the killing effect of MNNG (data not shown). More than 50 deletion derivatives were prepared from both sides of the insert DNA of pYG7034 according to the exonuclease III-mung bean nuclease digestion protocol. Among these deletion plasmids, 24 subcloned plasmids were introduced into a Δ*ada-25* strain and checked for the ability to make the host strain resistant to the cell-killing effect of MNNG, using MNNG-containing agar plates.

The four longest subclones whose insert DNA was deleted from the *Eco*RV site on the right in Fig. 4 could complement a Δ*ada-25* strain, while the plasmids which had about a 0.7-kb deletion from the same site no longer complemented the *ada* mutation. In the same way, the four longest subclones whose insert DNA was deleted from the *Eco*RV site on the left in Fig. 4 could complement the *ada* mutation, whereas the plasmids whose insert DNA was deleted more than about 0.6 kb from the same site no longer complemented the mutation. Thus, we concluded that the 1.3-kb region shown as an open box in Fig. 4 is the minimum region essential for encoding the MTase of *S. typhimurium*.

Identification of the MTase of *S. typhimurium*. To identify the MTase of *S. typhimurium*, proteins synthesized in maxicells containing pYG7034 and pYN3059 were labeled with [^{35}S]methionine and were subjected to SDS-polyacrylamide gel electrophoresis and fluorography. Plasmid pYG7034 produced a protein with an approximate molecular mass of 38 kDa (Fig. 5). The minor band of a protein with an approximate molecular mass of 15 kDa was also observed. Plasmid pYN3059 carrying the *E. coli ada* gene produced a protein with a molecular mass of 39 kDa, as expected. No bands around the molecular mass of 19 kDa corresponding to that of the Ogt protein of *E. coli* were observed. Thus, we suggest that the MTase encoded by the cloned DNA is analogous to the *E. coli* Ada protein but not to the Ogt protein and tentatively refer to the cloned gene as ada_{ST}.

DNA sequence of the ada_{ST} gene. We have determined the nucleotide sequences of both strands of the 1.3-kb region which carries the ada_{ST} gene (Fig. 6). Since the first cloned plasmid, pYG7001, was selected on MNNG-containing agar plates, it was possible that mutations could arise in the cloned gene. To avoid such a complication, we recloned the DNA which was not exposed to MNNG and confirmed that the 1.3-kb region shown in Fig. 6 has no mutational changes induced by MNNG. The nucleotide sequence of the 1.3-kb region contains an open reading frame (ORF) of 1,056 bp and part of the other ORF spanning from nucleotides 1192 to 1266, which is homologous to the 5′ region of the *alkB* gene of *E. coli* (14). We did not determine the nucleotide sequence of the rest of the 3′ region of the latter ORF. The former ORF potentially encoded a protein with a calculated molecular weight of 39,217, and the nucleotide sequence showed 70% similarity to the *ada* gene of *E. coli*. The similarity at the amino acid level between the two gene products was 75%. Thus, we suggest that the former ORF is the coding region of the ada_{ST} gene and the latter is that of the *alkB*-like gene of *S. typhimurium*. The termination codon of ada_{ST} and the initiation codon of the *alkB*-like gene were separated by 2 bp, whereas the *ada* and *alkB* genes of *E. coli* overlap by 1 bp (14). Upstream of the ada_{ST} gene, there was a potential *ada* box sequence (5′-GAATTAAAACGCA-3′), which differs from the *E. coli ada* box (5′-AAANNAAAGCGCA) by 2 bp (Fig. 7). Possible −35, −10, and Shine-Dalgarno regions were also observed. The distance between the −10 region and the Shine-Dalgarno region of the ada_{ST} gene was 10 bp longer than that of the *E. coli ada* gene. The amino acid sequence homology between Ada_{ST} and *E. coli* Ada was not restricted to a specific region but spanned all of the coding region (Fig. 8). *E. coli* and *S. typhimurium* are about 80% homologous in DNA sequence (40). The *N*-terminal halves of the Ada_{ST} and the *E. coli* Ada proteins shared homology with the AdaA protein of *Bacillus subtilis* (27), whereas the C-terminal halves were homologous to the AdaB and Dat proteins of *B. subtilis* (13, 26, 27), the Ogt protein of *E. coli* (39), and the human MTase (MGMT) (10, 57). The ada_{ST} gene encodes Cys-68 and Cys-320, which are highly conserved in other MTases and are possible acceptor sites for the methyl groups from the damaged DNA. The gene also encodes the sequence of Lys-178 and Glu-179, which corre-

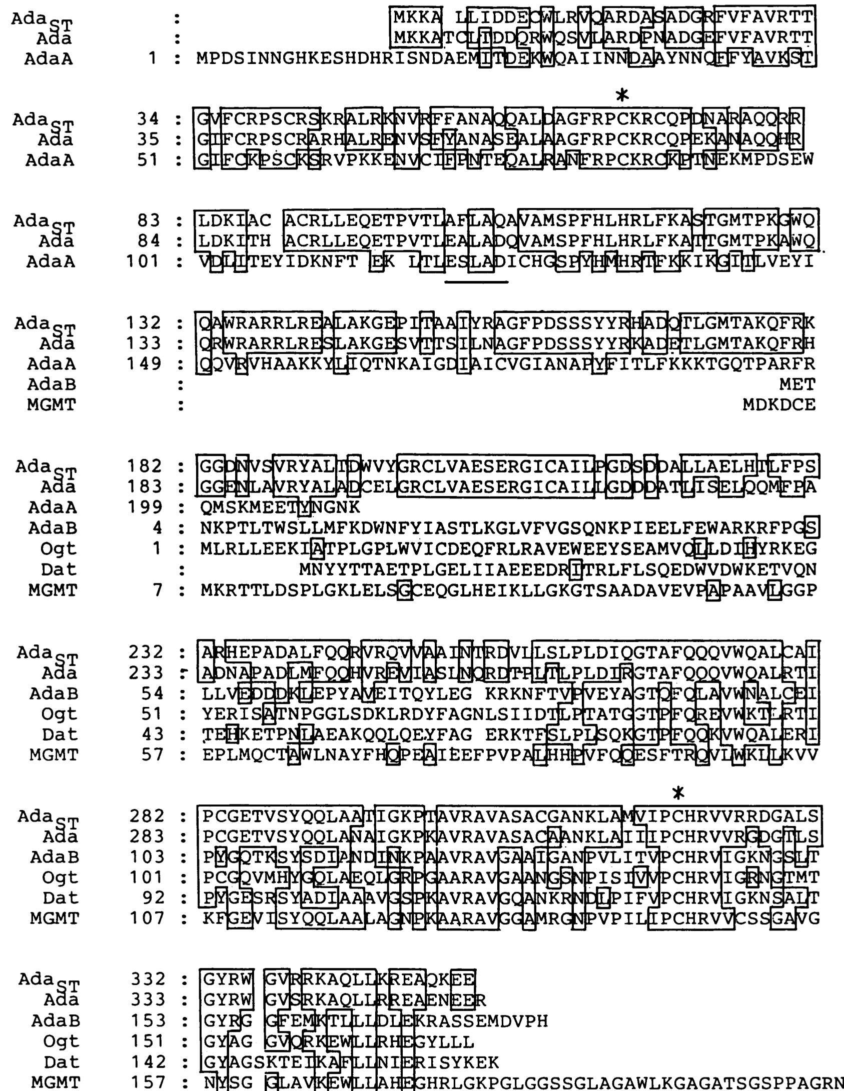

FIG. 8. Homology among the *S. typhimurium* (Ada_{ST}), *E. coli* (Ada and Ogt), *B. subtilis* (AdaA, AdaB, and Dat), and human (MGMT) MTases. Amino acid residues that are conserved between Ada_{ST} and at least one of other MTases are boxed. *, possible methyl acceptor sites from alkylated DNA; ——, amino acid sequences of the AdaA and Ada proteins that are similar to the sequence Glu-Ser-Val-Ala-Asp of the lambda CI repressor.

sponds to a main cleavage site by an endogenous protease in *E. coli*.

Low ability of Ada_{ST} to act as a transcriptional activator. To characterize the ability of Ada_{ST} to function as a transcriptional activator, we introduced plasmid pYG7001 carrying the ada_{ST} gene into a strain of *E. coli* containing a chromosomal *ada'-lacZ* fusion integrated at the *ada* locus (GW5354) (53). In this strain, the *ada* gene is joined to the *lacZ* gene so that levels of expression of the gene can readily be determined simply by assaying β-galactosidase activity. As shown in Table 2, *ada* transcription was induced about fivefold by MNNG if the cells harbored pYG7001 carrying the ada_{ST} gene. In contrast, *ada* transcription was induced more than 40-fold by MNNG if the cells had pYG7050, a low-copy-number plasmid carrying the *E. coli ada* gene. To more accurately compare the effects of the Ada_{ST} and *E. coli* Ada proteins on *ada* transcription, we subcloned the ada_{ST} gene and the *E. coli ada* gene onto the same low-copy-

TABLE 2. Effect of Ada_{ST} on *ada* transcription

Plasmid[a]	Relevant gene	Parent vector[b]	β-Galactosidase activity[c]	
			Uninduced	Induced
pBR322			107	101 (0.9)
pHSG576			84	73 (0.9)
pYG7001	ada_{ST}	pBR322	187	855 (4.6)
pYG7050	*ada*	pSE101	72	3,069 (42.6)
pYG7070	ada_{ST}	pHSG576	111	1,413 (12.7)
pYG7090	*ada*	pHSG576	93	3,609 (38.8)

[a] Detailed descriptions of pYG7001, pYG7070, pYG7090, and pYG7050 are given in Table 1.

[b] pBR322 is a high-copy-number plasmid. Both pHSG576 and pSE101 are low-copy-number plasmids.

[c] Expressed as units per optical density at 600 nm (24). Numbers in parentheses represent relative values when the activities of uninduced cells are assigned a value of 1.0. β-Galactosidase assays were performed on strains containing a chromosome *ada'-lacZ* fusion (GW5354) and a plasmid carrying the ada_{ST} or the *E. coli ada* gene (53). Induced cultures were assayed 2 h after the addition of 1 μg of MNNG per ml. Uninduced cultures were assayed after 2 h of growth without MNNG.

number plasmid, pHSG576. The resulting plasmids, pYG7070 and pYG7090, were introduced into strain GW5354, and β-galactosidase activities were determined. As shown in Table 2, *ada* transcription was induced about 13-fold by MNNG if the cells had pYG7070 carrying the *ada*$_{ST}$ gene, while *ada* transcription was induced about 40-fold by MNNG if the cells had pYG7090 carrying the *E. coli ada* gene. From these results, we suggest that Ada$_{ST}$ can activate *ada* transcription in *E. coli* to some extent but that its ability to function as a transcriptional activator is much lower than that of the *E. coli* Ada protein.

DISCUSSION

Although an adaptive response to alkylation damage has been observed in a number of microorganisms, several reports suggest that this response does not occur in *S. typhimurium* (8, 9, 17). Since chromosome DNA sequences that hybridize with the *E. coli ada* gene have been detected in *S. typhimurium* (50), it was assumed that *S. typhimurium* had the *ada*-like gene but that its function might be much weaker than that of the *E. coli ada* gene (17). To clarify the molecular nature of the *ada* gene of *S. typhimurium* and its relation to the apparent lack of the adaptive response of this microorganism, we have searched for this gene by screening for the gene which makes a Δ*ada-25* strain of *E. coli* resistant to the cell-killing and mutagenic effects of MNNG. DNA sequence analysis indicated that the cloned gene encodes a protein with a molecular mass of 39 kDa, which is consistent with the value deduced from the [^{35}S]methionine-labeling experiments using the maxicell technique (Fig. 5). The 15-kDa protein identified in maxicells containing pYG7034 might be produced by the *alkB*-like gene of *S. typhimurium* (Fig. 6). Since the molecular weight of the *E. coli* AlkB protein is 23,900 (14), we have apparently cloned only a fragment of the *alk*B-like gene of *S. typhimurium*. The DNA sequence of the cloned gene encoding the 39-kDa protein shows 70% similarity to the *E. coli ada* gene at the nucleotide level, and there was an *ada* box-like sequence upstream of the gene (Fig. 6 to 8). Thus, we conclude that the gene we have cloned is an analog of the *E. coli ada* gene and tentatively refer to the cloned gene as *ada*$_{ST}$.

The multicopy plasmid carrying the *ada*$_{ST}$ gene (pYG 7001) significantly reduced the mutation frequency induced by MNNG in *E. coli* and *S. typhimurium* (Fig. 2A and C). These results suggest that the Ada$_{ST}$ protein is functional at least as a repair enzyme in *E. coli* and in *S. typhimurium*. In fact, the crude extract of a Δ*ada-25* strain containing pYG7001 showed detectable repair activity for O^6-MeG. In accordance with these results, the predicted amino acid sequence of Ada$_{ST}$ contains the sequence Pro-Cys-His-Arg-Val around Cys-320 (Fig. 6). This amino acid sequence is highly conserved in all MTases whose genes have been cloned and sequenced (Fig. 8). Cys-321 of the *E. coli* Ada protein, which corresponds to Cys-320 of the Ada$_{ST}$ protein, is regarded as a methyl acceptor site from O^6-MeG and O^4-MeT in damaged DNA (17, 52, 55). In the accompanying report (60a), Vaughan and Sedgwick report that the Ada$_{ST}$ protein repairs O^6-MeG in DNA as efficiently as does the *E. coli* Ada protein.

In contrast to the DNA repair activity, the ability of the Ada$_{ST}$ protein to function as a transcriptional activator was much lower than that of the *E. coli* Ada protein (Table 2). The Ada$_{ST}$ protein encoded by pYG7001 increased expression of the *ada'-lacZ* chromosome fusion about 5-fold when an *E. coli* strain carrying both the fusion operon and the plasmid was exposed to a low concentration of MNNG, whereas the Ada protein of *E. coli* encoded by pYG7050 increased it about 40-fold under the same conditions. Even with the same copy number, the Ada$_{ST}$ protein encoded by pYG7070 showed a much smaller effect on *ada* transcription than did the *E. coli* Ada protein encoded by pYG7090 (Table 2). Vaughan and Sedgwick (60a) directly measured the extent of induction of the *ada*$_{ST}$ gene in *S. typhimurium* by using the immunoblotting technique and reported that the 39-kDa protein was weakly induced by pretreating the *S. typhimurium* cells with MNNG. The low ability of Ada$_{ST}$ to function as a transcriptional activator could account at least in part for the apparent lack of the adaptive response to alkylating agents in *S. typhimurium* (Fig. 1).

In unadapted cells of *E. coli*, about 30 to 40 molecules of MTase are expressed per cell, and more than 95% of them constitute the 19-kDa protein, probably the Ogt protein (19, 42). On the other hand, Rebeck et al. identified an active 19-kDa MTase in *S. typhimurium* and estimated that about 30 molecules of this MTase are constitutively expressed per cell (42). Morohoshi and Munakata also identified a similar-size MTase in unadapted cells of *S. typhimurium* (27a). These results raise the possibility that *S. typhimurium* has the Ogt-like protein and that this form of MTase is predominant in unadapted cells. If so, the apparent lack of the adaptive response of *S. typhimurium* would be due to the low concentration of the Ada$_{ST}$ protein in unadapted cells as well as to its low ability to function as a transcriptional activator.

Why is the extent of induction of the *ada*$_{ST}$ gene in *S. typhimurium* much lower than that of induction of the *ada* gene in *E. coli*? The following are possible factors which reduce the inducibility of the *ada*$_{ST}$ gene in *S. typhimurium*.

(i) In the *E. coli* Ada protein, transfer of the methyl group from the methylphosphotriester to Cys-69 converts Ada from a weak to a strong DNA-binding protein and transcriptional activator (17, 42, 52). If Ada$_{ST}$ had a poor repair activity to the methylphosphotriester, it would have a low ability to convert itself to a transcriptional activator. This possibility, however, seems to be unlikely since *ada*$_{ST}$ encodes the sequence Phe-Arg-Pro-Cys-Lys-Arg-Cys, which is conserved in the *E. coli* Ada and *B. subtilis* AdaA proteins (Fig. 8). Cys-68 at the fourth position of the sequence corresponds to Cys-69 of the *E. coli* Ada protein. In fact, Vaughan and Sedgwick (60a) report that the Ada$_{ST}$ protein shows considerable repair activity to the methylphosphotriester.

(ii) If the promoter sequence of the *ada*$_{ST}$ gene diverged greatly from the corresponding sequences of the *E. coli ada* and *alkA* genes, it would have a low ability to interact with a transcriptional activator. The nucleotide sequence of the promoter region of the *ada*$_{ST}$ gene is, however, fairly similar to that of the region of the *E. coli ada* gene (Fig. 7). The *ada* box-like sequence (5'-GAATTAAAACGCA-3') of *ada*$_{ST}$ is identical to the *ada* box sequence (5'-AAANNAAAGCGC A-3') of the *E. coli ada* and *alkA* genes (52) except for two nucleotides, i.e., the first G and the ninth A of the *ada* box-like sequence. However, Nakamura et al. reported that removal of the first five nucleotides of the *ada* box sequence or the change of G to A at the ninth position of the sequence had little effect on expression of the *E. coli ada* gene (33). If a similar regulation mechanism exists in *S. typhimurium*, changes of these nucleotides will not seriously affect the function of the *ada* box-like sequence of the *ada*$_{ST}$ gene. The only marked difference in the DNA sequence of the promoter region between the *ada*$_{ST}$ and *E. coli ada* genes is that

the ada_{ST} has an extra 10-bp sequence at the 3′ side of the −10 region (Fig. 7). Since the size of 10 bp corresponds to a full turn of the DNA helix, it would be interesting to determine the actual initiation site of transcription of the ada_{ST}.

(iii) Changes of some amino acids might directly affect the ability of the Ada_{ST} protein to bind the specific DNA sequence or to activate the RNA polymerase. Studies on the *E. coli* Ada protein suggest that the region essential for the specific DNA binding resides on the *N*-terminal half of the Ada protein (1). Recently, Morohoshi et al. reported that a portion of the AdaA protein of *B. subtilis* spanning from amino acids 117 to 137 has the potential to form a helix-turn-helix motif (27, 36). Dodd and Egan suggested that the *E. coli* Ada protein also has a potential helix-turn-helix motif at the corresponding region (5). The AdaA protein shows similarity to the lambda CI repressor, which is a typical DNA-binding protein (27, 36). The sequence Glu-Ser-Leu-Ala-Asp, which is present in the AdaA protein from amino acids 119 to 123, is almost identical to the corresponding sequence, Glu-Ser-Val-Ala-Asp, of the repressor. Of the five amino acid residues of the sequences, two acidic residues, the first Glu and the fifth Asp, are regarded as crucial for the ability of the lambda CI repressor to function as a transcriptional activator (2). Interestingly, Ada_{ST} contains the sequence Ala-Phe-Leu-Ala-Gln from amino acids 102 to 106, whereas the *E. coli* Ada protein has the sequence Glu-Ala-Leu-Ala-Asp at the corresponding region (Fig. 8). Thus, it might be interesting to determine the effect of changes of the first Ala and the fifth Gln residues of the Ada_{ST} sequence to acidic residues on the ability of the protein to function as a transcriptional activator.

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