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Inducible repair of phosphotriesters in *Escherichia coli*

(adaptive response/alkylation repair/methyltransferase)

JAMES G. MCCARTHY, BRENT V. EDINGTON, AND PAUL F. SCHENDEL*†

Genetics and Cell Biology Section, Biological Science Group, The University of Connecticut, Storrs, CT 06268

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ABSTRACT **Extracts from *Escherichia coli* cells induced for the adaptive response have been prepared that are capable of repairing O^6-methylguanine, O^4-methylthymine, and the phosphotriesters produced on the DNA backbone by alkylating agents such as *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG). The phosphotriesters are repaired by a methyltransferase distinct from the one that demethylates O^6-methylguanine. We propose that this increased capacity to repair phosphotriesters accounts for much of the increased resistance to MNNG toxicity seen in cultures induced for the adaptive response.**

Escherichia coli exposed to sublethal doses of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) soon become substantially more resistant to both the mutagenic and the cytotoxic effects of MNNG and related alkylating agents (1). This phenomenon is referred to as the adaptive response (2) and is effected by the induction of several proteins. Resistance to the mutagenic effects of alkylation is mediated by the induction of a protein that rapidly removes one of the major alkylation products, O^6-methylguanine (O^6-MeGua), from the DNA of the alkylated cells (3). This protein is unusual in that it repairs the alkylated guanine base by simply transferring the methyl group from the O^6 position of guanine to one of the cysteines of the protein (4, 5). This process leads to inactivation of the enzyme, and any further repair of O^6-MeGua requires synthesis of another whole new protein. Such a protein is called a methyltransferase (4).

The mechanism by which cells develop resistance to the toxicity of MNNG is less well understood. This resistance develops only if the cells have a full complement of DNA polymerase I (6), implicating excision repair in the process in some way. Recently, it has been reported that a DNA glycosylase that is active on 3-methylpurines is induced as part of the adaptive response (7). This glycosylase is the product of the *alkA* gene (8) and cells mutant in this gene are unable to develop resistance to the toxic effects of methyl methanesulfonate (MMS) (8). These results led to the suggestion that 3-methylguanine might be a potent lethal lesion and its removal, in cells induced for the adaptive response, might be responsible for the increased survival of these cultures after exposure to alkylating agents (7, 8).

An alternative explanation for why cells induced for the adaptive response become more resistant to MNNG toxicity is presented in this paper. We have found that cells apparently produce a second methyltransferase in response to MNNG. This protein does not repair O^6-methylguanine but removes methyl groups from the phosphate oxygens of the DNA backbone. We suggest that this dealkylation facilitates a variety of excision-repair reactions the composite effect of which is increased survival.

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MATERIALS AND METHODS

Bacterial Strains. The strains used were *E. coli* B strains F26 (*His⁻ Thy⁻*) (2) and two derivatives of this strain—BS21, which is constitutive for the adaptive response (9), and BS21R, which is an Ada⁻ revertant of BS21.

Reagents. Salmon sperm DNA, alkaline phosphatase, spleen phosphodiesterase, DNase I, protease VI, and all buffers and reagents used in the methyltransferase assay were from Sigma. The DNA polymers poly(dA) and poly(dT) and the dithymidine monophosphate used to synthesize the dithymidine monophosphate methyl ester [dTp(Me)dT], chromatography standard, were from P-L Biochemicals. Venom phosphodiesterase was from Millipore. The ^{14}C- and ^{3}H-labeled *N*-methyl-*N*-nitrosourea (methyl labeled) were from New England Nuclear.

Chromatographic Standards. Authentic O^4-methylthymidine (O^4-MedT) and 3-methylthymidine (3-MedT) were gifts from G. Margison. O^2-Methylthymidine (O^2-MedT) and dTp(Me)dT were prepared by treating thymidine or dithymidine monophosphate with *N*-methyl-*N*-nitrosourea. Reactions were carried out in the dark at 37°C in 0.1 M sodium cacodylate [pH 8.0 for the O^2-MedT synthesis and pH 7.0 for the dTp(Me)dT synthesis]. Products were separated by HPLC on a C-18 column. From the thymidine reaction, we isolated O^2-MedT that eluted between thymidine and 3-MedT. dTp(Me)dT was recovered from the dithymidine monophosphate reaction as a base-labile product that formed a double peak that is retained on the column much longer than any of the methylated nucleosides.

Synthesis of Alkylated DNA Substrates. Salmon sperm DNA or the DNA polymers poly(dT) and poly(dA) was dissolved in 0.01 M sodium acetate (pH 5.0). Radioactively labeled *N*-methyl-*N*-nitrosourea [^{14}C at 16.2 mCi/mmol (1 Ci = 37 GBq) or ^{3}H at 1.5 Ci/mmol] was added and the pH was adjusted to 7.0 with cacodylate buffer (final concentration, 0.1 M). Salmon sperm DNA was labeled with ^{14}C and the DNA polymers with ^{3}H. The reactions were allowed to proceed at 37°C for 3 hr at which time the mixtures were dialyzed extensively. The salmon sperm DNA was heat depurinated as described (10). Analysis of this template showed ≈30% of the radioactivity in O^6-MeGua, 60% in phosphotriester, and 10% in other labeled species. The alkylated poly(dA) and poly(dT) were mixed with equimolar amounts of nonalkylated complementary polymer to produce poly(dA)(methylated)·poly(dT) and poly(dT)(methylated)·poly(dA).

Methyltransferase Assay. Alkylated DNA was mixed with various amounts of cell extract containing methyltransferase in

Abbreviations: MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; ENNG, *N*-ethyl-*N'*-nitro-*N*-nitrosoguanidine; MMS, methyl methanesulfonate; O^6-MeGua, O^6-methylguanine; O^4-MeThy, O^4-methylthymine; O^2-MeThy, O^2-methylthymine; O^4-MedT, O^4-methylthymidine; O^2-MedT, O^2-methylthymidine; 3-MedT, 3-methylthymidine; dTp(Me)dT, dithymidine monophosphate methyl ester; kDa, kilodalton(s).

* Present address: Genetics Institute, 225 Longwood Avenue, Boston, MA 02115.

† To whom reprint requests should be addressed.

70 mM Hepes, pH 7.8/1 mM dithiothreitol/1 mM EDTA. The mixture was maintained at 37°C for 10 min to allow for the transfer of methyl groups from DNA to protein. Protease VI (700 μg/ml) was then added and incubation was continued for 2 hr to digest the extract proteins. Carrier DNA and perchloric acid (final concentration, 7%) were added and the DNA was removed by centrifugation. Finally, the supernatant liquid was tested for radioactivity. Details of this procedure are presented elsewhere (11).

Partial Purification of Methyltransferase Activities. A crude lysate was prepared by sonicating a culture of BS21 cells and spinning down the cell debris. This lysate was partially purified as described (10). All steps through the phosphocellulose column were carried out. The material recovered from this column was used as the source of methyltransferase as indicated.

Analysis of Methylated Nucleosides and Dinucleoside Monophosphates. Reaction mixtures (0.21 ml) were prepared containing 70 mM Hepes, pH 7.8/1 mM EDTA/1 mM dithiothreitol and 5–10 × 10^3 cpm of poly(dT)(methylated)·poly(dA). To these mixtures, either 0.04 ml of 70 mM Hepes, pH 7.8/1 mM EDTA/1 mM dithiothreitol/40% glycerol or 0.04 ml of methyltransferase extract was added. The mixtures were incubated at 37°C for 10 min. To each mixture, *E. coli* DNA labeled with [^{14}C]thymidine was added (3.9 × 10^3 cpm). The mixtures were made 20 mM in $MgCl_2$, and 100 Kuntz units of DNase I was added. After 1 hr at 37°C, 50 μg of alkaline phosphatase and 50 μg of both venom and spleen phosphodiesterase were added. The enzymes were allowed to react for 3 hr at 37°C. The reaction mixture was then frozen at −70°C.

HPLC analysis of the digested DNA was carried out on a Supelco LC-18 column. The sample was loaded in 5% methanol/H_2O and eluted with a linear 5–50% methanol/H_2O gradient. The order of elution of authentic marker compounds determined prior to the analysis was O^2-MedT, 3-MedT, O^4-MedT, dTp(Me)dT. Nevertheless, 3-MedT and O^4-MedT were added to each digest to fix with certainty the positions of elution during the analysis. UV absorbance was monitored, fractions were collected, and radioactivity was determined. All ^{3}H radioactivities were normalized to the recovery of ^{14}C from the column. In all cases, ^{14}C recoveries were 75–80%.

RESULTS

Detection of Phosphotriester Repair in Crude Lysates. The demonstration of an inducible methyltransferase activity that repairs O^6-MeGua (4) promoted the search for similar activities. A crude lysate was prepared by sonicating a culture of *E. coli* BS21, a strain that constitutively produces a high level of O^6-MeGua methyltransferase (9). This lysate was assayed for methyltransferase activity by the protease assay using alkylated and partially depurinated salmon sperm DNA as the labeled substrate. The protein dependence of this experiment is shown in Fig. 1*A*. Very little radioactivity is released with protease alone or with high levels of lysate alone but when lysate and protease are both present, some of the radioactivity becomes acid soluble. The extent of release is initially proportional to the amount of extract added, but a plateau level of release corresponding to 60–70% of the radioactivity is reached. O^6-MeGua comprises only about 30% of the radioactivity in the template; thus, these results suggest that methyl groups are being removed from sites other than the O^6 position of guanine, and the magnitude of this additional release strongly suggests repair of another major alkylation species, presumably phosphotriesters. A similar reaction was carried out using alkylated poly(dT), a DNA substrate containing mostly phosphotriesters and no guanine bases. Fig. 1*B* shows that radioactivity is released from this template

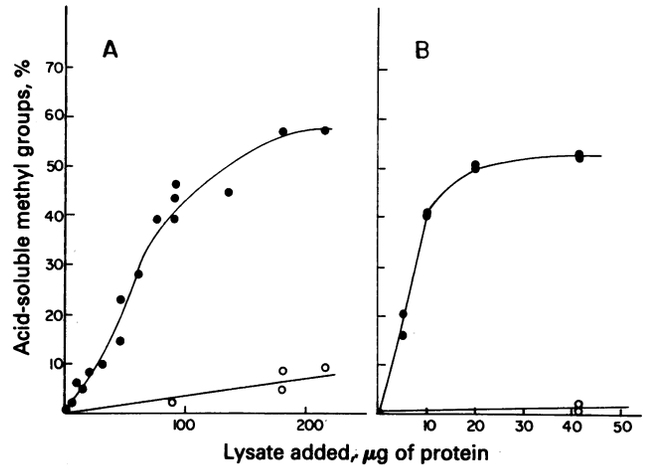

FIG. 1. Release of radioactivity from methylated DNA by an activity in a crude extract of BS21. (*A*) Partially depurinated methylated salmon sperm DNA (^{14}C labeled, 16.2 mCi/mmol, 1,800 cpm per reaction mixture). (*B*) Poly(dT)(methylated)·poly(dA) (^{3}H labeled, 1.5 Ci/mmol, 2,000 cpm per reaction mixture). ●, With protease; ○, without protease.

as well. The plateau value in this case is 50–55% of total radioactivity released.

The results in Fig. 1 strongly suggest that there is an activity in strain BS21 that can repair phosphotriesters. To determine whether this activity is part of the adaptive response, lysates were prepared from BS21; from strain F26, the wild-type parent of BS21; and from strain BS21R, a revertant of BS21 isolated in our laboratory that is incapable of mounting the adaptive response. The propensity of BS21 to revert to an Ada$^-$ phenotype has been reported previously (9). Lysates from either F26 or BS21R were unable to carry out any detectable protease-dependent release of radioactivity from methylated poly(dT)·poly(dA) regardless of the amount of extract used. Lysates prepared from BS21, or from either BS21 or F26 cultures that had been treated with a low level of MNNG to induce the adaptive response, had significant protease-dependent repair capacity. All three of these lysates could release up to 50% of the radioactivity from methylated poly(dT)·poly(dA). In contrast, pretreatment of BS21R cultures with MNNG did not induce any detectable repair activity. These results are consistent with the repair of phosphotriesters being part of the adaptive response.

Whereas BS21 and MNNG-treated F26 lysates ultimately released similar amounts of radioactivity from methylated poly(dT)·poly(dA), the specific activity of the phosphotriester repair activity in a BS21 lysate was 3–4 times greater than that of the induced F26 lysate and was not increased by pretreatment with MNNG (Table 1).

Characterization of Partially Purified Phosphotriester Repair Activity. The phosphotriester repair activity was partially purified to rid the extract of nucleic acids and much of the protein. Fig. 2 shows that this partially purified extract can release radioactivity from both methylated poly(dA) and methylated poly(dT) strands. Interestingly, release is almost as great from poly(dA) (methylated)·poly(dT), a template containing no alkylated oxygens except in its phosphate backbone, as from poly(dT)(methylated)·poly(dA). Most of this release is dependent on the addition of protease, but a small fraction was seen with extract alone. This protease-independent release is of unknown origin but it was not inhibited by the addition of 50 μg of either single- or double-stranded DNA to the reaction mixture. When single-stranded polymers were used as substrates,

Table 1. Methyltransferase activity in crude extracts of *E. coli* B

Strain	Pre-treatment	Phenotype	Specific activity*
F26	None	Not adapted	<0.5
	MNNG (1 μg/ml), 90 min	Adapted	29
BS21	None	Adapted	114
	MNNG (1 μg/ml), 90 min	Adapted	114
BS21R	None	Not adapted	<0.5
	MNNG (1 μg/ml), 90 min	Dead	<0.5

*Reported as fmol of methyl groups released per μg of protein.

a much smaller amount of protease-independent release was detected and no protease-dependent release of radioactivity was seen. Thus the repair activity requires a double-stranded substrate.

The direct demonstration of phosphotriester repair is shown in Table 2. The extract was incubated with poly(dT)(methylated)·poly(dA). The DNA was then digested enzymatically to nucleosides and analyzed by HPLC. When the results were normalized for losses during the analysis, the data show that ≈50% of the dTp(Me)dT was lost during incubation with the extract. Furthermore, the small amount of O^4-MedT in the polymer was completely removed. Other nucleosides such as 3-MedT and O^2-MedT were unaffected by the extract.

A second incubation of poly(dT)(methylated)·poly(dA) with a saturating amount of extract was carried out, and a protease assay carried out on a sample of the reaction mixture indicated that ≈50% of the radioactivity had been transferred to protein during the reaction. The proteins in the remaining sample were digested to amino acids as described (4) and then analyzed on an amino acid analyzer. Greater than 95% of the radioactivity released in the protease assay could be accounted for in a peak that comigrated with methylcysteine. Thus, the activity that repairs phosphotriesters appears to be a methyltransferase.

Physical Characterization of the Phosphotriester Repair Activity. The repair of phosphotriesters is either mediated by the same protein that repairs O^6-MeGua or by a different protein or proteins. The O^6-MeGua repair activity is known to be stable at 45°C (12). We incubated the extract at 45°C for various

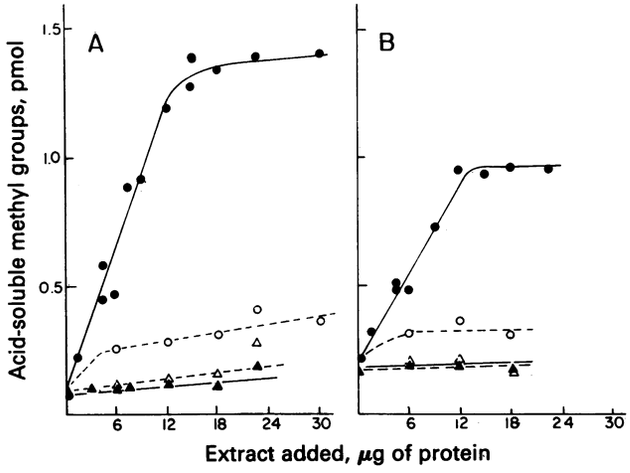

FIG. 2. Reaction of methylated DNA polymers with the partially purified extract from BS21. (*A*) ● and ○, poly(dT)(methylated)·poly(dA); ▲ and △, single-stranded poly(dT)(methylated). (*B*) ● and ○, Poly (dA)(methylated)·poly(dT); ▲ and △, single-stranded poly(dA). ● and ▲, with protease; ○ and △, without protease.

Table 2. Analysis of methylated nucleosides found in alkylated poly(dT) after repair by partially purified extract from BS21

Alkylated nucleoside	Control, fmol	After reaction with extract, fmol	% remaining after reaction
O^2-MedT	129	129	100
3-MedT	506	539	107
O^4-MedT	183	10	6
dTp(Me)dT	6,343	2,978	47

times prior to addition of poly(dT)(methylated)·poly(dA). The percentage of methyltransferase activity as a function of time at 45°C prior to commencement of the reaction is shown in Fig. 3. There is a rapid loss of ≈25% of the activity in the first few minutes, but the remaining 75% is relatively stable. These results suggest that a component of the activity that repairs phosphotriesters is heat stable but a minor heat-labile activity is also present.

The O^6-methylguanine–DNA methyltransferase has a molecular mass of 18 kilodaltons (kDa) (12). Reactions of the extract with methylated salmon sperm DNA or poly(dT)·poly(dA) were physically analyzed by separating the proteins on an 11.5% $NaDodSO_4$/polyacrylamide gel (13). When salmon sperm DNA was used, a major band comigrating with an 18-kDa protein and four minor bands were seen. The faint bands ran at the front and at the positions of 20-, 27-, and 39-kDa proteins (data not shown). A fluorograph of a gel on which the proteins from heated and control extracts that had been incubated with radioactively labeled poly(dT)·poly(dA) were separated is shown in Fig. 4. The polymers were methylated either on the poly(dT) strand (lanes A–C) or the poly(dA) strand (lanes D–F). With both alkylated templates, the unheated reactions gave two major bands, a darker band near the dye front and a lighter band at a position where a 39-kDa protein migrates. When extracts that had been heated at 45°C prior to the reaction were used, the 39-kDa radioactive band disappeared. With the poly(dT)(methylated)·poly(dA) template, two faint bands at positions corresponding to proteins of 18 and 20 kDa were also seen. These were not seen when the polymer had been methylated on the poly(dA)

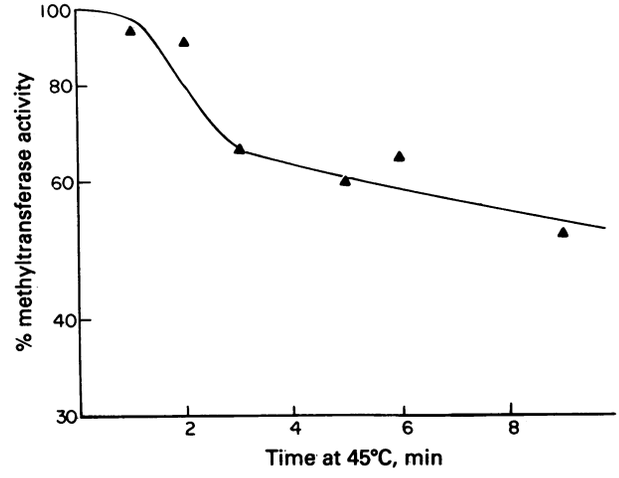

FIG. 3. Heat lability of phosphotriester repair activities. Mixtures of 8 μg of partially purified extract protein and 0.24 ml of reaction buffer were heated at 45°C for the times indicated. The mixtures were then put on ice and poly(dT)(methylated)·poly(dA) (1,800 cpm per reaction mixture) was added. The reactions were carried out at 37°C as usual. The extent of release of radioactivity in a reaction mixture without heating was set at 100% and the release in the heated reaction mixtures was compared with it.

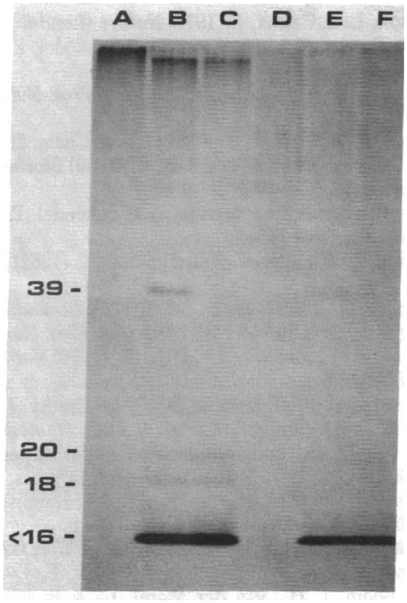

FIG. 4. Fluorography of a $NaDodSO_4$/polyacrylamide gel. Numbers designate molecular masses (in kDa) of proteins expected to migrate to the positions indicated. Lanes: A, alkylated poly(dT)-(methylated)-poly(dA); B, alkylated poly(dT)(methylated)-poly(dA) plus extract; C, alkylated poly(dT)(methylated)-poly(dA) plus heated extract; D, alkylated poly(dA)(methylated)-poly(dT); E, alkylated poly(dA)(methylated)-poly(dT) plus extract; F, alkylated poly(dA)-(methylated)-poly(dT) plus heated extract.

strand. Thus, the extract contains at least two species that can accept methyl groups from phosphotriesters and possibly two species that accept methyl groups from alkylated thymine. At present, it is not clear whether these activities are related to one another or are different gene products. In either case, the phosphate repair activities are distinct from the 18-kDa protein that accepts methyl groups from O^6-MeGua.

DISCUSSION

The results presented in this paper show that *E. coli* has the ability to repair the phosphotriesters produced by alkylating agents on the DNA backbone. The repair activity is inducible and is produced as part of the adaptive response. Apparently, the repair is carried out by two or more methyltransferases. (The radioactivity near the gel front could represent many proteins of <16 kDa.) The relationship of these proteins to one another is not clear. They may be related and have different sizes because of some aspect of the isolation procedure, or they may really be distinct gene products. In any event, these proteins are probably distinct from the methyltransferase that repairs O^6-MeGua because they do not have the same molecular weight as the O^6-MeGua repair enzyme and the larger triester repair protein is very heat labile. In addition, we have been able to separate the O^6-MeGua repair activity from the triester repair activities by using a phosphocellulose column (unpublished results).

In addition to the repair of phosphotriesters, we have shown that O^4-MedT lesions can be repaired by extracts of *E. coli*. Because comparatively few of these alkylated bases are produced, it has not been possible to show with any certainty that this repair is mediated by a methyltransferase. The fact that the protease assay consistently shows more release of radioactivity from alkylated poly(dT) strands than from alkylated poly(dA) strands suggests that the poly(dT) substrate contains more sites for methyltransferase attack than are found in poly(dA). These results are consistent with the hypothesis that O^4-MedT is repaired by a methyltransferase that is distinct from the one that repairs phosphotriesters. Other laboratories also have evidence that an O^4-methylthymine-DNA methyltransferase is present in *E. coli* (J. Laval, personal communication). One of the faint bands at the position of 18- and 20-kDa proteins in Fig. 4 may be this methyltransferase.

To date, we have been unable to find evidence for the repair of O^2-methylthymine (O^2-MeThy). Recently evidence for a glycosylase that may repair this lesion has been reported (T. McCarthy, personal communication). This does not rule out the possibility that a methyltransferase may also be found that can repair O^2-MeThy.

One puzzling aspect of the results presented in Figs. 1 and 2 is that the release of radioactivity reaches a maximum value far below the level expected for full repair of phosphotriesters. In fact, the reaction stops when about half of the triesters have been lost (Table 2). This seems to be true for both the synthetic polymers and the native DNA templates. Since single-stranded DNA is not a substrate for triester repair, it is conceivable, but unlikely, that half of the DNA is either single-stranded or some other unusual form. An interesting alternative possibility is that the methyltransferases act only in the major groove of double-stranded DNA. From there, they would have access to O^4-MeThy and O^6-MeGua but not to O^2-MeThy. Furthermore, they may only be able to repair the phosphotriesters that are oriented toward that groove. This hypothesis, if correct, raises many interesting questions about the repair of altered forms of DNA such as Z-DNA (14), which does not have a normal major groove.

As mentioned in the *Introduction*, cells induced for the adaptive response are less sensitive to the lethal effects of alkylating agents such as MNNG. We suggest that this resistance is due mainly to the induction of the phosphotriester methyltransferase. The involvement of DNA polymerase I in the adaptive response (6) strongly implicates excision repair in the repair of potentially lethal lesions. An obligatory part of excision-repair processes is the exonucleolytic degradation of the DNA segment to be repaired. Phosphotriesters prevent hydrolysis of the phosphodiester bond and thus block the action of exonucleases. One can easily imagine that DNA that contains many such lesions would be difficult to repair by any excision-repair mechanism. Thus, the action of glycosylases would lead to breaks in the DNA which could not readily be rejoined by DNA polymerase I and DNA ligase. The rapid removal of the methyl groups from the phosphotriesters might well alleviate much of this problem.

There are several reasons why we favor the repair of triesters over the repair of 3-methylguanine (7, 8) as an explanation for the enhanced resistance of adapted cells to the toxic effects of MNNG. First, as we have argued above, the repair of triesters should enhance all forms of excision repair. Second, this explanation does not require singling out any particular lesion as "the toxic lesion". Instead, it recognizes that many kinds of lesions can be a problem and the repair of any of them may be important. Third, ethylating agents are known to be better at producing phosphotriesters than are methylating agents (15). Ethylnitrosoguanidine (ENNG) is much more toxic per mutant induced than is MNNG (data not shown), and induction of the adaptive response reduces the lethality of ENNG to a greater extent than it reduces the effect of MNNG.

Finally, if exonucleases are blocked by phosphotriesters, breaks or gaps might accumulate at these points. Such lesions are known to block DNA replication (16) and to induce SOS functions (17). The induction of the adaptive response allows DNA synthesis to proceed (18) and suppresses the induction of SOS functions (19). Both of these results would aid in cell sur-

vival. We feel that these are compelling arguments for the suggestion that triester repair is the primary cause of enhanced survival of adapted *E. coli*.

This explanation of the increased survival of cells induced for the adaptive response does not necessarily exclude the previously suggested hypothesis that induction of the *alkA*-encoded glycosylase may be an important biochemical event that in some cases causes enhanced survival of adapted cultures of *E. coli* (7, 8). The *alkA* mutations make cells very sensitive to MMS (20) and it is the enhanced survival of cells exposed to this chemical that has been reported to be blocked in alkA mutants (8). MMS is very good at alkylating nitrogen atoms within DNA bases but produces very few phosphotriesters (15, 21). Thus excision repair is not blocked by this chemical and, given the possibility to perform this kind of repair, the increased survival seen in adapted cultures could be due to induction of the alkA glycosylase. Nevertheless, this does not mean that 3-methylguanine or any other particular alkylated base is the primary killing lesion in cells treated with MNNG because the cells are being forced to cope with a very different set of lesions when responding to MNNG instead of MMS.

This paper reports that more than one methyltransferase is induced in *E. coli* as part of the adaptive response. As yet, their relationship to one another and their specific tasks in DNA repair are unclear. Nevertheless, it seems certain that this kind of repair activity is more common than originally thought and many other similar activities may be found.

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