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Active site and complete sequence of the suicidal methyltransferase that counters alkylation mutagenesis

(*Escherichia coli*/O^6-methylguanine/DNA repair/adaptive response/thymidylate synthase)

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ABSTRACT **The inducible resistance to alkylation mutagenesis and killing in *Escherichia coli* (the adaptive response) is controlled by the *ada* gene. The Ada protein acts both as a positive regulator of the response and as a DNA repair enzyme, correcting premutagenic O^6-alkylguanine in DNA by suicidal transfer of the alkyl group to one of its own cysteine residues. We have determined the DNA sequence of the cloned ada^+ gene and its regulatory region. The data reveal potential sites of *ada* autoregulation. Amino acid sequence determinations show that the active center for the O^6-methylguanine–DNA methyltransferase is located close to the polypeptide COOH terminus and has the unusual sequence -Pro-Cys-His-, preceded by a very hydrophobic region. These same structural features are present at the active site of thymidylate synthase, suggesting a common chemical mechanism for activation of the cysteine.**

Monofunctional alkylating agents such as *N*-methyl-*N*-nitrosourea (MNU), *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), and the metabolically activated form of dimethylnitrosamine are efficient mutagens and carcinogens. The major mutagenic lesion introduced into DNA by these agents is O^6-methylguanine (O^6MeGua), which mispairs with thymine residues during DNA replication (1, 2), resulting in G·C → A·T transition mutations (3). The same base change has been shown to occur during the *in vivo* neoplastic activation of a rat *ras* proto-oncogene in MNU-induced mammary carcinomas (4).

The main biochemical features of O^6MeGua repair are the same in bacteria (5) and mammalian cells (6, 7). The methyl group is transferred from an O^6MeGua to a cysteine residue located within the methyltransferase itself. The generation of an *S*-methylcysteine residue leads to inactivation of the enzyme both *in vivo* (8) and *in vitro* (9, 10). The ability of a cell to counteract alkylation mutagenesis is, therefore, limited by the number of available methyltransferase molecules. The *Escherichia coli* methyltransferase is induced on exposure of the cells to MNNG (11, 12). This antimutagenic "adaptive response" to alkylating agents contrasts with the mutagenic SOS response and is not under control of the *recA* and *lexA* genes (13, 14). Instead, the adaptive response is regulated by the *ada* gene, located at 47 min on the standard genetic map of *E. coli* (15–17). The O^6MeGua–DNA methyltransferase is a product of the cloned *ada* gene itself (18). A model for the adaptive response to alkylating agents is shown in Fig 1. Exposure of cells to alkylating agents generates an inducing signal, probably a modified form of the methyltransferase. In response to this signal, the *ada* gene is increasingly expressed. The induced gene product repairs O^6MeGua, the minor lesion O^4-methylthymine (19), and methyl phosphotriesters (ref 20; unpublished data) by direct methyl group transfer. The Ada protein is very susceptible *in vitro* to specific cleavage by a cellular protease (18). Consequently, the methyltransferase is usually isolated as an active fragment one-half of the size of the *ada* gene product (9, 18). The Ada protein also acts as a positive regulator of its own synthesis (17, 21) and of the expression of at least two genes located elsewhere on the chromosome: the *alkA* gene (12, 22), which encodes a DNA glycosylase that counteracts cell killing by removing several different lesions (19), and the *aidB* gene (23), which alters cellular resistance to alkylating agents (Fig. 1). In addition, *ada* forms a small operon together with *alkB* (17), a gene that counteracts lethal alkylation damage (by an unknown mechanism) independent of the adaptive response (24). We report here the complete primary structure of the ada^+ gene and its protein product. We have located the Ada methyltransferase active site and show that it bears a strong similarity to the active center of another enzyme with an active cysteine sulfhydryl group—i.e., thymidylate synthase (5,10-methylenetetrahydrofolate:dUMP *C*-methyltransferase, EC 2.1.1.45) (25).

MATERIALS AND METHODS

Sequencing Procedures. The ada^+ gene was isolated from the recombinant plasmid pCS68 (18) by *Hin*dIII cleavage and polyacrylamide gel electrophoresis. DNA sequencing was performed by the dideoxy method of Sanger *et al.* (26) on pEMBL subclones (27) using synthetic oligonucleotides as primers or by the chemical method of Maxam and Gilbert (28). O^6MeGua–DNA methyltransferase was purified to homogeneity as described (9). Tryptic and chymotryptic peptides of the fully reduced and iodoacetamide-alkylated protein were purified by reversed-phase HPLC, and their amino acid sequences were determined by microscale sequencing (29).

Isolation of Active Site Peptides. The homogeneous 19-kDa methyltransferase (100–200 μg) was incubated with DNA containing 0.15–0.3 nmol of ^{3}H-labeled O^6MeGua residues (40,000–80,000 cpm) in a 1-ml standard reaction mixture (10). After 5 min at 37°C, 1.5 mg of MNU-treated nonradioactive DNA (containing 5–10 nmol of O^6MeGua) was added, and the incubation was continued. The reaction was stopped after 10 min by addition of crystalline guanidine·HCl to a concentration of 5 M. The solution (2–3 ml) was incubated at 37°C for 20 min to denature the protein and then chromatographed at 20°C on a column (1 × 108 cm) of Sepharose CL-6B (Pharmacia) equilibrated with 6 M guanidine·HCl/50 mM Tris·HCl, pH 8.2/10 mM dithiothreitol/1 mM EDTA. The automethylated protein eluted at 1.8 times the void volume (9) and appropriate fractions were pooled. The material was concentrated by vacuum dialysis using Spectrapor 2 tubing

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Abbreviations: MNU, *N*-methyl-*N*-nitrosourea; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; O^6MeGua, O^6-methylguanine.

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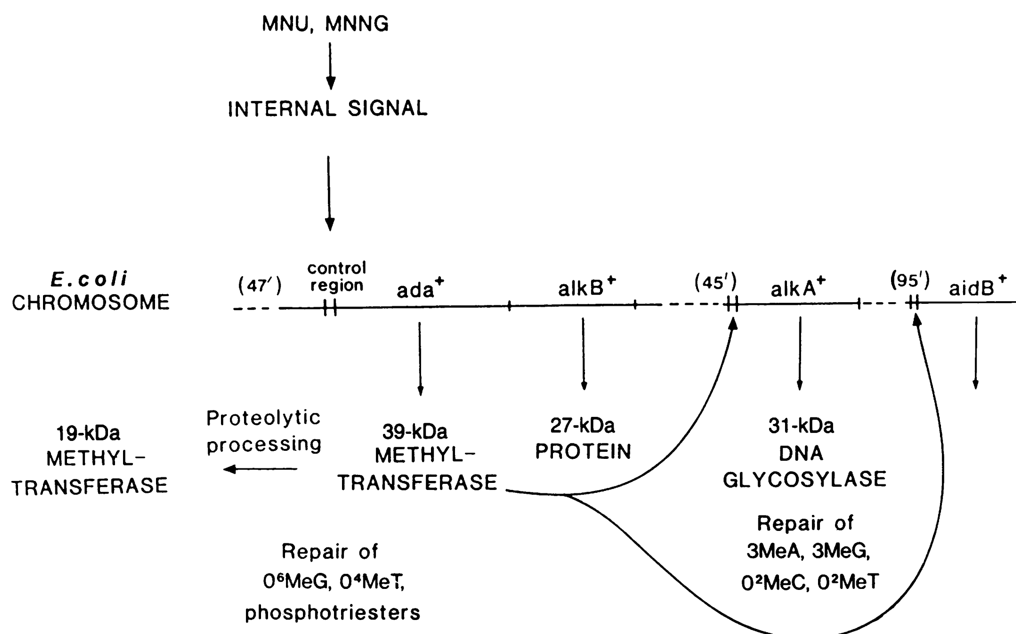

FIG. 1. Schematic model for the positive regulation of the adaptive response to alkylating agents in *E. coli* (see text).

(Spectrum Medical Industries, Los Angeles), and the remaining unmodified cysteine residues were blocked by addition of twice-recrystallized iodoacetamide to 20 mM. After 3 hr at 0°C, the solution was dialyzed extensively against 0.1% trifluoroacetic acid (which caused loss of up to one-half of the radioactive material) and dried under vacuum at room temperature. The resulting residue was redissolved in 100 μl of 0.1% trifluoroacetic acid and stored at −20°C. For protease digestion, 30 μl of the methylated protein was diluted to 360 μl containing 1 M urea and 0.1 M NH_4HCO_3 and incubated at 37°C for 5 min before addition of 1 mg of chymotrypsin A_4 (Boehringer Mannheim) per ml in 1 mM HCl to 1–2% of the methyltransferase concentration by weight. The reaction mixture was incubated at 37°C for 150 min; then a second equal aliquot of chymotrypsin was added and the incubation was continued for 90 min. The resulting peptide digest was separated by HPLC (29) on a Dupont Zorbax C_8 reversed-phase column (0.45 × 25 cm) using a linear gradient of 0–60% acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 1 ml·min^{-1}. The effluent was monitored for absorbance at 206 nm and 280 nm, and 0.5-ml fractions were collected into polypropylene tubes.

RESULTS

Sequence of the *ada* Gene. The ada^+ gene with its inducible promoter was cloned by inserting a *Hin*dIII/*Sma* I restriction fragment (1320 base pairs) of *E. coli* B DNA into the

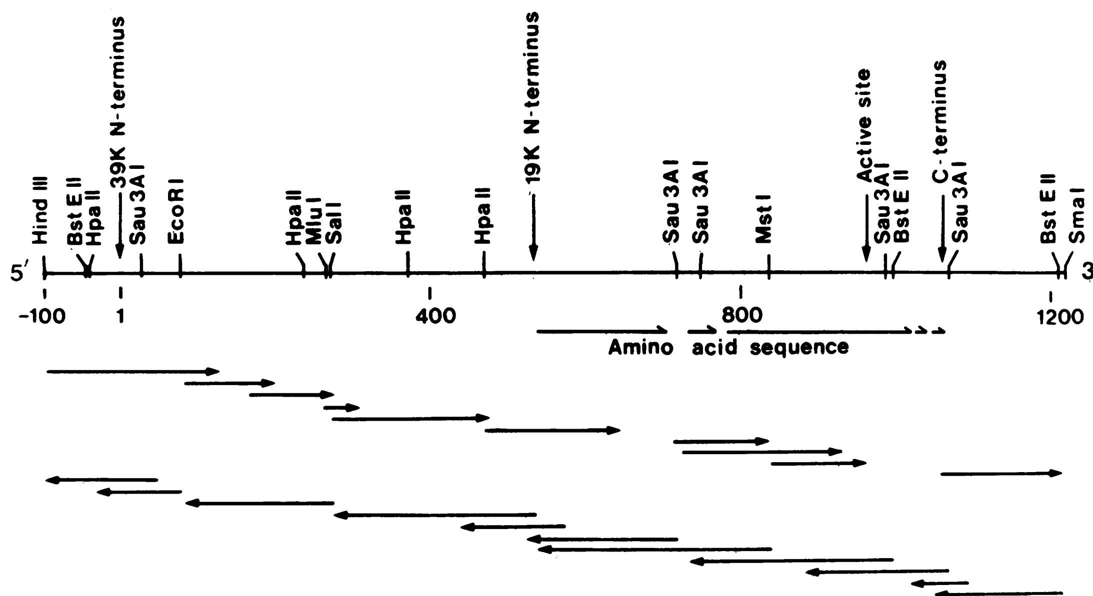

FIG. 2. DNA and protein sequencing strategy. A cloned *Hin*dIII/*Sma* I restriction enzyme fragment encompassing the ada^+ gene of *E. coli* B is shown. The horizontal arrows show the direction of DNA sequence analysis. The interrupted line indicates the partial amino acid sequence for the homogeneous 19-kDa domain (9) obtained by peptide sequencing that was carried out prior to DNA sequence analysis. The data obtained in this way account for 85–90% of the total 19-kDa protein sequence, but the final ordering of the non-overlapping peptides was carried out by comparison with the DNA sequence. Over 99% of the DNA sequence has been determined either from both DNA strands or from one DNA strand and the verifying amino acid sequence data.

pAT153 multicopy plasmid (17, 18) and its sequence was determined. The experimental strategy is shown in Fig. 2. The origin of *ada* transcription is located between the *Hin*dIII and *Eco*RI cleavage sites (left side of Fig. 2), as indicated by subcloning experiments and by the molecular weights of several protein fragments derived from *ada* genes with mapped Tn*1000* inserts (ref. 17 and unpublished data). A single open reading frame of >1 kilobase, which is of sufficient length to encode the entire Ada protein, follows this region. The amino acid sequence of the 19-kDa domain of O^6MeGua–DNA methyltransferase, determined along the protein, agrees with the distal half of the polypeptide predicted from the DNA sequence (Fig. 3). These results define the open reading frame as the *ada* structural gene. There is only one translation initiation codon at the 5′ end of the open reading frame, an ATG at position 1–3, which is preceded by an identifiable Shine–Dalgarno sequence (30) for a ribosome binding site. This has recently been verified independently as the start of the structural *ada* gene by NH_2-terminal amino acid sequence analysis of the Ada protein (Y. Nakabeppu and M. Sekiguchi, personal communication). The first translational stop codon encountered at the 3′ end (TAA at nucleotides 1063–1065) is preceded by a predicted heptapeptide sequence identical with that determined for a tryptic peptide of the 19-kDa O^6MeGua–DNA methyltransferase. The TAA termination codon overlaps an ATG codon that may be the translation start of the *alkB* gene (17, 24). A downstream sequence corresponding to a transcriptional stop signal is not apparent and was not expected because the *ada* gene and the distal *alkB* gene apparently form a small operon (17).

The Promoter Region. The presumed sequence for a ribosome binding site at residues −10 to −5 in Fig. 3. is preceded by a noncoding region with translational termination signals in all reading frames. Two possible RNA polymerase binding sites, each with 4 of 6 nucleotides agreeing with the canonical regions "−35" and "−10" from a transcription initiation site (31), are present close to the Shine–Dalgarno sequence, as shown in Fig. 3. If these assignments are correct, synthesis of the *ada* mRNA would start at a purine nucleotide only 1 or 2 residues removed from the ribosome binding site. The distance between the two tentative RNA polymerase binding segments is just 15 residues, probably allowing only weak transcription (32) in the absence of a positive effector. The sequenced *E. coli* B *ada* gene contains the regulatory region that allows induction of the adaptive response to alkylating agents (17). Immediately upstream of the apparent promoter

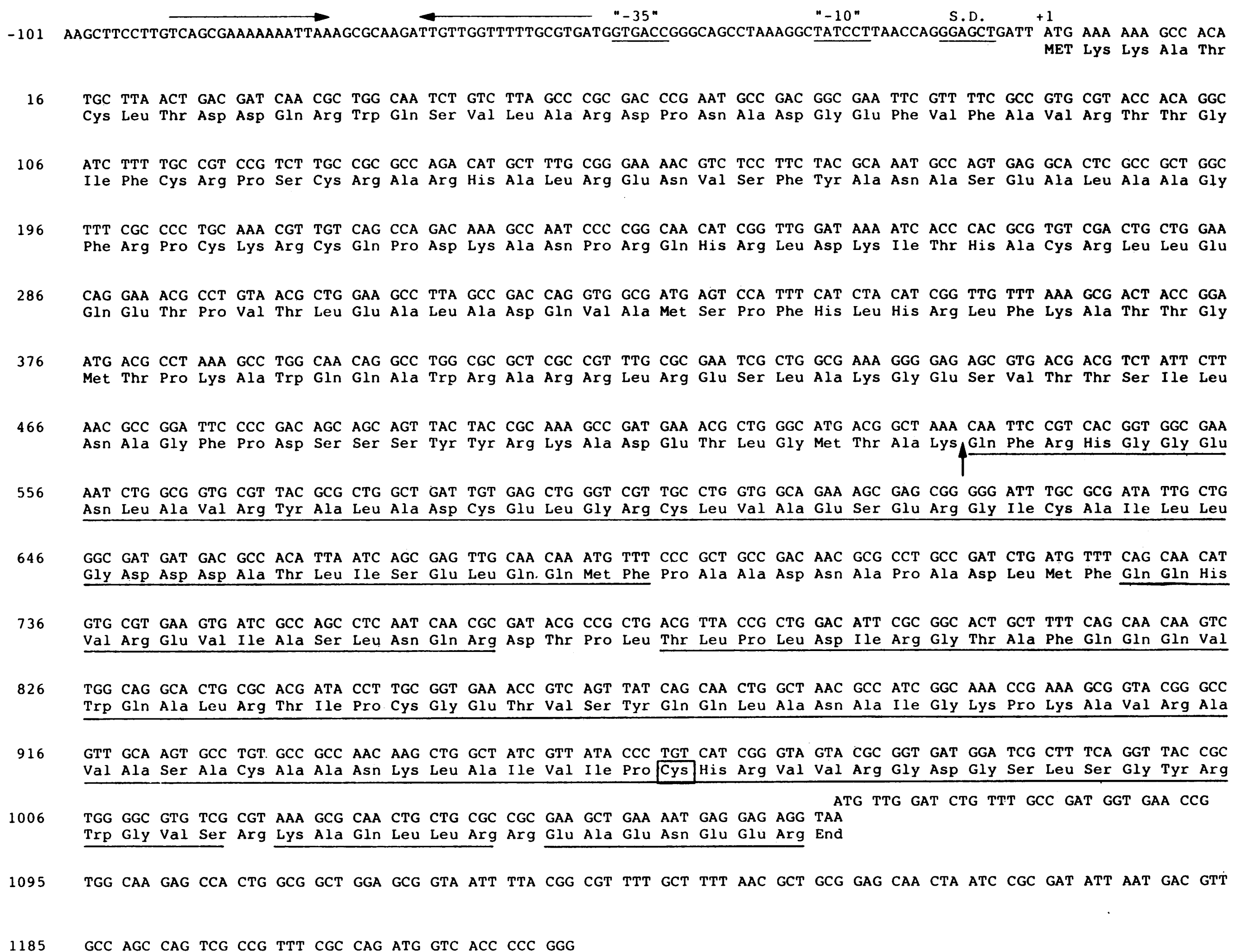

FIG. 3. Sequence of the *ada* gene. The suggested RNA polymerase and ribosome binding sites are underlined and designated by conventional notation. Horizontal arrows indicate a stretch of dyad symmetry in the promoter region. The predicted protein sequence for the 354-residue-long gene product is also shown. The NH_2-terminal and chymotryptic and tryptic peptide sequences of the active 176-residue domain are underlined. The longest contiguous polypeptide sequence (encoded by nucleotide residues 781–1017) was determined from eight overlapping chymotryptic and tryptic peptides. The proteolytic processing site (vertical arrow) and the active site cysteine (box) are indicated. The nucleotide residue 1065 is shown twice.

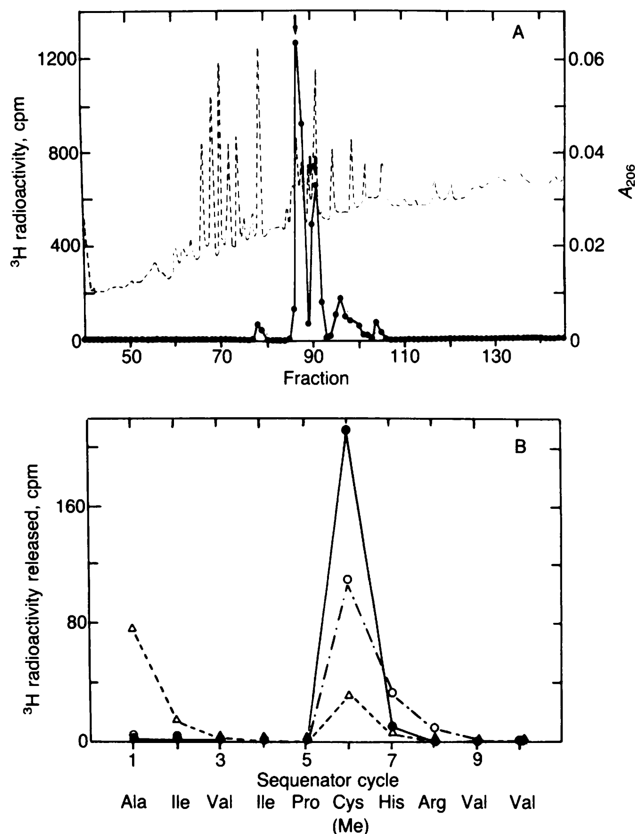

FIG. 4. Active site sequence of *E. coli* O^6MeGua–DNA methyltransferase peptides from [^{3}H]methyl-labeled transferase. (*A*) HPLC of chymotryptic peptides from the active 19-kDa domain. Twenty micrograms of protein containing 5100 cpm was digested with chymotrypsin. Amino acid analysis was performed on an aliquot of each radioactive fraction (indicated by arrows) as well as on major unlabeled peptides. The dotted line indicates A_{206}. (*B*) Location of *S*-[^{3}H]methylcysteine. Radioactively labeled peptides were subjected to microscale sequencing by automated Edman degradation (29), and the resulting phenylthiohydantoin derivatives were quantitated by HPLC and scintillation spectroscopy. The peak fraction of the second of the two labeled peptides, (fraction 91, ●) contained a single major sequence, as shown; similar results were obtained with the adjacent fraction 90 (○), although the latter also contained a contaminating peptide sequence. The first of the two labeled peptides, fraction 87 (△), had the same features as the peptide in fraction 91, with an NH_2-terminal alanine residue and release of the radioactive material in cycle 6.

is a region of dyad symmetry (residues −47 to −90). Close to the center of symmetry is an octanucleotide sequence, 5′ A-A-A-G-C-G-C-A 3′ that is also present in the promoter region of the *alkA* gene, adjacent to the putative RNA polymerase −10 binding site (22). Since the Ada protein positively controls both its own synthesis and that of the AlkA protein, the region including this octanucleotide sequence seems an attractive candidate for a protein recognition site.

The promoter region of the *ada* gene has also been sequenced by Le Motte and Walker (42) and by Y. Nakabeppu and M. Sekiguchi (personal communication), who employed *E coli* K-12. The DNA sequences (residues −100 to +150) are identical. The DNA cytosine at position −13 would occur as a 5-methylcytosine residue in strain K-12 while remaining unmethylated in strain B (33), but this difference does not appear to affect the inducibility of the Ada protein (our unpublished data).

Site of Proteolytic Cleavage of the Ada Protein. The DNA sequence predicts that the Ada polypeptide comprises 354 amino acids and has a molecular weight of 39,291. The protein is rapidly cleaved after extraction (18) to generate a specific 176-residue active fragment of molecular weight 19,289. The proteolytic cleavage site is

(NH_2)-Thr-Ala-Lys↓Gln-Phe-Arg-(COOH).

This is the only Lys-Gln bond in the Ada protein. The sequence has no resemblance to the -Ala-Gly- site cleaved in the LexA or λ repressors in the presence of activated RecA protein (21). The Ada protein is not cleaved by the Lon protease or by a serine protease (18). NH_2-terminal amino acid sequence analysis of the isolated 19-kDa fragment has indicated that there is no (<1%) simultaneous cleavage at the arginine that is only 3 residues removed from the susceptible lysine. It is of interest that specific cleavage of a Lys-Gln bond also occurs during the conversion of preprogastrin to gastrin (34) in mammalian cells.

Site of Self-Alkylation. The O^6MeGua–DNA methyltransferase is the only known example in which a protein cysteine residue is used by an enzyme as a final methyl group acceptor. The 19-kDa methyltransferase contains 6 cysteine residues, only 1 of which acts as a methyl acceptor (9). To identify the active residue, the 19-kDa protein was incubated with alkylated DNA that contained [^{3}H]methyl-labeled O^6MeGua residues. After transfer of a [^{3}H]methyl group to the protein, sequencing from the NH_2 terminus ruled out the three cysteines 18, 23, and 33 residues from this end as the methyl acceptor, since none of them was associated with ^{3}H radioactivity. To obtain [^{3}H]methyl-labeled peptides from other regions, the denatured polypeptide was digested with chymotrypsin. After HPLC separation, the peptides were identified by their absorption at 206 nm. More than 90% of ^{3}H radioactive material was recovered as a split peak in the chromatogram, representing two major labeled chymotryptic peptides (Fig. 4*A*). Both peptides had the same NH_2-terminal amino acid sequence (consistent with a chymotryptic cleavage at the leucine encoded by DNA residues 943–945), with a *S*-[^{3}H]methylcysteine residue being released in the expected amount of phenylthiohydantoin derivative during the sixth sequencing cycle (Fig. 4*B*). Separate peptides containing the two remaining internal cysteines of the 19-kDa protein (encoded by DNA residues 850–852 and 928–930, respectively) were also located and sequenced, and neither contained a [^{3}H]methyl group derived from alkylated DNA. These results show that the cysteine encoded by nucleotides 961–963 in the *ada* gene (Fig. 3) is the sole O^6MeGua methyl acceptor in the protein. In separate experiments, we have also found that ethyl groups from the guanine O^6 position are transferred to this same site (unpublished data), despite the rate of repair of ethylated DNA that is slower by a factor of 100 (35). The two long [^{3}H]methyl-labeled chymotryptic peptides were not sequenced completely and may have differed at their COOH termini due to incomplete proteolysis. Alternatively, the chromatographic heterogeneity observed might have been due to partial oxidation of the *S*-methylcysteine residue (5).

Active Site Homology with Thymidylate Synthase. Comparison of the Ada protein sequence with about 3000 protein sequences in two 1984 data banks (36) by the procedure of Wilbur and Lipman (37) failed to reveal any closely related species. Such a search using only the active center tripeptide of the O^6MeGua methyltransferase proved more interesting. Maley *et al.* (25) have shown that the amino acids at the active site of thymidylate synthases from prokaryotes and eukaryotes are highly conserved. A -Pro-Cys-His- sequence is centrally located in this region, with an array of hydrophobic short-chain amino acids present on the immediate NH_2-terminal side. These features also occur in the O^6MeGua–DNA methyltransferase (Fig. 5). In the reaction catalyzed by thymidylate synthase, an intermediate ternary complex is apparently formed, involving covalent binding between the

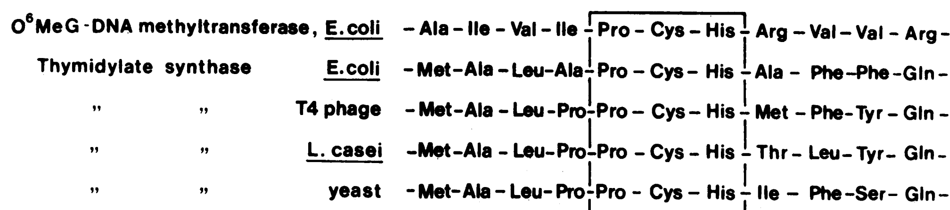

FIG. 5. Active site region of *E. coli* O^6MeGua–DNA methyltransferase compared with those of thymidylate synthases (ref. 25 and F. Maley, personal communication) from several sources. *L. casei, Lactobacillus casei.*

cysteine at the active site and the 6-carbon position of dUMP (25).

DISCUSSION

The Ada protein acts as a positive regulator of its own synthesis (17, 21) as well as that of other proteins (22, 23). There is no genetic evidence for a separate repressor protein acting on *ada* (15–17), and the tentative promoter structure of the *ada* gene predicts weak transcription in the absence of protein effectors. It is thus likely that the Ada protein (or a derivative of it) binds in the promoter region of *ada* to augment transcription by direct interaction with RNA polymerase (38).

The similarity between the active sites of thymidylate synthase and O^6MeGua–DNA methyltransferase probably reflects a common mechanism for obtaining a reactive cysteine within a protein. The similar sequences are likely products of convergent evolution. The proline residue might be of conformational importance for cysteine activation in the polypeptide by causing the cysteine to protrude or by bringing the nearby hydrophobic residues into its proximity. The histidine residue could act as a proton acceptor in the generation of a reactive thiolate anion, as seen in papain (39). The appropriately positioned nucleophile would then attack the substrate carbon atom, with direct transfer of a methyl group to the protein in the case of the O^6MeGua–DNA methyltransferase. One might predict that a potentially reactive -Pro-Cys-His- sequence would be discriminated against as part of a structural region of a protein. From a survey of ≈3000 protein sequences this peptide appeared only 7 times, compared to an expected 14 times. (The reversed sequence, -His-Cys-Pro-, occurs 16 times in the data bank.) Moreover, in some cases (such as a high-sulfur fraction of keratin) the cysteine within the -Pro-Cys-His- tripeptide would be expected to occur as a nonreactive half-cystine residue. The -Pro-Cys-His- sequence also occurs in the *E. coli sn*-glycerol-3-phosphate acyltransferase (EC 2.3.1.15) (40) and the bacteriophage T7 gene 1.7 protein, which is an early but nonessential viral function (41).

The suicidal self-methylation of the O^6MeGua–DNA methyltransferase results in threshold values for cellular damage by small amounts of alkylating agents, with mutations accumulating only above a certain level of drug exposure (8). The delineation of the primary structure around the active cysteine allows more detailed mechanistic studies on this unusual methyl transfer reaction. In addition, the general structure of the regulatory region of the *ada* gene has been defined, and its particular elements can now be located by mutagenesis and protein binding experiments.

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