Site-Directed Mutation of the *Escherichia coli* *ada* Gene: Effects of Substitution of Methyl Acceptor Cysteine-321 by Histidine in Ada Protein

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Oligodeoxynucleotide-mediated mutagenesis of the *ada* gene of *Escherichia coli* was used to produce two mutant Ada proteins. In mutant I the methyl acceptor Cys-321 for *O*⁶-methylguanine was replaced by histidine; and in mutant II the positions of Cys-321 and His-322 of the wild-type protein were inverted. Neither mutant protein had *O*⁶-methylguanine-DNA methyltransferase activity, but both retained the phosphotriester-DNA methyltransferase activity involving methyl group transfer to Cys-69. Under the control of the endogenous promoter, synthesis of mutant I protein was undetectable before or after adaptation treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. This appeared to be due to both inhibition of transcription of the mutant gene and degradation of the synthesized protein. On the other hand, mutant II protein was inducible by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, although to a smaller extent than the wild-type protein was, and the phosphotriester-DNA methyltransferase activity appeared to reside in 24- to 30-kilodalton cleavage products. Mutant I protein could be produced under *lac* promoter control, and its cleavage products, unlike those of mutant II protein, tended to aggregate. These results indicate that (i) Cys-321 cannot be replaced or transposed with the nucleophilic amino acid histidine for *O*⁶-methylguanine-DNA methyltransferase function, (ii) single amino acid replacement or transposition at the *O*⁶-methylguanine methyl acceptor site can have a profound effect on the *in vivo* stability and regulatory function of the Ada protein, and (iii) the integrity of the protein may not be absolutely needed for its transcription-activation function.

The 39-kilodalton (kDa) Ada protein of *Escherichia coli* has a central role in the adaptive response of the bacteria to DNA methylating agents (27, 39). The protein accepts methyl (and ethyl) groups from DNA phosphotriesters at its Cys-69 residue and from *O*⁶-alkylguanine (or *O*⁴-alkylthymine) in DNA at the Cys-321 position (7, 34, 37). The Cys-69-methylated protein is a transcriptional activator for genes of the *ada* regulon when it binds to a conserved DNA sequence 5' upstream of *ada* and *alkA* in a situation similar to that of the cyclic AMP-cyclic AMP receptor protein-mediated activation of the *lac* promoter (1, 27, 39). However, there are important differences between these two cases. For example, the methylated Ada protein is a monomer in solution, while the other known sequence-specific DNA-binding proteins, including the cyclic AMP-cyclic AMP receptor protein complex, are dimers or tetramers in their native states (1, 3, 31). The Ada protein is also unusual in that it has two domains with distinct activities and a hinge region near the middle (3, 34, 40). The protein is cleaved *in vitro* at a unique site in the hinge region by a novel protease (2; P. R. Caron and L. Grossman, J. Cell. Biochem. 12A[Suppl.]:259, 1988). In fact, the *O*⁶-methylguanine-DNA methyltransferase (MGMT) originally purified from a constitutive *ada* mutant (*E. coli* BS21) was the 19-kDa C-terminal cleavage product of the Ada protein (6). The N-terminal and C-terminal fragments are independently active as methyl acceptors from methyl phosphotriesters and *O*⁶-methylguanine in DNA, respectively (for a review, see reference 20). However, the two domains are expected to be in close proximity because the intact Ada protein is globular (3).

The *E. coli ada* gene was first cloned by B. Sedgwick (33) and subsequently by others (18, 22, 26). Its nucleotide sequence indicates the presence of a regulatory region 5' upstream of the promoter which is recognized by the Ada protein (7, 27).

In order to understand the mechanistic role of the different domains and amino acid residues in the various activities of the Ada protein, we initiated a systematic introduction of site-directed mutations in the *ada* gene to investigate the biochemical properties of the mutant proteins. The effects of Cys-321 substitution by another nucleophilic amino acid, histidine, on methyltransferase activities and *ada* activator function are described.

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* GW5352 and GW5354 were derived from AB1157 and contained *ada-10::Tn10 del-16 del-17* and $\Phi(ada'-lacZ^+)$ 1, respectively (18). F' *lacI*^H/Tn5 was crossed into GW5352 from GC2438, a gift from S. Gottesman (National Cancer Institute) to produce the strain KT100. For site-directed mutagenesis, a methylation-deficient strain, GM373 (F⁻ 42 [F' *lac*]/*dam-3 dcm-6 hsdR17 tonA31 lacY1 tsx-78 supE44 galK2 galT22 thi-1*), and a mismatch repair-deficient strain, GM374 (F-42 [F' *lac*]/*mutL25 thr-1 leuB6 thi-1 argE3 hisG4 proA2 lacY1 galK2 mtl-1 xyl-5 ara-14 rpsL31 tsx-33 glnV44 rfbD1 kdgK51*) (23), were used.

pCS70 (33) carrying the *ada* gene was the parental plasmid that was used for mutagenesis. Plasmid pSM31 carried the *HindIII-XmaI ada* fragment from pSC70 under the control of *lacOP* in pUC9. To overproduce Ada protein, pSM41 was constructed by deleting the promoter region of the *ada* gene

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(85-base-pair [bp] fragment). The construction of these plasmids has been described previously (38).

Synthesis of oligonucleotides. The deoxynucleotide oligomers (Fig. 1) that were used to produce His-321 and His-321 Cys-322 mutations in the *ada* gene were synthesized by the phosphoramidite method (35) by using an automated DNA synthesizer (model 1450; Systec).

Oligonucleotide-directed mutagenesis of the *ada* gene. The *Hind*III-*Xma*I fragment (1.3 kbp) containing the *ada* gene in pSC70 was ligated at the corresponding site in the polylinker of M13 mp19 replicative-form (RF) DNA. M13 single-stranded DNA carrying the *ada* gene was annealed with synthetic oligonucleotide carrying the mutation and was converted into RF DNA with DNA polymerase I (Klenow fragment) and T4 DNA ligase in two stages (28, 44). After purification of RF type I DNA by band sedimentation in an alkaline sucrose gradient (29), it was dialyzed against 10 mM Tris hydrochloride–1 mM EDTA (pH 8.0) and used to transfect *E. coli* GM374. Mini-preparations of phage DNA obtained from single plaques were screened for the desired mutation by dot blot hybridization on nitrocellulose by using as probes the same oligonucleotides that were used for mutagenesis after they were labeled with ^{32}P at the 5' termini with polynucleotide kinase and [γ - ^{32}P]ATP (44). The mutations were then verified by sequence analysis of the single-stranded DNA of M13 mp19-*ada* recombinant phages as described by Sanger et al. (32). Finally, the mutant sequences of pSM32 and pSM33 were also confirmed by direct sequencing of the duplex DNA (43). In both cases, the oligonucleotide 5'-TTTACGCGACACGCC corresponding to nucleotides 1123 to 1009 of *ada* (7) was used as the primer.

Recombinant DNA techniques. Standard recombinant DNA techniques were performed essentially as described by Maniatis et al. (21) and were used for the isolation of plasmid DNA, restriction enzyme digestion, agarose gel electrophoresis, DNA ligation reactions, transformation, and hybridizations.

Preparation of crude extract. The plasmid-containing *E. coli* cells were grown overnight at 37°C in L-broth (1% tryptone [Difco Laboratories, Detroit, Mich.], 0.5% yeast extract, 1% NaCl [pH 7.0]) containing 100 µg of ampicillin per ml. The overnight cultures were diluted 1:50 with the same fresh medium and incubated at 37°C with shaking until the A_{550} reached about 0.2. Isopropyl- β -D-thiogalactopyranoside (IPTG) was added to final concentration of 0.5 mM, and the cultures were incubated for another 4 h. In the case of adaptive treatment, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) was added at 5 µg/ml when the cell density reached about 0.6 at an A_{550} , and the cultures were incubated for another 1 h at 37°C with shaking. After the cells were harvested by centrifugation, cells were suspended in 20 mM Tris hydrochloride (pH 8.2)–10% glycerol–1 mM EDTA–1 mM dithiothreitol (buffer A) containing 0.4 M NaCl (26) and sonicated. The extracts were centrifuged at 125,000 $\times g$ for 10 min at 4°C to remove cell debris. The supernatants were used as crude extracts for the assay of methyltransferase activity and Western blot (immunoblot) analysis.

Assay of methyltransferase activity. For the measurement of total methyltransferase activity, i.e., transfer of methyl groups for both *O*⁶-methylguanine and methylphosphotriesters, substrate DNA was prepared by reacting *N*-[^3H]methylnitrosourea (0.8 Ci/mmol) with calf thymus DNA by a previously described procedure (30). The substrate had a specific activity of about 10⁵ cpm/mg of DNA, of which 55% of the radioactivity was present in *O*⁶-methylguanine and the remainder of which was mostly present in phospho-

triesters, as determined by high-pressure liquid chromatography of an acid hydrolysate (12). The method used to assay the activity was a modification of the procedure of Waldstein et al. (41). Portions of crude extracts were mixed with buffer A containing 320 fmol of substrate in a total reaction volume of 200 µl. After incubation at 37°C for 1 h, the reaction was terminated by the addition of sodium dodecyl sulfate (SDS) to a final concentration of 1%. The radiolabeled protein was digested by incubating it with 100 µg of proteinase K at 37°C for 2 h, the nucleic acids were precipitated with alcohol, and the supernatants were counted to measure the released radioactivity.

For the assay of MGMT activity specifically, synthetic poly(dC-dG-[^3H]m⁶dG) was used as the substrate (11, 12). The extracts were incubated (37°C for 1 h) with substrate containing 1 pmol (2,900 cpm) of [^3H]O⁶-methylguanine, in 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.9), 2 mM dithiothreitol, 2 mM EDTA, and 0.1 mM spermidine in a total volume of 100 µl. After the reaction was carried out, the nucleic acids were isolated by phenol-chloroform (1:1) extraction and alcohol precipitation with carrier calf thymus DNA and redissolved in H₂O (90 µl). The purines were liberated from the substrate by adding HCl to a final concentration of 20 mM and heating the solution at 70°C for 30 min. They were then separated by high-pressure liquid chromatography on a column (Aminex A-6). The experimental details of this assay have been described elsewhere (12). The molar amount of O⁶-methylguanine that was demethylated was calculated from the fraction of radioactivity in guanine and is equal to the amount of methyltransferase (19, 25).

β -Galactosidase assay. The β -galactosidase assay was performed as described by Miller (24). Cells were grown overnight in supplemented M9 glucose medium (18) containing 100 µg of ampicillin per ml. Overnight cultures were diluted 1:100 with the same medium containing 2 µg of MNNG per ml for adaptive treatment.

DNA-RNA dot blot hybridization analysis. Total cellular RNA was prepared by the hot phenol extraction procedure (9). After harvesting, *E. coli* cells were suspended in 20 mM sodium acetate (pH 5.5)–0.5% SDS–1 mM EDTA. After the addition of an equal volume of phenol that was saturated with the same buffer, the mixture was incubated at 60°C for 10 min with vigorous shaking and centrifuged, and the aqueous layer was then reextracted with phenol at room temperature. Finally, total RNA was precipitated by adding 2.5 volumes of ethanol in the presence of 0.3 M sodium acetate. For dot blot analysis, RNA was purified by centrifugation through a cushion of 5.7 M CsCl to remove contaminating DNA (13). Portions of purified RNA were serially diluted and spotted onto nylon filters by using a dot blot manifold (Bio-Rad Laboratories, Richmond, Calif.) and the filters were air dried and baked for 2 h at 80°C. The filter-bound RNA was then hybridized with the 1.3-kbp *Hind*III-*Xma*I *ada* gene fragment that was labeled with [α - ^{32}P]dCTP by DNA polymerase I (Klenow fragment) by using random deoxyhexanucleotides as primers (10).

Determination of relative copy number of plasmids. Plasmid DNA was extracted with an SDS-alkaline lysis preparation (21). The plasmid molecules were linearized with *Hind*III, and portions were serially diluted and subjected to electrophoresis in 0.8% agarose gels. Gels were stained with ethidium bromide and photographed. Relative copy numbers of plasmids were estimated by comparing intensities of the bands of linearized molecules (36).

Gel filtration of Ada proteins. The methyltransferase pro-

	318	319	320	321	322	323	324
Template DNA:	5'-GTT	ATA	CCC	TGT	CAT	CGG	GTA
	Val	Ile	Pro	Cys	His	Arg	Val
Primer (I):	3'-CAA	TAT	GGG	<u>GTA</u>	GTA	GCC	CAT
			Pro	His	His		
Primer (II):	3'-CAA	TAT	GGG	<u>GTA</u>	<u>ACA</u>	GCC	CAT
			Pro	His	Cys		

FIG. 1. Synthetic primers used for site-directed mutagenesis. The predicted amino acid sequences are shown under the DNA sequences, and mutation sites are underlined.

teins were partially purified by a modification of the procedure described by Bhattacharyya et al. (3). The bacterial suspensions in buffer A containing 0.4 M NaCl were sonicated and centrifuged at $125,000 \times g$ for 30 min. The supernatants were treated with polyethyleneimine (18 μ g per A_{260} unit of extract) and centrifuged to remove DNA. Ammonium sulfate was added to 70% saturation, and the precipitated proteins were collected by centrifugation and redissolved in 1 ml of buffer A containing 0.1 M NaCl before they were loaded onto a gel filtration column (74 by 0.9 cm; AcA44 column). The methyltransferase activity of the fractions was determined by assaying them with $\text{CH}_3\text{-}^3\text{H}$ -labeled, methylated calf thymus DNA substrate as described above.

Western blotting. Portions of crude bacterial extracts, which were prepared as described above, were electrophoresed on SDS-15% polyacrylamide slab gels (17), and the separated proteins were then electrophoretically transferred to a nitrocellulose membrane (4). Unbound sites on the membrane sheets were blocked during overnight incubation with Bovine Lacto Transfer Technique Optimizer (BLOTTO) (16). The sheets were then incubated for 2 h with polyclonal rabbit antibodies (pretreated with *E. coli* *ada* mutant PJ6 [15] extract and diluted 200 times with BLOTTO) that were raised against either whole 39-kDa Ada protein (denatured with 1% SDS) or its 19-kDa C-terminal fragment. After the membranes were rinsed to remove excess antibody, they were incubated with ^{125}I -labeled protein A (10^7 cpm) for 1 h. After thorough washing, the membrane was air dried and exposed to X-ray film at -70°C .

Amino acid analysis of methylated proteins. Extracts containing methyltransferase activity were incubated with $\text{CH}_3\text{-}^3\text{H}$ -labeled, methylated DNA in 10 mM sodium citrate (pH 7.4) for 30 min at 37°C . Proteinase K (500 $\mu\text{g}/\text{ml}$) was then added and incubation was continued for 2 h. Nucleic acids were precipitated by adding HCl to 1 N and then were chilled in ice for 15 min. After centrifugation the supernatants were lyophilized in a vacuum centrifuge. Samples containing 7×10^3 to 2.1×10^4 cpm were acid hydrolyzed in 6 N HCl-10 mM 2-mercaptoethanol at 110°C for 21 h and chromatographed on an amino acid analyzer (121 M; Beckman Instruments, Inc., Fullerton, Calif.). Fractions were collected for the determination of radioactivity.

RESULTS

Construction of plasmids. The nucleotide sequences of synthetic oligomers that were used to create the His-321 and His-321 Cys-321 mutations of the cloned *ada* gene are shown in Fig. 1. The *HindIII-XmaI* fragments containing the whole *ada* gene and carrying the mutation sites were introduced into plasmid pUC9 from M13 mp19-*ada* RF DNA. The

resultant plasmids were named pSM32 (His-321) and pSM33 (His-321 Cys-322), respectively (Fig. 2).

Introduction of the whole *ada* gene into pUC9 resulted in the poor induction of the Ada protein that was under the control of *lacOP*, as mentioned above. We have previously developed a high-level Ada protein expression plasmid, called pSM41, by deletion of the regulatory sequence of the *ada* gene (38). To overproduce the mutant proteins, the 0.95-kbp *MluI-XmaI* (7) fragments containing the mutant sites were introduced into the *MluI-XmaI* large fragment of pSM41. The resultant plasmids were termed pKT42 (His-321) and pKT43 (His-321 Cys-322), respectively (Fig. 2).

Induction of MGMT in *E. coli* harboring mutant *ada*-containing recombinant plasmids. Table 1 shows the MGMT activity in GW5352 harboring wild-type or mutant *ada* plasmid with and without adaptation treatment. MGMT activity was significantly induced by MNNG treatment of GW5352 harboring the wild-type, *ada*-containing plasmid pSM31. In GW5352 harboring pSM32 (His-321) (mutant I) or pSM33 (His-321 Cys-322) (mutant II), the levels of MGMT of both treated and untreated cells were the same as those in GW5352 containing pUC9.

Table 1 also shows the levels of MGMT activity in KT100 harboring plasmids for high-level expression of Ada proteins. The parental high-level expression plasmid (pSM41) containing the wild-type *ada* gene from which the promoter region was deleted can produce the Ada protein by induction with IPTG but not with MNNG (38). In KT100 cells containing either mutant plasmid pKT42 (His-321) or pKT43 (His-321 Cys-322), the levels of the activity were the same as those in host cells harboring pUC9.

Methylphosphotriester-DNA methyltransferase activity of mutant proteins. Based on results of amino acid sequence analysis (34) and the site-directed mutagenesis approach (39), it has been shown that Cys-69 in the N-terminal fragment of the Ada protein is the methyl acceptor site for methyl phosphotriesters in alkylated DNA. In our construction of site-directed mutants, we did not introduce any amino acid substitutions in the N-terminal region. To test whether these two *ada* mutant proteins had phosphotriester methyltransferase (PMT) activity, we measured the total methyltransferase activity by using [^3H]methylnitrosourea-treated calf thymus DNA substrate, which contained both *O*⁶-methylguanine and methylphosphotriesters.

The transfer of methyl groups from methylated DNA to protein was observed in extracts of GW5352 harboring pSM33 (His-321 Cys-322) after adaptation treatment (Table 1). The level of induced activity was about 4% of that observed with the wild-type *ada* plasmid. However, for the mutant I plasmid (His-321), no detectable methyltransferase activity could be induced by MNNG treatment. As ex-

FIG. 2. Construction of plasmids. pSM31 (4 kbp) contained the wild-type *ada* gene in pUC9. pSM32 (His-321) and pSM33 (His-321 Cys-322) contained the mutant I and mutant II *ada* genes, respectively. The 85-bp *ada* promoter region (hatched area) was deleted from pSM31 to produce pSM41. A 950-bp *Mlu*I (M)-*Xma*I (Xm) fragment containing the mutation sites for mutants I and II were recombined into the *Mlu*I-*Xma*I large fragment of pSM41 to produce pKT42 and pKT43, respectively. ▽, wild-type acceptor site; ▼, mutant I site; arrowheads, mutant II site.

pected, amino acid analysis of the MNNG-induced mutant II protein following its reaction with [*methyl*-³H]DNA revealed a single radioactive peak corresponding to authentic S-methylcysteine (data not shown).

On the other hand, when the *lac* promoter-driven expression system was used, methyltransferase activity was induced with both mutant plasmids pKT42 (His-321) and pKT43 (His-321 Cys-322). Thus, both mutant Ada proteins were capable of accepting methyl groups from DNA phosphotriesters at Cys-69, despite the fact that there was no MNNG-induced adaptive response in the mutant I *ada* gene that was under the control of the endogenous promoter.

Transcription activation by mutant Ada proteins in vivo. To investigate the effects of mutant Ada proteins on the activation of the *ada* promoter in vivo, we used an *ada*⁺-*lacZ*⁺ chromosomal fusion *E. coli* GW5354, carrying a single copy of the fused gene (18; D. E. Shevell, P. K. LeMotte, and G. C. Walker, J. Cell. Biochem. 12A[Suppl.]:325, 1988), and measured *ada* gene transcription-promoting activities of mutant I and II proteins by determining the expression of β-galactosidase activity. A considerable increase of enzymatic activity was observed in GW5354 with pSM31 carrying the wild-type *ada* gene after MNNG treatment (Fig. 3). In contrast, in cells with pSM33 (His-321 Cys-322) carrying the mutant II *ada* gene, only a relatively small increase of enzymatic activity was observed following MNNG treatment; and in the case of cells with the mutant I plasmid pSM32 (His-321), no increase of enzymatic activity was detected even after 3 h of incubation with MNNG. These results support the conclusions that the mutant I protein does not activate the *ada* promoter and that the mutant II

TABLE 1. Methyltransferase activities in extracts of cells harboring wild-type and mutant *ada* plasmids

Strain (plasmid)	<i>ada</i> mutation	Treatment	Concn (pmol/mg of protein) of MGMT	Total concn (pmol/mg protein) of methyltransferase ^a
GW5352(pUC9)		-MNNG	<0.1	0.4
		+MNNG	0.3	0.9
GW5352(pSM31)	Wild type	-MNNG	38	79
		+MNNG	368	736
GW5352(pSM32)	His-321 (mutant I)	-MNNG	<0.1	0.4
		+MNNG	0.2	0.8
GW5352(pSM33)	His-321 Cys-322 (mutant II)	-MNNG	<0.1	8.2
		+MNNG	0.4	26.8
KT100(pUC9)		-IPTG	<0.1	1.0
		+IPTG	0.2	2.0
KT100(pSM41)	Wild type	-IPTG	34	56
		+IPTG	580	607
KT100(pKT42)	His-321 (mutant I)	-IPTG	<0.1	7.3
		+IPTG	<0.1	40.4
KT100(pKT43)	His-321 Cys-322 (mutant II)	-IPTG	<0.1	5.0
		+IPTG	<0.1	34.9

^a The total methyltransferase activity should be about 40% higher than that of MGMT in the wild-type Ada protein. The lack of such exact correlation may have been because of experimental errors and the presence of inhibitors in crude extracts.

FIG. 3. Induction of β -galactosidase activity in strains containing an *ada-lacZ*⁺ chromosomal fusion. To induce β -galactosidase under *ada* control, MNNG (2 μ g/ml) was added at time zero. (A) GW5352(pSM31). (B) GW5352(pUC9). (C) GW5352(pSM32 [His-321]). (D) GW5352(pSM33 [His-321 Cys-322]). Solid and dotted lines represent cultures with and without MNNG, respectively. OD₆₀₀, Optical density at 600 nm.

protein is less effective than the wild-type protein as a transcriptional activator.

Western blot analysis of Ada proteins. In order to confirm the synthesis of mutant proteins under the control of either *lac* or endogenous promoter and the sizes of these proteins, we tested extracts of *E. coli* carrying various plasmids for Ada antigens in Western blots. By using antibody against the denatured Ada protein, we detected bands at a position of 39 kDa in extracts of MNNG-treated cells harboring pSM31 and IPTG-treated cells carrying pSM41 and pKT42 (His-321) (Fig. 4). Several other minor bands with smaller sizes (20 to 36 kDa) were also present in pSM31- and pKT42 (His-321)-containing extracts but not in pSM41-containing extracts. The strongest among these bands corresponded to about 36 kDa. No antibody-positive bands were detected in cell extracts harboring pSM32, pSM33, pKT43, and pUC9. Essentially identical results were obtained with antibody raised against the 19-kDa C-terminal fragment of Ada protein (data not shown).

Gel filtration of Ada proteins. There was an apparent discrepancy in that for both pSM33 and pKT43 (His-321 Cys-322) plasmids, no antigenic signal for the mutant II protein was detectable in Western blots of *E. coli* extracts, even though these extracts had significant levels of PMT activity. In order to resolve this issue, we investigated the

size distribution of wild-type and mutant proteins possessing PMT activity by gel filtration under non-denaturing conditions. The PMT activity in partially purified extracts of *E. coli* harboring the wild-type plasmid pSM41 was eluted from the column as a single major peak with an apparent molecular weight of about 35,000 (Fig. 5). In contrast, the activity from the mutant I plasmid pKT42 (His-321) was eluted over a wide range of molecular weights (15,000 to 66,000), indicating that both aggregated and fragmented proteins were present. The activity from the mutant II plasmid pKT43 (His-321 Cys-322) was fractionated into at least three major peaks (molecular weights, 24,000 to 30,000), with no evidence of a 39-kDa intact protein. Thus, rapid degradation of the mutant II protein may result in non-cross-reacting fragments which still retain PMT activity.

Analysis of transcript. The absence of PMT activity and antigenic signals for the mutant I protein in extracts of MNNG-treated *E. coli* carrying pSM32 (His-321) suggested that the protein represses its own promoter instead of activating it. We therefore assayed the transcriptional activator functions of mutant plasmids by comparing the steady-state levels of *ada* transcripts after adaptation treatment by RNA-DNA dot blot hybridization. The level of transcript from pSM32 (His-321) was about fourfold less than that from pSM31 (wild type) (Fig. 6). The amount of transcript from

FIG. 4 Western blot analysis of wild-type and mutant Ada proteins. Lane 1, GW5352(pUC9), control; lane 2, GW5352(pSM31); lane 3, GW5352(pSM32 [His-321]); lane 4, GW5352(pSM33 [His-321 Cys-322]); lane 5, KT100(pSM41); lane 6, KT100(pKT42 [His-321]); lane 7, KT100(pKT43 [His-321 Cys-322]). The positions of the protein markers are indicated by their molecular sizes. KT100 strains harboring plasmids were induced with IPTG, and GW5352 strains were induced with MNNG.

pSM33 (His-321 Cys-322) was somewhat less than that from pSM31. We could not detect any difference in plasmid copy number among the various strains of *E. coli* that we used (data not shown).

DISCUSSION

Three distinct activities of the *E. coli* Ada protein, namely, its methyltransferase reactions with (i) O^6 -methylguanine (and O^4 -methylthymine) and (ii) methyl phosphotriesters in DNA and (iii) its transcriptional activation of the *ada* gene, were investigated by site-directed mutagenesis of the cloned *ada* gene. Our initial experiments were designed to determine whether Cys-321, the alkyl acceptor from O^6 -alkylguanine in DNA, can be effectively replaced by histidine, which is also nucleophilic, and whether it can undergo methylation of its ring nitrogens in certain posttranslational reactions (14). Demple et al. (7) have noted that the Pro-320 Cys-321 His-322 sequence of the Ada protein is also present in thymidylate synthetases and is responsible for a specific methyltransfer reaction (8). We therefore reasoned that His-322, while it is not the ultimate methyl acceptor, may have a role in the methyltransferase reaction.

Experiments with mutant *ada* genes I (His-321 His-322) and II (His-321 Cys-322) under *ada* regulation showed no O^6 -methylguanine-specific methyltransferase activity in extracts of *E. coli* harboring the mutant *ada*-containing plasmids. These results demonstrate that histidine cannot replace Cys-321 as the methyl acceptor for O^6 -methylguanine and that the exact position of cysteine in the acceptor site is critical. More surprisingly, no PMT activity above that of the control could be detected in the extract of *E. coli* carrying the mutant I plasmid with or without adaptation treatment. In the case of mutant II, the PMT activity was present, although at a lower level than that of the wild type, and this activity was inducible by MNNG. Thus, as expected, the PMT activity and transcription activator function were conjoined. However, the PMT activity by itself may not be

FIG. 5. Gel filtration profiles of wild-type and mutant Ada proteins. The details are described in the text. Symbols: Dashed line, wild-type protein (from pSM41); solid line, mutant I protein from pKT42 (His-321); dotted line, mutant II protein from pKT43 (His-321 Cys-322). The eluting positions of protein markers, namely, bovine serum albumin (BSA) (66 kDa), ovalbumin (45 kDa), and lysozyme (14 kDa), are indicated.

FIG. 6. Dot blot analysis of transcripts. RNA was prepared from GW5352-harboring plasmids with MNNG pretreatment. Blots: 1, GW5352(pUC9); 2, GW5352(pSM33 [His-321 Cys-322]); 3, GW5352(pSM32 [His-321]); 4, GW5352(pSM31). The dots contained 2.0, 1.0, 0.5, 0.25, 0.125, and 0.0625 μ g of total cellular RNA from left to right in the rows, respectively.

sufficient for an adaptive response because certain *E. coli* *ada* mutants (15) are noninducible by MNNG (25) and yet have normal PMT levels (5). It is interesting in this context that we consistently observed a small increase in methyltransferase activity in the GW5352 control after MNNG treatment, even though the basal level in this bacterium was very low (Table 1). Thus, the transposon-insertion mutant behaves differently from the original *ada* mutants.

We also measured indirectly the transcription activation of the wild-type *ada* gene by the mutant proteins by using the *ada'*-*lacZ*⁺ fusion system (18; Shevell et al., J. Cell. Biochem., 1988). Again, mutant I was inactive while mutant II was less active than the wild-type Ada protein. These results are consistent with the methyltransferase activity data.

The complete lack of PMT and transcription activation activities in the case of mutant I plasmid-containing cells was intriguing. Based on the construction of the *ada* plasmids, we expected that the full-length mutant protein would be produced and that it would retain the PMT activity which resides in the N-terminal domain. To our surprise, we did not detect any specific protein bands in Western blots of extracts of *E. coli* with pSM32 (His-321) and pSM33 (His-321 Cys-322) using polyclonal antibodies raised against either the intact wild-type Ada protein or the 19-kDa MGMT fragment. We also carried out the rapid lysis of plasmid-bearing cells that were treated with MNNG by boiling them in the buffer described by Laemmli (17) and analyzed the proteins by SDS-polyacrylamide gel electrophoresis. In contrast to the presence of a major 39-kDa protein band from *E. coli* bearing the wild-type plasmid, no such band was detected in extracts of cells carrying pSM32 and pSM33 (unpublished data). We considered the likely reasons for the absence of the mutant proteins to be inhibition of transcription from the *ada* promoter, rapid degradation of the mutant protein once it was synthesized, or both.

We explored both possibilities. This was done by directing the synthesis of the proteins under the *lac* promoter in the plasmids pKT42 (His-321) and pKT43 (His-321 Cys-322). It was evident from the PMT activity of extracts that both mutant I and II proteins were produced. The Western blot confirmed the presence of a 39-kDa band for mutant I, although we never observed any bands for mutant II.

The fact that a 39-kDa band of the mutant I protein was produced in *E. coli* under the control of the *lac* promoter and not from the *ada* promoter suggests that the mutant protein or its fragments may act as inhibitors of transcription of its own gene. Such inhibitory effects have already been observed in some terminally deleted mutant Ada proteins (Shevell et al., J. Cell Biochem., 1988). The quantitation of transcripts of the wild-type and mutant *ada* genes at the

steady-state level showed that transcription of the mutant I gene in pSM32 (His-321) was inhibited severalfold relative to those of the wild-type and mutant II genes in pSM33 (His-321 Cys-322) after adaptation treatment. This was not caused by a gene dosage effect, because the copy number of the plasmids was similar in all strains. The lower level of transcription of the mutant I gene may be due to the fact that the mutant protein acts as a transcription inhibitor. Alternatively, it is possible that the unmethylated Ada protein acts as the activator for basal transcription and that this feedback loop is absent in the case of the mutant I protein because of its extreme lability.

The unusual sensitivity of both mutant Ada proteins to proteolysis is interesting in view of the fact that the point mutations in these proteins represent a minor change in the primary sequence. Results of early studies indicated that the wild-type Ada protein is easily degraded *in vitro* into several discrete fragments, with the 19-kDa C-terminal MGMT fragment being one of the relatively stable end products (26, 34, 40). Recently, a protease inducible by heat shock has been implicated in this breakdown (2; Caron and Grossman, J. Cell. Biochem., 1988). In our hands, the purified wild-type Ada protein is quite stable with respect to its integrity (3). Even in crude extracts containing the wild-type Ada protein, Western blots routinely showed a predominant 39-kDa band with a few minor antigenic bands, among which a strong 36-kDa band was often observed (Fig. 5). It is not clear why the 36-kDa band was not observed earlier by others (26, 34, 40).

In the case of the mutant proteins, no antigenic band was detected in cell extracts harboring pSM32 (His-321), pSM33 (His-321 Cys-322), or pKT43 (His-321 Cys-322). There are two possible reasons for this. First, the mutant proteins may be extremely sensitive to degradation. Second, because significant PMT activity was observed in pSM33- and pKT43 (His-321 Cys-322)-containing extracts, it appears that the N-terminal breakdown products of the mutant proteins were not recognized by the antibody preparation, even though it was raised against the denatured 39-kDa Ada protein.

It is not clear whether heat shock protease is responsible for the *in vivo* degradation of the mutant proteins. The presence of a 36-kDa band in extracts of cells containing either the wild-type or the mutant I gene (Fig. 5) suggests the involvement of the same protease for both wild-type and mutant proteins. The absence of the 39-kDa protein band after SDS-polyacrylamide gel electrophoresis of rapid lysates of both *lon*⁻ (42) and *lon*⁺ hosts harboring pSM32 (His-321) suggests that the *lon* protease is not responsible for degradation of the mutant proteins (unpublished data).

The gel filtration profiles revealed an interesting difference in the two mutant Ada proteins encoded by pKT42 (His-321) and pKT43 (His-321 Cys-322). While the PMT of mutant I was heterogeneously distributed, indicating both aggregation and degradation, the mutant II protein had only a few discrete peaks with PMT activity, all of which were smaller than the peaks of the wild-type protein. It is likely that both degradation of the nascent mutant polypeptides by endogenous proteases and aggregation of the resulting fragments occur because of their incorrect or incomplete folding caused by the substitution or transposition of cysteine with the bulkier histidine residue.

Two other groups of investigators have studied the effect of site-directed mutations of the *ada* gene of *E. coli*. Takano et al. (37) have shown that the Ala-321 mutant acts as a constitutive transcriptional activator of the gene. Shevell et al. (J. Cell. Biochem., 1988) have observed that different

deletions of the *ada* gene at the 3' end yield mutant proteins that either activate or repress the *ada* promoter function. Results of these studies, as well as our observations that the mutant II Ada protein activated *ada* transcription while the mutant I Ada protein did not, indicate that a minor perturbation in the C-terminal region of the protein can profoundly affect its transcription activation function.

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