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REGULATION AND EXPRESSION OF THE ADAPTIVE RESPONSE TO ALKYLATING AGENTS

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PERSPECTIVES AND SUMMARY

Inducible DNA repair pathways enable cells to display increased resistance to the deleterious effects of chemical mutagens and radiation. Several such networks have been delineated recently in *Escherichia coli,* including the SOS response (1), the adaptive response to alkylating agents (2, 3), and inducible responses to oxygen radical damage in DNA (4–7). These various circuits are generally under positive regulatory control, but the biochemical strategies employed to generate specific protein activators differ between the pathways.

In the adaptive response to alkylating agents, bacteria acquire increased resistance to the mutagenic and cell-killing effects of a large group of chemical mutagens. Alkylating agents, of which methylating agents appear to be widespread environmental mutagens, act through covalent modification of the cellular genome to generate miscoding base derivatives and lesions that block DNA replication. All oxygens and nitrogens in DNA can be modified by methylating agents, except for the nitrogens forming a glycosyl bond with deoxyribose, oxygens in phosphodiester bonds, and the exocyclic amino groups, resulting in 14 different types of primary lesions. However, the most relevant adducts are O^6-methylguanine, which is a miscoding base, and 3-methyladenine, which is a cell-killing lesion. The main function of the adaptive response is to improve the repair of these two harmful base derivatives. To remove 3-methyladenine, cells employ the same strategy as for excision of anomalous bases such as uracil from DNA. The base-sugar bond is cleaved by a DNA glycosylase to release the altered base residue in free form and generate a repairable apurinic/apyrimidinic (AP) site (8). In contrast, O^6-methylguanine is corrected by direct reversal of damage, accomplished by transfer of the methyl group to a cysteine residue in the repair enzyme itself. The protein is not regenerated and undergoes suicide inactivation as a consequence of the DNA repair event. The strategies for repair of these two important DNA lesions have been conserved during evolution. Thus, similar repair functions for these damaged purine residues are present in mammalian cells, although the responsible enzymes appear to be constitutively rather than inducibly expressed in higher cells. The same situation seems to hold true for the key component of the bacterial SOS response, the RecA protein: similar proteins that promote DNA strand transfer have been found in mammalian cells, but there is no evidence for their inducibility (9–10a).

Methylating agents trigger the adaptive response in *E. coli* by generating an intracellular signal for its induction. This signal has been identified recently as one of the minor DNA methylation products, one of the two stereoisomers of a methyl phosphotriester (2). The regulatory Ada protein transfers this particular methyl group to one of its own cysteine residues in a self-methylation

reaction analogous to that employed for repair of O^6-methylguanine, and this posttranslational modification event converts the protein from a weak to a strong transcriptional activator. The methylated protein binds tightly to a specific DNA sequence in the promoter regions of genes induced in the response, and thereby facilitates the initiation of transcription. Models of transriptional activation by posttranslational modification of preexisting regulatory proteins have been proposed recently for several other systems (11–15).

THE ADAPTIVE RESPONSE IN VIVO

Simple alkylating agents, such as N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and N-methyl-N-nitrosourea, are toxic and mutagenic to cells and also carcinogenic in mammalian systems. It was, therefore, of great interest when an inducible DNA repair pathway that protects specifically against the damaging effects of these agents was discovered in *E. coli* (16). After a 30 min exposure to low levels of MNNG, bacteria showed resistance to the mutagenic and cell-killing effects of this agent when challenged with higher doses (17). The resistant phenotype was not due simply to detoxification of the damaging agent, but to an inducible DNA repair pathway requiring de novo protein synthesis (16). This response also provides protection against the mutagenicity of ethylation and propylation damage and the toxicity of even bulkier alkylation lesions (18, 19). As the size of the adducts is increased, however, other cellular repair systems play increasing roles in the removal of the adducts from DNA (19). The adaptive response is quite distinct from the SOS response, a DNA repair pathway induced by a wide variety of damaging agents including ultraviolet light, and regulated by the *recA* and *lexA* gcnc products (1); the adaptive response is induced only by alkylation damage, and is independent of the latter gene products (17, 20).

O^6-methylguanine is the major mutagenic lesion produced in DNA by simple methylating agents. This altered base directs the incorporation of either thymine or cytosine without blocking DNA replication, resulting in G·C to A·T transition mutations (21–23). NMR studies on double-stranded oligonucleotides have shown that O^6-methylguanine causes a small helical distortion and altered hydrogen bonding when opposite either cytosine or thymine (24, 25). This agrees with genetic experiments (26) that indicate the active removal by mismatch repair of both cytosine and thymine when opposite an O^6-methylguanine residue in newly replicated DNA. It is presumably a matter of chance which pyrimidine residue finally escapes this correction system.

An activity that specifically catalyzes the repair of O^6-methylguanine is present at a very low level in *E. coli* (27–29). This repair function is induced

as part of the adaptive response and accounts for the increased resistance to alkylation mutagenesis (30). The kinetics of disappearance of O^6-methylguanine residues in vivo revealed, surprisingly, that the repair function could only act once. The response was saturated on exposure of cells to high doses of methylating agent, and new protein synthesis was required before additional repair could take place (31). Nevertheless, the protection against low and moderate doses of alkylating agents by induction of the repair pathway is extremely efficient and reduces the mutation frequency several thousand–fold in comparison with nonadapted cells. It may be concluded that O^6-methylguanine must be repaired rapidly after cellular exposure to alkylating agents to prevent mutation fixation by DNA replication past these miscoding lesions (32).

Mutants defective in various components of the adaptive response have been isolated by exploiting their altered sensitivity to the mutagenicity and/or toxicity of alkylating agents. As a result, the response is now known to involve the increased expression of at least four genes: *ada, alkA, alkB,* and *aidB* (see Table 1). The *ada* mutants are sensitive both to the mutagenicity and toxicity of methylating agents (33). Mutations resulting in constitutive overexpression of the inducible activities also map at this locus (34, 35). The Ada protein is the key component of the adaptive response, because it has a dual function, serving both as a DNA repair enzyme to remove O^6-methylguanine and as the positive regulator of the response (36). The *alkA* mutants are sensitive to killing but not to mutagenesis by alkylating agents due to their inability to remove the toxic lesion 3-methyladenine from DNA (37, 38). *E. coli polA* mutants are also unable to adapt against alkylation

Table 1 Inducible genes of the adaptive response to alkylation damage

Inducible gene	Chromosomal location	Mutant phenotype	Gene product	Lesions repaired	Other properties
ada	47′	Sensitive to mutagenesis and killing	39-kd DNA methyltransferase	O^6 MeG O^4 MeT p(Me)	Positive regulator of adaptive response
alkB	47′	Sensitive to killing	24-kd protein	?	—
alkA	45′	Sensitive to killing	31-kd DNA glycosylase	3MeA 3MeG O^2 MeC O^2 MeT	—
aidB	95′	Resistant to killing	?	—	—

toxicity because of the participation of DNA polymerase I in excision-repair after the removal of 3-methyladenine lesions (39). The *alkB* mutants are sensitive to cell-killing by alkylating agents, but are proficient in repairing 3-methyladenine (40). The AlkB protein has been purified, but its physiological role remains unknown (41). The *aidB* mutant was isolated as an in vivo *lacZ* gene fusion inducible by alkylating agents, and has enhanced resistance to alkylation damage (42). The *ada* and *alkB* genes form a small operon, which maps at 47 min on the *E. coli* genetic map (35, 43). The *alkA* and *aidB* genes map at 45 and 95 min, respectively (37, 44). Our present knowledge of the roles of these gene products in DNA repair and regulation of the adaptive response will be elaborated in the following sections.

REPAIR OF O^6-METHYLGUANINE

Methyl Group Transfer

Biochemical studies have clarified the main molecular mechanisms of DNA repair involved in the adaptive response. Cell-free extracts from MNNG-treated *E. coli* were found to remove O^6-methylguanine from radioactively labeled alkylated DNA more efficiently than control cell extracts, and this provided a system for purification of the active factor (45). The O^6-methyl group is not liberated as free O^6-methylguanine or in an oligonucleotide, but is transferred directly from the alkylated DNA to a protein. The methyl group is accepted by a cysteine residue (46), and an unmodified guanine is simultaneously regenerated in DNA (47). Purification of the activity to apparent homogeneity showed that the methyltransferase and the acceptor are contained in the same protein (29). This is in contrast to signal transduction for chemotaxis of *E. coli,* in which a methyl receptor protein is covalently modified by a separate transferase using S-adenosylmethionine as methyl group donor (48). The O^6-methylguanine-DNA methyltransferase does not require Mg^{2+} or other cofactors, but overcomes a significant energy barrier because an O^6-methylguanine residue is a relatively stable entity at neutral pH. Thus, nonenzymatic demethylation only occurs in strong acid or alkali (49), and does not seem to be promoted at neutral pH by low-molecular-weight compounds such as free cysteine or glutathione.

The methyltransferase undergoes irreversible inactivation in its reaction with an O^6-methylguanine residue, because no mechanism appears to exist to demethylate the resulting S-methylcysteine moiety, which is chemically very stable. The initial part of this unusual reaction resembles that of an enzyme acting by covalent catalysis, but the methylated protein accumulates as a dead-end product instead of being regenerated (50). The lack of turnover of the activity is in agreement with the in vivo data, which showed that the repair

function is expended in the reaction (31). In consequence, the repair capacity is saturated when a threshold dose of alkylating agent forms a larger number of O^6-methylguanine residues in DNA than the number of available intracellular transferase molecules. This is the critical point at which cells start to exhibit greatly increased susceptibility to alkylation mutagenesis.

The direct reversal of an O^6-methylguanine residue by an active transferase is a rapid and error-free process, occurring in less than one second at 37°C (50). This reaction, in which an entire protein molecule is consumed in order to achieve the correction of a single mutagenic base, may have evolved to provide a particularly efficient defense against a common and highly mutagenic lesion (32, 51). Thus, *E. coli* apparently expends considerable amounts of energy on repairing these potentially mutagenic alterations. Similarly, correction of a replicative mismatched base-pair often requires excision and resynthesis of tracts of more than one thousand adjacent nucleotide residues (52, 53).

N-methylated purine residues in DNA such as N^6-methyladenine or 7-methylguanine are not recognized by the transferase. Moreover, the transferase is sensitive to DNA conformation and demethylates O^6-methylguanine residues in single-stranded DNA markedly more slowly than in double-stranded DNA (50). Single-stranded DNA regions close to replication forks may, therefore, be particularly susceptible to mutagenesis by simple alkylating agents, since an O^6-methylguanine residue generated within such a region would not be corrected efficiently. This may be one of the factors that contributes to the known property of MNNG to generate mutations in clusters at replication forks (54–56). In addition to O^6-methylguanine, the minor but potentially mutagenic alkylation lesion O^4-methylthymine is also a substrate for the *E. coli* transferase, although the demethylation reaction proceeds less efficiently than for O^6-methylguanine (57). Similarly, higher forms of O^6-alkylguanine (O^6-ethylguanine, O^6-hydroxyethylguanine) are repaired at relatively slow rates by the transferase and may be regarded as substrate analogues to O^6-methylguanine. An interesting substrate is O^6-chloroethylguanine, because this derivative is a long-lived reaction intermediate in the interstrand cross-linking of DNA by bifunctional chloroethylnitrosoureas. Consequently, the active transferase can reduce cell-killing by such agents by preventing the formation of lethal cross-links (58, 59).

Methyl Acceptor Sites

The major O^6-methylguanine-DNA methyltransferase of *E. coli* was found unexpectedly to be the product of the regulatory *ada* gene itself (36). Extracts of noninduced cells contain a very low level of such a transferase activity, which could be due to the small amount of Ada protein present in noninduced

cells (36) and/or a second enzyme. Recently, preliminary observations on the occurrence of low levels of a second transferase activity, an apparently noninducible 19–20-kd protein, have been obtained (62; D. Shevell, P. Le Motte, G. C. Walker, unpublished data). A dual cellular defense may exist against each of the two important alkylation lesions, 3-methyladenine (see below) and O^6-methylguanine, in that separate inducibly and constitutively expressed repair enzymes could occur in both cases.

Sequencing of the *ada* gene and a radioactive methylated peptide derived from the Ada protein after repair of an O^6-methylguanine residue allowed the localization of the cysteine acceptor in the protein sequence (60). This residue, Cys-321, is the closest of the 12 cysteines to the C-terminus of the 39-kd Ada protein (Figure 1). The same residue is also the apparent acceptor site for alkyl groups derived from O^6-ethylguanine and O^4-methylthymine. However, the intact Ada protein has the ability to abstract two, rather than one, methyl groups from DNA to internal cysteine residues. The second methyl group is not derived from O^6-methylguanine, but from an apparently innocuous lesion, a methylphosphotriester (57, 61, 62). The reaction of an alkylating agent with either of the two oxygen atoms not involved in the phosphodiester bond can generate a pair of isomers, in the R and S configuration. Only the S diastereoisomer is recognized and repaired by the Ada protein (57, 63, 64). The methyl group is transferred to a cysteine residue, Cys-69, in the N-terminal part of the Ada protein (65). The assignments of methyl acceptor sites in the Ada protein have been confirmed by site-specific mutagenesis (K. Takano, M. Sekiguchi, unpublished data). Substitution of residue Cys-321 with alanine abolishes the acceptor activity for O^6-methylguanine and O^4-methylthymine, whereas that for methylphosphotriesters is retained. Conversely, substitution of Cys-69 with alanine removes the phosphotriester repair function, but allows demethylation of the two DNA base residues.

A comparison of the two scavenger sites in the protein reveals considerable sequence homology in the immediate vicinity of the active cysteines: residues 68–71 are -Pro-Cys-Lys-Arg-, whereas residues 320–323 are -Pro-Cys-His-Arg-, in both cases preceded by a short array of hydrophobic amino acids (60, 65). A possible mechanistic model for methyl group abstraction is that the proline residue causes a bend in the protein chain with protrusion of the adjacent cysteine, whereas a basic residue on the other side of the cysteine may serve as proton acceptor in a charge transfer reaction to generate a reactive thiolate anion (60). It is of interest that thymidylate synthase from a variety of sources also contains a -Pro-Cys-His- sequence at the center of its active site, preceded by a number of hydrophobic residues (66). This arrangement may reflect a common strategy for generating a particularly reactive cysteine residue within a protein sequence.

Proteolytic Processing

The Ada amino acid sequence does not contain any extensive strongly basic or acidic regions, or long hydrophobic or repeated sequences. The protein is unusually susceptible to proteolytic enzymes, however, and is cleaved preferentially by several reagent proteases (trypsin, chymotrypsin, subtilisin, V8 protease) within a stretch of 10 amino acid residues at the center of the molecule. The distinct transferase activities for O^6-methylguanine and methylphosphotriesters are retained by the separate C-terminal and N-terminal fragments, respectively. These data strongly indicate that the Ada protein is composed of two functional domains of similar size united by a short hinge region (65). A schematic figure of the Ada protein with the locations of the cysteine acceptors and the hinge region is shown in Figure 1.

The central region of the Ada protein is also very susceptible to an endogenous protease of *E. coli,* and the protein is cleaved into its two domains in crude cell extracts (36, 67). For this reason, the O^6-methylguanine-DNA methyltransferase activity was initially isolated as the C-terminal domain of the Ada protein from cell extracts of a constitutive mutant strain (29, 60), and purification of intact Ada protein was only achieved after cloning of the *ada* gene into expression vectors (57, 67). The *E. coli* enzyme that cleaves the Ada protein is not the Lon protease or a number of other partly characterized proteases (68). Moreover, it is not susceptible to a variety of standard inhibitors of proteolytic enzymes. This endogenous protease activity cleaves the Ada protein at only two sites adjacent to Lys-178 and Lys-129. There is considerable amino acid sequence homology between these two regions of cleavage (68). Interestingly, a site in the UvrB protein susceptible to endogenous proteolytic processing is also surrounded by a similar sequence (68, 69). The primary structures at the sites of cleavage are shown in Figure 2. These sequences show some similarity to targets of processing of certain precursors of peptide hormones in higher

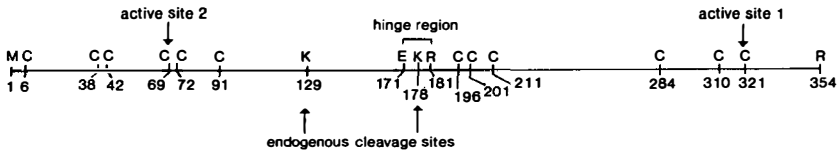

Figure 1 Diagram of the 39-kd Ada protein. The 12 cysteine residues and the amino acid residues at sites particularly sensitive to proteolytic cleavage are indicated. The positions of the two active-site cysteine residues and the central hinge region are also shown. The figure is from Sedgwick et al (65).

organisms, where chain scission may occur at monobasic residues preferentially preceded by proline (70). It seems likely that a single *E. coli* protease is responsible for the cleavage at the sites shown in Figure 2, because the proteolytic enzyme has been purified and shown to cut the homogeneous Ada and UvrB proteins into large fragments of the predicted sizes (L. Grossman, personal communication).

REPAIR OF 3-METHYLADENINE

E. coli induced for the adaptive response attains increased resistance to both the mutagenic and killing effects of alkylating agents. The induced activity, which renders cells resistant to the toxic effects, is 3-methyladenine-DNA glycosylase II (38, 71). *E. coli* contains two enzymes that liberate 3-methyladenine from alkylated DNA, designated 3-methyladenine-DNA glycosylases I and II (72–76). They are distinguishable by their molecular sizes and enzymatic properties. Glycosylase I (M_r = 21,100) releases only 3-methyladenine, and its activity is inhibited by the reaction product, free 3-methyladenine. On the other hand, glycosylase II (M_r = 31,400) liberates 3-methylguanine, O^2-methylthymine, O^2-methylcytosine, and (at a very low rate) 7-methylguanine in addition to 3-methyladenine, and the activity is not product-inhibited. Moreover, the regulation of the expression of the two

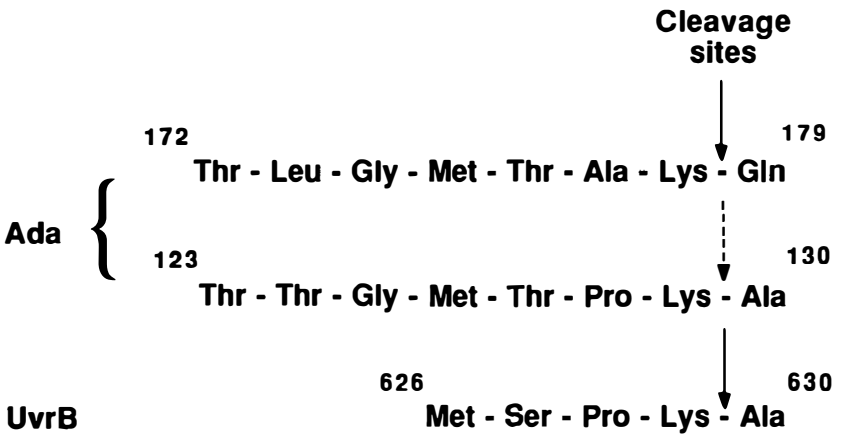

Figure 2 Cleavage sites in the Ada and UvrB proteins. Ada protein is cleaved between Lys-178 and Gln-179 by an endogenous *E. coli* protease yielding 20-kd and 19-kd fragments (60). Fragmentation generating a 24-kd fragment is proposed to occur between Lys-129 and Ala-130, which are preceded by an amino acid sequence similar to that of the known cleavage sites. Cleavage of UvrB protein by an endogenous activity at the site shown (68, 69) appears to be due to the same protease. The solid and dashed arrows indicate known and proposed cleavage sites, respectively. The figure is modified from Teo (68).

enzymes differs in response to alkylating agents. The activity of glycosylase II is induced to a 20-fold higher level in cells exposed to low concentrations of such agents, whereas the level of glycosylase I remains unchanged.

E. coli alkA mutants, which are unable to induce glycosylase II (37, 38, 77) are anomalously sensitive to the toxicity of methylating agents. They cannot adapt against the killing effects of the agents, but gain resistance to the mutagenic effects through the induction of O^6-methylguanine-DNA methyltransferase. The *alkA* gene has been cloned and its nucleotide sequence determined (78–82). The amino acid composition and amino-terminal sequence of purified 3-methyladenine-DNA glycosylase II verified that *alkA* is the structural gene for this enzyme (81).

3-Methyladenine-DNA glycosylase I is the product of the *tag* gene, which has been cloned and sequenced (78, 83–85). Mutants in *tag* are slightly sensitive to methylating agents. However, *tag alkA* double mutants are markedly more sensitive than the single mutants (80, 86), and are presumably totally unable to excise 3-methyladenine from their DNA. Overexpression of the cloned *tag* gene can suppress the increased sensitivity of *alkA* mutants to methylating agents (78). These results strongly suggest that both enzymes are responsible for repair of the toxic lesion 3-methyladenine. The biological effects of 3-methyladenine appear to be due to blocking of DNA replication (86). In agreement with results on other blocking lesions such as pyrimidine dimers and apurinic sites, there is evidence that 3-methyladenine may cause mutations in bacteria after induction of the SOS response, presumably due to an inducible error-prone read-through mechanism (87).

The *alkA* gene product can remove several different methylated bases from DNA. This broad substrate specificity was an unexpected finding for a DNA glycosylase, because most other enzymes of this class, such as uracil-DNA glycosylase, hypoxanthine-DNA glycosylase, and 3-methyladenine-DNA glycosylase I, seem to recognize only a single type of base lesion (8, 72, 73, 76). However, the ability of the AlkA protein to liberate a variety of minor alkylation lesions in addition to 3-methyladenine may not be of great biological significance, because the physiological effects of an *alkA* mutation can be almost completely suppressed by overexpression of the *tag* gene. There is little or no relevant homology between the amino acid sequences of the *tag* and *alkA* gene products (83, 84). Therefore, it seems likely that the two genes evolved from different origins and the mechanisms for substrate recognition by these enzymes may be different, although both enzymes are DNA-binding proteins that only cleave base-sugar bonds in double-stranded alkylated DNA. 3-Methyladenine DNA glycosylase II presumably recognizes a specific structural feature absent in native DNA, such as a methyl group protruding into the minor groove of the DNA double helix (76). In contrast, glycosylase I may show a more precise interaction with a 3-methyladenine residue, as reflected by its low K_M for this substrate and its product inhibition by free

3-methyladenine. A difference in the interactions of the two enzymes with DNA is also suggested by the observation that massive overproduction of the AlkA protein is deleterious to the cell, whereas no such effect is seen with the Tag protein (88). A similar negative effect on survival by enzyme overproduction has been described for the *uvrD*-encoded DNA helicase (89). The presence of large quantities of the AlkA and UvrD proteins bound to DNA in vivo might conceivably interfere with replication and transcription.

ADDITIONAL INDUCIBLE GENES

The AlkB Protein

The *alkB* gene of *E. coli* encodes another factor that is part of the adaptive response to alkylating agents. AlkB mutants are highly sensitive to cell-killing by the simple alkylating agent methyl methanesulfonate. The *alkB* gene maps close to the *ada* gene at 47 min of the *E. coli* K-12 chromosome (43). Cloning and nucleotide sequence analysis revealed that these two genes overlap by one nucleotide residue in the termination codon of *ada* and the initiation codon of *alkB* (40, 41, 60). The two genes form an operon, because the expression of the *alkB* gene is controlled by the promoter of the *ada* gene. In spite of the fact that the product of the *alkB* gene has been purified and the primary structure determined, its biochemical function remains unknown. However, a number of observations have been made concerning the *alkB* gene. The AlkB protein has a molecular weight of 24,000. The *alkB* mutants exhibit decreased host cell reactivation of phage λ treated with methyl methanesulfonate, indicating that *alkB* encodes a DNA repair activity rather than a protein for detoxification of the alkylating agent. Induction of the *ada* and *alkA* genes is not affected in *alkB* mutants, so the latter gene is unlikely to play a regulatory role in the adaptive response. The *alkB* mutants are relatively more sensitive to killing by methyl methanesulfonate than by MNNG. This is paradoxical, because the former agent is known to introduce the same N-methylated base lesions as MNNG into DNA while being less effective for methylation of oxygens. However, the phenotype of an *alkB* mutant is generally similar to that of an *alkA* or *tag* mutant (40, 41). These data would appear to indicate a role for the AlkB protein in the removal of a potentially toxic alkylation lesion such as 1-methyladenine or 3-methylpyrimidines. However, a direct search by enzyme assays for several DNA glycosylase activities and examination of HPLC patterns of alkylated bases released from DNA by extracts of wild-type and *alkB* mutant cells have so far failed to reveal a specific function of the protein.

The AidB Protein

Several of the components of the SOS response were first described as damage-inducible *(din)* genes, detected by screening of large numbers of

random insertion mutants constructed by using phage Mu-*dlac* (90). Volkert and coworkers have investigated the adaptive response to alkylating agents by a similar approach. In addition to MNNG-inducible genes mapped at the *ada/alkB* and *alkA* loci, another gene under *ada* control was detected, *aidB* (42, 44). The latter mutant has two unusual properties. First, it can be induced by exposure to alkylating agents, but it can also be induced in an *ada*-independent fashion by anaerobic culture conditions (M. Volkert, personal communication). Second, about one half of the mutants deficient in the *aidB* gene become more resistant, rather than more sensitive, to exposure to alkylating agents (42). In this regard, it is noteworthy that inactivation of one of the inducible genes of the SOS response, *sfiA,* also provides cells with a more damage-resistant phenotype. The SfiA protein is an inhibitor of cell division (91). A different gene induced by alkylating agents, *aidC,* is not under *ada* control (44) and also differs from the genes involved in the adaptive response by being efficiently induced by ethylating and propylating agents (M. Volkert, personal communication). The *aidC* gene conceivably is involved in direct detoxification of alkylating agents.

THE REGULATORY FUNCTION OF THE ADA PROTEIN

The Ada Protein as Transcriptional Activator

The *ada* gene is the regulatory gene of the adaptive response to alkylation damage. *E. coli ada* mutants have been isolated that are either noninducible for the adaptive response, or express it constitutively (33, 34). The *ada* gene has been cloned by several different strategies, including direct complementation of an *ada* mutation by selecting for an MNNG-resistant phenotype (92, 36), cloning of an *ada*::Tn10 mutation (93), cloning of the *alkB* gene with adjacent *ada* sequences (94), and nonselective screening of cell extracts for methyltransferase activity (62). The product of the *ada* gene cloned into plasmids of high copy number induces partial expression of the cellular *alkA* gene and also of *ada-lacZ* and *alkA-lacZ* fusions in the absence of alkylating agents. The Ada protein is, therefore, a weak positive regulator of expression of its own gene and of *alkA*. When cells carrying the cloned *ada* gene are exposed to alkylating agents, the cellular expression of *alkA* and *ada* is increased due to generation of an inducing signal (92, 2, 93). Since *alkB* forms an operon with *ada,* it is also regulated by the Ada protein (60, 41). In addition, induction of *aidB*::Mu-*dlac* expression by alkylation damage requires a functional *ada* gene product (42).

The molecular nature of the inducing signal that arises in cells exposed to simple alkylating agents has been an intriguing problem. The Ada protein itself becomes methylated after transferring methyl groups from O-alkylated lesions in DNA to two of its own cysteine residues. This self-methylation

converts the Ada protein from a weak to a strong activator of transcription of both the *ada* and *alkA* genes in DNA-dependent protein synthesis and run-off transcription experiments as observed by Teo et al (2). Nakabeppu et al (3) also found that methylation converted the Ada protein to a strong activator of run-off transcription of the *ada* gene, but did not observe this for the *alkA* gene. They proposed that the increased cellular levels of unmethylated Ada protein, after induction of *ada* gene expression by cellular alkylation, might be sufficient to switch on expression of the *alkA* gene. The repair of methylphosphotriesters in DNA by the intact Ada protein, with concomitant methylation of Cys-69, was found to be the event that converts the protein to a strong activator of gene expression (2). A scheme is shown in Figure 3.

DNA Binding by the Activated Ada Protein

The control of *ada* and *alkA* gene expression has been demonstrated in vivo (93, 94), and also in the in vitro experiments described above, to occur at the transcriptional level. Moreover, the Ada protein, after methylation at Cys-69, binds specifically to the promoter of both these genes as determined by DNase I protection experiments (2, 95). The poor protection afforded by the unmethylated protein agrees with it being a weak activator of transcription. Thus, the available footprinting experiments support the concept that only methylated Ada protein is an efficient activator of the *ada* and *alkA* genes. The sites of initiation of transcription of the *ada* and *alkA* genes have been determined by primer extension cDNA synthesis and S1 nuclease mapping (3, 67). The DNA sequence protected by the methylated protein is located immediately upstream of the putative RNA polymerase–binding site in the *ada* promoter, and overlaps with this site in the *alkA* promoter. The bound methylated Ada protein may facilitate recognition of these promoters by RNA polymerase, either by direct contact with the polymerase or by causing a change in DNA conformation. The Ada protein–binding site of the *ada* promoter is in a region of dyad symmetry, and such regions have often been implicated in the control of gene expression. The Ada-binding sites of the *ada* and *alkA* genes contain a common DNA sequence, AAANNAAAGCGCA (2). This sequence does not occur in any other known *E. coli* promoters. It remains to be determined whether the promoter of the *aidB* gene, whose expression is also regulated by the Ada protein (42), contains a similar DNA sequence. Recent site-specific mutagenesis experiments (T. Nakamura, M. Sekiguchi, unpublished data) on the promoter region of the *ada* gene have shown that conservation of a part of the sequence, AAAGCGCA, is indeed necessary for the regulated expression of the *ada* gene. Moreover, constitutive mutants with single-base substitutions near this region were obtained.

DNase I protection experiments have shown that the DNA-binding site of the Ada protein is contained within the 20-kd N-terminal domain, represent-

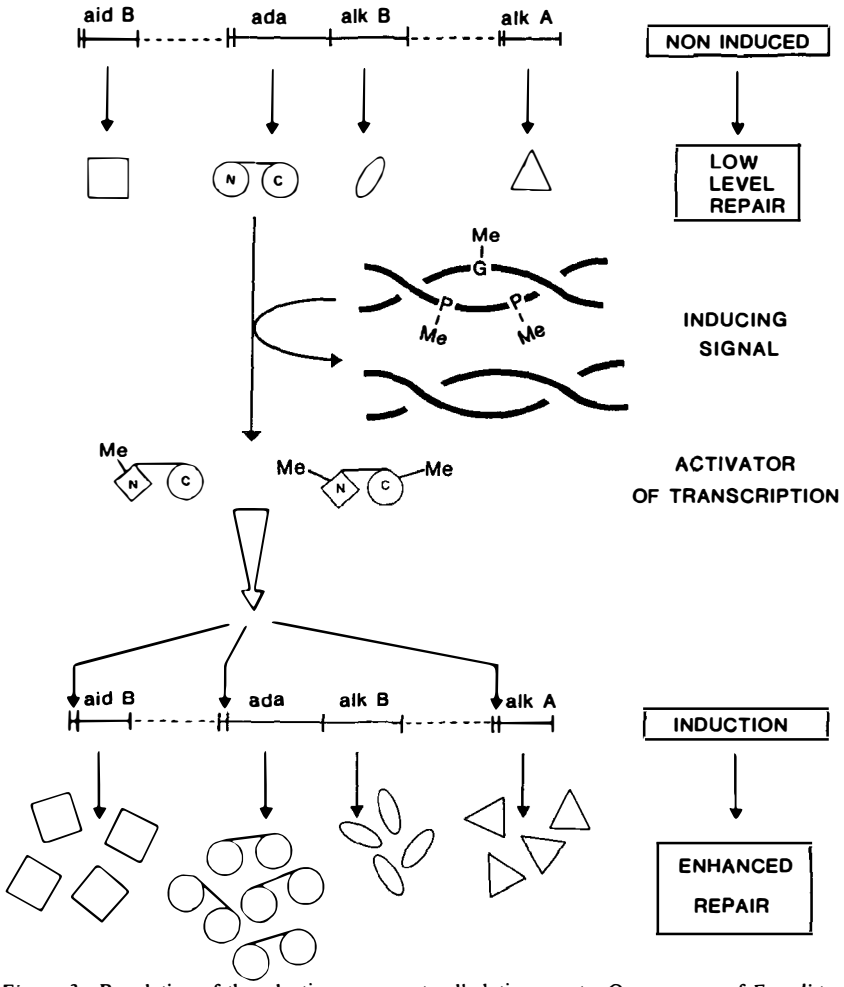

Figure 3 Regulation of the adaptive response to alkylating agents. On exposure of *E. coli* to simple methylating agents, the cellular DNA is alkylated at many sites. The Ada protein, present at a low level in uninduced cells, transfers a methyl group from a methylphosphotriester in DNA to its own N-terminal domain. This covalent modification, resulting in a conformational change, converts the Ada protein from a weak to a strong transcriptional activator of the inducible genes. Enhanced repair of methylated DNA by the increased cellular levels of these gene products conveys cellular resistance to alkylation mutagenicity and toxicity.

ing 50% of the intact protein (B. Sedgwick, unpublished data). This fragment, however, is unable to induce run-off transcription, indicating that a putative local change in DNA conformation due to protein binding is not sufficient to account for the increase in transcription. A small 10-kd N-terminal fragment capable of DNA methylphosphotriester repair inhibits in-

duction of the adaptive response in vivo, presumably by removing the inducing signal and competing with the intact protein for the Ada binding site (96). In contrast, larger N-terminal fragments composed of 80–88% of the Ada protein are strong activators of expression from the *ada* promoter, and must contain the transcriptional activating functions while lacking the cysteine acceptor site for O^6-methylguanine repair (93; D. Shevell et al, unpublished data). Site-specific alteration of the latter cysteine residue to alanine, however, also generates an Ada protein with increased activating properties (K. Takano, M. Sekiguchi, unpublished data). Other single-site mutations in the C-terminal domain can reduce the efficiency of induction of the adaptive response, and it has been proposed that this domain may be important in direct interactions between the Ada protein and RNA polymerase (97). The general picture that emerges from these experiments is that the Ada protein has separate recognition sites for binding to a specific DNA sequence and for interaction with RNA polymerase, as observed for a number of other regulatory proteins (98).

Methylation of the Ada protein at Cys-69 may cause a conformational change of the N-terminal domain that allows it to bind to the AAAGCGCA sequence. A reduced sensitivity to trypsin of the N-terminal region of the methylated intact protein (65), and altered sensitivity of this protein to the endogenous *E. coli* protease (T. Yoshikai, M. Sekiguchi, unpublished data) provide support for this concept. Four independently derived constitutive mutants of the adaptive response, isolated as methylnitrosourea-resistant strains, have mutations in the *ada* structural gene and not in the promoter region (34; S. Hughes, unpublished data). These mutant Ada proteins may have a changed conformation that mimics that of the methylated protein. Of these four constitutive mutants, three overexpress both the *ada* and *alkA* genes. The fourth overexpresses *ada* to a high level, but *alkA* expression is only slightly increased (71). A truncated Ada protein missing 12% of the total sequence at the C-terminal end also conveys this latter phenotype (D. Shevell et al, unpublished data). Such mutated Ada proteins may have a changed conformation that allows efficient binding to the *ada* but not the *alkA* promoter.

Overproduction of the Ada protein appears somewhat deleterious to the cell. Thus, the alkylation-resistant mutants constitutively expressing the adaptive response revert spontaneously to an alkylation-sensitive phenotype at a high frequency (34). Moreover, these constitutive *ada* mutant genes as well as the strongly activating truncated *ada* genes, which contain 80–88% of the original *ada* sequence and a short tail of vector-derived sequence, can only be cloned using vectors with low copy number, presumably because massive overproduction of such mutant Ada proteins is a lethal event (D. Shevell et al, unpublished data; S. Hughes, unpublished data). The regulation of the

adaptive response by modification of the Ada protein is reminiscent of that of the cyclic AMP receptor protein, which undergoes a conformational change on binding cyclic AMP, enabling it to bind to promoters of catabolite-sensitive operons close to their RNA polymerase–binding sites (99, 100). The cAMP-induced alteration, however, is noncovalent and reversible. Methylation of the Ada protein is an example of activation of a regulatory protein by covalent posttranslational modification (2, 3). Recently the *E. coli* NtrC protein involved in the regulation of nitrogen metabolism was shown to be activated/deactivated by phosphorylation/dephosphorylation of its N-terminal domain (11).

Many regulatory proteins of *E. coli,* including the cyclic AMP receptor protein, bind to operator DNA as dimers using α-helixes to contact the major groove (101). The purified Ada protein shows no evidence of dimerization, and its sequence does not contain an apparent helix-turn-helix motif. A second known mechanism of protein-DNA recognition occurs by extended metal-binding fingers (102–104). Pairs of cysteine/histidine residues are held together by a zinc ion forming a DNA-binding loop of amino acids. Pairs of cys/his residues do occur in the Ada protein and could form one or conceivably two such fingers. In this case, Cys-69 would be postulated to be involved in zinc binding, and its methylation might cause a change in the conformation of a putative DNA binding structure. It seems uncertain, however, whether the Ada protein has the necessary structural requirements for this type of interaction with DNA (104). Alternatively, the Ada protein may employ a mode of DNA recognition that is different from these particular types of interactions.

Switching Off the Response

The methylated Ada protein is not actively demethylated, and is therefore irreversibly converted into a transcriptional activator. The adaptive response may be switched off simply by dilution of the activator during growth in the absence of alkylating agents. Any other mechanism for down-regulation would involve inactivation of the methylated Ada protein, or competition with its activity as a positive gene regulator. The Ada protein is sensitive to cleavage by a cellular protease in cell extracts (see Figure 1) (36). The methylated N-terminal 20-kd domain, a product of this cleavage, can bind to the *ada* promoter, but is unable to activate transcription. Proteolytic cleavage of the intact protein was therefore suggested as a possible mechanism for negative modulation of the adaptive response (2, 3, 65). This switch-off could be accelerated by competition of the methylated Ada fragments with the activated intact protein in binding to promoter sequences (105). In support of such models, cloned, truncated *ada* genes encoding 42–66% of the intact protein are dominant inhibitors of the wild-type *ada* gene (D. Shevell et al,

unpublished data). The proposals for an active switch-off mechanism, however, require evidence of proteolytic cleavage of the methylated Ada protein in vivo, which as yet has not been clearly demonstrated. The unmethylated Ada protein remains at a high cellular level for 1–2 hours after its induction (36).

Ethylating agents are less effective inducers of the adaptive response than methylating agents, as observed by the induction of cellular resistance to alkylation mutagenicity and toxicity (18), and more recently by the use of *alkA-lacZ* and *ada-lacZ* gene fusions (82, 94). Since ethylating agents introduce a larger proportion of triesters than do methylating agents (106), and ethylphosphotriesters are repaired by the Ada protein (62), it may be postulated that ethylation of Cys-69 instead of methylation is less efficient for conversion of Ada to a transcriptional activator. In addition to the induction of the response by formation of alkyl phosphotriesters in DNA, the adaptive response can also be activated to some extent by protein-methylating agents such as methyl iodide, which may act by directly modifying the reactive Cys-69 residue in the Ada protein (107).

Comparison with Other Inducible Responses

In addition to the adaptive response, the SOS response can be induced by alkylating agents but is initiated by a different alkylation product. Strains containing *alkA* and *tag* mutations, which are deficient in 3-methyladenine-DNA glycosylase activities, are very sensitive to induction of the SOS response by alkylation damage. The persisting 3-methyladenine residues in DNA of such strains are presumably the SOS-inducing signal, because they block DNA replication (86, 108). Conversely, an observed inhibition of SOS induction in cells induced for the adaptive response to alkylating agents (109) may be due to enhanced 3-methyladenine removal by high cellular levels of 3-methyladenine-DNA glycosylase II (87).

The adaptive response, the SOS response, and the heat-shock response in *E. coli,* which are all induced by environmental stress, have quite different mechanisms of positive regulation. The activated RecA protein enhances proteolytic cleavage of the repressor of the SOS genes (110, 1). The HtpR protein is an alternative sigma factor that stimulates selective transcription of the heat-shock genes (111, 112). Neither of these mechanisms bears any resemblance to the covalent modification of the Ada protein that triggers the adaptive response to alkylating agents. However, there is a certain parallelism between the strategy of Ada protein–mediated control and that employed by bacterial transmembrane proteins with regulatory functions that respond to environmental signals such as limitation of an essential nutrient. These proteins in several cases are composed of an N-terminal periplasmic domain and a C-terminal cytoplasmic domain. The former has the role of a specific sensor that transmits a signal to the cytoplasmic sequence, which is a DNA-binding

domain that accounts for transcriptional activation (113). With regard to the adaptive response, the sensor for cellular exposure to methylating agents is the intracellular nonenzymatic formation of a phosphotriester in DNA, whereas the regulator is contained in the N-terminal region of the Ada protein.

THE ADAPTIVE RESPONSE IN OTHER MICROORGANISMS

Several microorganisms have been tested for their ability to acquire enhanced resistance to MNNG toxicity and mutagenicity by treatment with low MNNG doses. Using this approach, an adaptive response to alkylation damage has been observed in *Bacillus subtilis* (114, 115), *B. thuringiensis* (116), *Micrococcus luteus* (117), and *Streptomyces frodiae* (118). Cell extracts from induced *B. subtilis* and *M. luteus* contain an increased level of O^6-methylguanine-DNA methyltransferase activity. Moreover, noninducible *B. subtilis ada* mutants have been isolated (119, 120). An adaptive response to alkylation damage does not seem to occur, however, in *Haemophilus influenzae* (121) or *Salmonella typhimurium* (122). The *S. typhimurium* phenotype is comparable to that of several *E. coli ada* point mutants in having a low, but significant, constitutively expressed cellular activity for repair of O^6-methylguanine (28, 36, 122). Chromosomal DNA sequences that hybridize with the cloned *E. coli ada* gene have been detected in *S. typhimurium* as well as in several other Gram-negative bacteria (123). *Saccharomyces cerevisiae* appears exceptional in not possessing an adaptive response (124) or even an O^6-methylguanine-DNA methyltransferase, perhaps reflecting a reliance on an effective cell wall barrier and recombinational DNA repair.

Recently, Morohoshi & Munakata (125) reported that constitutive and inducible methyltransferase activities for O^6-methylguanine and methylphosphotriesters occurred as three separate peptides in crude cell extracts of *B. subtilis*. Attempts are presently being made to clone the corresponding genes to ascertain the existence of three different gene products, but the enzyme activities have not yet been purified. Thus, at present it is unclear whether these interesting preliminary results could be partly explained by the presence of active protein fragments in cell extracts.

MNNG-induced mutations show extensive clustering at replication forks in *B. subtilis* and *E. coli,* but not in *H. influenzae* and *S. cerevisiae*. The absence of such clustering may be related to the lack of an inducible O^6-methylguanine DNA repair activity (114, 121). It seems possible that enhanced repair of O^6-methylguanine by induction of a methyltransferase activity may allow complete removal of these mispairing lesions before their replication, except for lesions occurring in DNA regions close to replication forks (50). This model is supported by the observed reduction in clusters of

mutants when *E. coli* is MNNG-treated in the presence of chloramphenicol to prevent synthesis of inducible proteins (126).

REPAIR OF O^6-METHYLGUANINE IN MAMMALIAN CELLS

The unusual mechanism for repair of O^6-methylguanine in DNA, uncovered in *E. coli,* seems to be widely distributed among other organisms. Thus, mammalian cells, fish cells (127), and *Drosophila melanogaster* (128) contain an activity with biochemical properties similar to that of the *E. coli* methyltransferase. The mammalian transferase employs one of its own cysteine residues as methyl group acceptor, and the protein is expended in the reaction (129–134). Human cells have 5–10 times higher levels of this activity than rodent cells (135). The properties of the mammalian enzyme as a defense mechanism against carcinogenic agents have been reviewed elsewhere (136–138). The human O^6-methylguanine-DNA methyltransferase has a molecular weight of only 24,000, compared with 39,000 for the *E. coli* Ada protein, and is unable to act on alkylphosphotriesters (96, 132, 139). In these respects, it resembles the Ada C-terminal domain, rather than the entire Ada protein. Thus, it would appear that the specific signal for induction of an inducible response by demethylation of a phosphotriester as seen in *E. coli* has not been retained in higher cells. The mammalian O^6-methylguanine-DNA methyltransferase has been difficult to purify and appears to be of low antigenicity, so the corresponding gene has not yet been cloned.

When human cells in tissue culture are exposed to low concentrations of MNNG, no induction of transferase activity occurs (139–141). However, a number of preliminary, although not entirely convincing reports (142–145) on adaptation to alkylation resistance in various animal cell lines have appeared. The enzyme can be induced severalfold in rat liver by apparently unspecific procedures such as exposure of animals to cytotoxic agents causing liver necrosis or by partial hepatectomy (146, 147). The mechanism for induction is likely to be quite different from that which accounts for the adaptive response to alkylating agents in *E. coli.*

A variety of widely employed mammalian cell lines do not express the O^6-methylguanine-DNA methyltransferase activity. Cells of this Mer^- (or Mex^-) phenotype are particularly susceptible to alkylating agents (132, 148–150). Conversion to the Mer^- phenotype usually occurs as a consequence of stress treatment or malignant transformation in tissue culture, but there may also be selection for the outgrowth of rare Mer^- cells from tissue explants. The molecular basis for the conversion is not known, but is believed to involve switching off transcription of the O^6-methylguanine-DNA methyltransferase gene. Furthermore, Mer^+ variants have been obtained at a low

frequency ($\sim 10^{-5}$) from Mer^- cell lines by chloroethylnitrosourea treatment; these Mer^+ variants exhibit a reduced frequency of malignant transformation after exposure to N-ethyl-N-nitrosourea (150a). It is also of interest that Mer^+ cells can be temporarily depleted of their transferase activity and sensitized to killing by chloroethylnitrosoureas by exposure to large but nontoxic levels of free O^6-methylguanine base in the culture media (138, 151–153).

EXPRESSION OF THE BACTERIAL *ada* GENE IN MAMMALIAN CELLS

The *ada* gene was initially cloned by conveying resistance to MNNG mutagenesis to *E. coli ada* mutants with recombinant DNA (92). It seemed possible that resistance to alkylating agents might be conveyed in an analogous fashion to alkylation-sensitive mammalian cell lines of the Mer^- phenotype by transfection with the *ada* gene in an SV40-derived vector. Several groups have recently described such experiments, and their results are in good agreement (96, 154–156). Integration and expression of the *ada* gene in mammalian cells stably conferred increased resistance to mutation induction and killing by a variety of simple alkylating agents. The C-terminal domain of the Ada protein, which repairs O^6-methylguanine, accounted for this resistance, whereas the N-terminal Ada domain was not sufficient (96, 105, 157, 158). Attempts are now being made to express the Ada protein in transgenic mice (155; T. Ishikawa and M. Sekiguchi, unpublished data), and such animals could be useful for evaluating various aspects of carcinogenesis by methylating agents (159).

Expression of a microbial gene encoding a second DNA-repair enzyme in repair-deficient human cells has been achieved recently. The bacteriophage T4 *denV* gene encodes a DNA glycosylase that acts on pyrimidine dimers; this enzyme can provide an alternative DNA-incision mechanism in ultraviolet-irradiated mammalian cells (160). The cloned *denV* gene (161) has been integrated and expressed in human cells and partially complements the radiation-sensitive phenotype of xeroderma pigmentosum (162).

FUTURE PROSPECTS

A few years ago, the major questions that needed clarification with regard to the adaptive response to alkylating agents seemed to be the elucidation of the mechanism of repair of the highly mutagenic DNA lesion O^6-methylguanine, and the definition of the intracellular signal for induction of the response. These two problems have been largely solved, and have revealed novel mechanisms for DNA repair and transcriptional activation of genes. As in the case of the SOS response, the general outline of the regulation of the inducible

pathway is now understood. However, in both the adaptive response and the SOS response, about one-half of the inducible genes have unknown functions. In the adaptive response these genes are *alkB* and *aidB*. While these functions may have only peripheral or accessory roles in the response, it is also possible that our present picture is an oversimplification and underestimates the regulatory contributions of these gene products.

A second insufficiently understood aspect of the adaptive response is the separation of the repair of O^6-methylguanine from the generation of a transcriptional activator by repair of an innocuous minor DNA lesion, one of the two stereoisomers of a methyl phosphotriester. Since both O^6-methylguanine and the phosphotriester are corrected in the same fashion by transfer of the methyl group to a cysteine residue in the Ada protein, it is unclear why the important antimutagenic repair of O^6-methylguanine could not also serve to generate the inducing signal. One possible explanation of this dichotomy might be that phosphotriesters are rarer lesions than O^6-methylguanine. Consequently, accidental exposure to low concentrations of alkylating agents in the environment might generate occasional O^6-methylguanine residues in DNA, which could be scavenged effectively by the uninduced cellular levels of methyltransferase activity. The cumbersome induction of the response would only occur at higher alkylation doses that generate significant amounts of DNA phosphotriesters.

An associated question is why an inducible pathway for repair of DNA methylation damage should exist in certain microorganisms. S-adenosylmethionine is a weak alkylating agent that can accidentally methylate nitrogens in DNA in a nonenzymatic reaction (163, 164), and this probably explains the need for a constitutively expressed 3-methyladenine-DNA glycosylase. However, since bacteria do not possess P-450 proteins for activation of compounds such as dimethylnitrosamine, the selection for an adaptive response during evolution strongly indicates that bacteria are frequently exposed to relatively high concentrations of direct-acting alkylating agents that cause DNA methylation at oxygens and nitrogens. The ecological niche in which these compounds occur, and their chemical identification, remain to be determined, but this would appear to be a challenging problem in environmental mutagenesis.

Finally, the direct transfer of methyl groups from DNA to a protein, and the accompanying conversion of the protein to a transcriptional activator, are not yet understood at a detailed mechanistic level. We hope that the current availability of relatively large amounts of pure Ada protein from expression vector systems, and the clarification of the domain structure of this protein, will encourage work by physical chemical techniques such as X-ray diffraction measurements, to provide a detailed three-dimensional structure for the interaction between damaged DNA and this unusual antimutagenic activity and regulatory factor.

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