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Induction of resistance to alkylating agents in *E. coli*: the *ada*$^+$ gene product serves both as a regulatory protein and as an enzyme for repair of mutagenic damage

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The expression of several inducible enzymes for repair of alkylated DNA in *Escherichia coli* is controlled by the *ada*$^+$ gene. This regulatory gene has been cloned into a multicopy plasmid and shown to code for a 37-kd protein. Antibodies raised against homogeneous O^6-methylguanine-DNA methyltransferase (the main repair activity for mutagenic damage in alkylated DNA) were found to cross-react with this 37-kd protein. Cell extracts from several independently derived *ada* mutants contain variable amounts of an altered 37-kd protein after an inducing alkylation treatment. In addition, an 18-kd protein identical with the previously isolated O^6-methylguanine-DNA methyltransferase has been identified as a product of the *ada*$^+$ gene. The smaller polypeptide is derived from the 37-kd protein by proteolytic processing.

Key words: mutagenesis/adaptive response to alkylating agents/O^6-methylguanine

Introduction

Two inducible pathways of DNA repair have been studied in detail in *Escherichia coli*, the SOS response (Little and Mount, 1982; Walker, 1984) and the adaptive response to alkylating agents (Cairns *et al.*, 1981). In the latter, *recA*-independent pathway, exposure of cells to non-lethal concentrations of N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) or N-methyl-N-nitrosourea (MNU) causes the induction of DNA repair enzymes that act specifically on alkylated DNA to counteract the mutagenic and toxic effects of the damaging agents (Lindahl, 1982). The response is governed by the *ada*$^+$ gene, positioned at 47 min on the *E. coli* K-12 genetic map; this gene has recently been cloned into the multicopy plasmid pAT153 (Sedgwick, 1982,1983). Mutants with a defect at this locus fail to induce resistance to mutagenic and lethal alkylation damage (Jeggo, 1979). Several genes and repair functions are under *ada*$^+$ positive control (McCarthy *et al.*, 1983; Sedgwick, 1983; Volkert and Nguyen, 1984). The best characterized gene is *alkA*$^+$, located at 45 min on the *E. coli* chromosome, that encodes a DNA glycosylase which initiates excision-repair by removing several killing lesions, including 3-methyladenine, 3-methylguanine, O^2-methylcytosine and O^2-methylthymine (Karran *et al.*, 1982; Evensen and Seeberg, 1982; Yamamoto *et al.*, 1983; McCarthy *et al.*, 1984). A different component of the adaptive response removes O^6-methylguanine, the main mutagenic lesion generated by simple alkylating agents (Schendel and Robins, 1978). In the latter reaction, the methyl group of O^6-methylguanine is transferred to a cysteine residue of the repair enzyme itself, effecting direct reversal of the DNA damage (Olsson and Lindahl, 1980; Foote *et al.*, 1980; Demple *et al.*, 1982). This activity, O^6-methylguanine-DNA methyltransferase, has been purified previously as a homogeneous 18-kd protein from an *E. coli* mutant constitutive for the adaptive response (Demple *et al.*, 1982). Both *in vivo* and *in vitro*, the enzyme undergoes suicide inactivation on self-methylation and consequently can act only once (Robins and Cairns, 1979; Lindahl *et al.*, 1982).

Previous attempts to identify the structural gene for O^6-methylguanine-DNA methyltransferase in *E. coli* K-12 using standard genetic techniques have not succeeded. Thus, although screening of bacterial mutants that cannot induce resistance to alkylation mutagenesis has resulted in the isolation of *ada* strains (Jeggo, 1979), no mutant deficient only in expression of the transferase activity has been found (our unpublished data). In the present work, we have used antibodies raised against the homogeneous methyltransferase to investigate its induction during the adaptive response. The antibodies specifically recognize in *E. coli* cell extracts two proteins which correspond to the 18-kd transferase and the 37-kd Ada protein. Both polypeptides have been shown to be products of the same gene. This may provide an explanation for the apparent absence of *E. coli* mutants defective in methyltransferase activity but retaining a regulatory function.

Results

Methyltransferase induction analyzed by immunoblotting

Rabbit antibodies against homogeneous O^6-methylguanine-DNA methyltransferase isolated from *E. coli* reacted efficiently with the immunogen on nitrocellulose (Western) blots of SDS-polyacrylamide gels, as revealed with ^{125}I-labelled anti-rabbit IgG (Figure 1, lane 1). When the anti-transferase antibody was used to probe Western blots of *E. coli* cell extracts, little or no antigenically reactive cellular protein was detected (Figure 1, lane 2). This was expected, since unadapted *E. coli* contain only ~20 methyltransferase molecules per cell (Demple *et al.*, 1982; Mitra *et al.*, 1982). Following exposure of the bacteria to a non-lethal amount of MNNG, a large increase of serologically reactive protein was observed in cell extracts (Figure 1, lane 3). Induction was accompanied by a corresponding increase in O^6-methylguanine-DNA methyltransferase activity. Thus, direct enzyme assays of crude cell extracts showed that the untreated cells yielded <1 transferase unit/mg protein, while the MNNG-adapted cells contained 10.5 transferase units/mg.

These results confirm previous data on the induction of the methyltransferase during the adaptive response. A surprising observation, however, was that two different polypeptides of 37 kd and 18 kd were recognized by the rabbit antiserum (Figure 1, lane 3). No 37-kd protein was detected in the methyltransferase preparation used as immunogen (Figure 1, lane 1). This agrees with the observation that the gel filtration step employed during the purification of the 18-kd transferase completely removed proteins of >25 kd (Demple *et al.*, 1982)

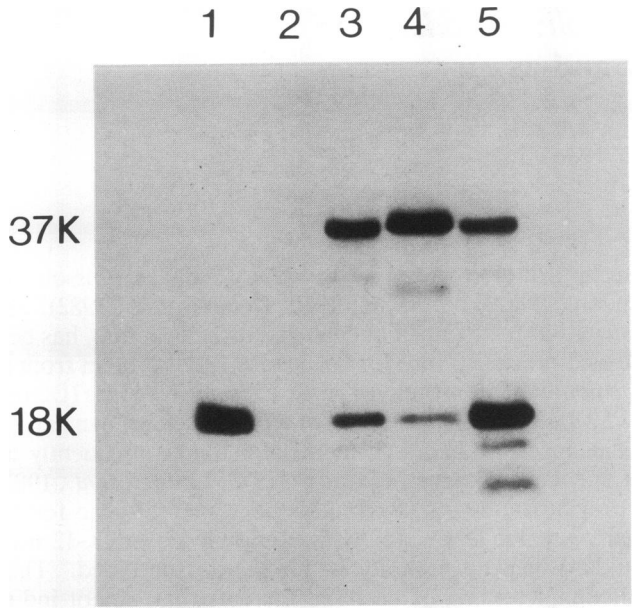

Fig. 1. Immunoblot of proteins in cell extracts of various *E. coli* strains. A nitrocellulose blot of an SDS-polyacrylamide gel was probed with rabbit antibody, followed by ^{125}I-labelled anti-rabbit IgG. **Lane 1:** control, purified O^6-methylguanine-DNA methyltransferase (0.1 μg); **lane 2:** *E. coli* F26; **lane 3:** F26, after treatment with 1 μg/ml MNNG for 1 h at 37°C; **lane 4:** *E. coli* F26 carrying plasmid pCS68, no MNNG treatment; **lane 5:** *E. coli* BS21, an *adc* mutant, constitutively expressing the adaptive response. **Lanes 2–5** each contained 100 μg cellular protein.

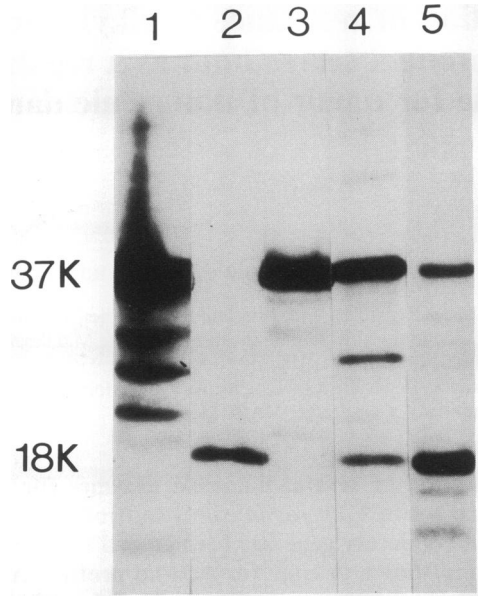

Fig. 2. Processing of the 37-kd protein in cell extracts of *E. coli* BS21. Experimental conditions for immunoblotting were as described in Figure 1. **Lane 1:** lysate of frozen cell pellet with hot 10% SDS. The trailing observed is due to the high viscosity of the lysate. **Lane 2:** purified 18-kd O^6-methylguanine-DNA methyltransferase. **Lane 3:** lysate of freshly collected cells with hot 10% SDS. **Lane 4:** cell extract, SDS added 15 min after disruption of cells and centrifugation at 4°C. **Lane 5:** extract of frozen cells, SDS added 30 min after cell disruption.

and rules out the possibility that the antibodies are recognizing a separate 37-kd protein contaminant in the apparently homogeneous methyltransferase preparation. Further, the 37-kd protein band was not due to a dimer of the 18-kd protein, since the protein was resistant to boiling in the presence of 2% SDS and 5% 2-mercaptoethanol. It was concluded that the 37-kd band must represent an inducible protein that cross-reacts serologically with the 18-kd methyltransferase.

During adaptation of wild-type cells with MNNG, the 37-kd protein could be detected in increased amounts 4–5 min after initiation of treatment; it reached a high level after 20 min and remained at this elevated plateau for 2–3 h after the removal of MNNG (data not shown). The high cellular levels of the activities associated with the adaptive response in wild-type cells treated with alkylating agents (Karran *et al.*, 1982) can also be obtained in bacteria that have not been exposed to such agents but instead transformed with a multicopy plasmid carrying the *ada*$^+$ gene (Sedgwick, 1983), and in *adc* mutants which constitutively express the adaptive response (Sedgwick and Robins, 1980). Extracts of the latter two types of cells were analysed with the anti-transferase antibody. In both cases, two distinct major bands corresponding to a 37-kd and an 18-kd protein were observed (Figure 1, lanes 4 and 5). Thus, both polypeptides were present in extracts of over-producing strains which had not been treated with alkylating agents. In addition to the two major polypeptides, longer autoradiographic exposures revealed a few minor bands of serologically cross-reacting material, all of which were smaller than the 37-kd protein, in particular in cell extracts of the constitutive strain (Figure 1, lane 5).

Proteolytic cleavage of the 37-kd protein

The Western blot in Figure 1 shows representative data on cell extracts of various *E. coli* strains. The extracts were prepared by standard biochemical techniques, that is, the bacteria were sonicated briefly in an ice-cold neutral buffer supplemented with EDTA and the serine protease inhibitor phenylmethylsulfonyl fluoride (PMSF). After removal of cellular debris by centrifugation, aliquots of the extracts were used immediately for SDS-gel electrophoresis and enzyme assays. During these studies, it became apparent that the relative proportion of 37-kd to 18-kd protein varied among different preparations, and that the 37-kd protein appeared to be rapidly processed to the 18-kd form after breakage of the cells (Figure 2). Freezing and thawing of bacteria or cell extracts promoted this conversion. When living cells were lysed directly with a hot 10% SDS solution, the 37-kd protein was the predominant form (Figure 2, lane 3). Even in such cell lysates, smaller forms of serologically cross-reacting material were observed, indicative of some proteolytic processing of the 37-kd protein, but there was little or none of the 18-kd form present (Figure 2).

Attempts to inhibit the processing of the 37-kd protein to an 18-kd fragment in cell extracts by specific protease inhibitors were negative. Thus, both polypeptides were observed on Western blots of extracts made in the presence of 0.5 mM PMSF, 0.5 mM tosyl-lysine chloromethyl ketone, 10 μg/ml pepstatin, 50 μg/ml leupeptin, or 10 μg/ml chymostatin, and the relative proportion of 18-kd material increased on storage of the extracts. Further, the conversion of the 37-kd to the 18-kd form occurred in a similar fashion in cell extracts of an adapted *E. coli lon* mutant, so the processing could not be ascribed to the Lon protease (Mizusawa and Gottesman, 1983).

Subcloning of the ada$^+$ gene and characterization of the gene product

A small region of the *E. coli* chromosome containing the

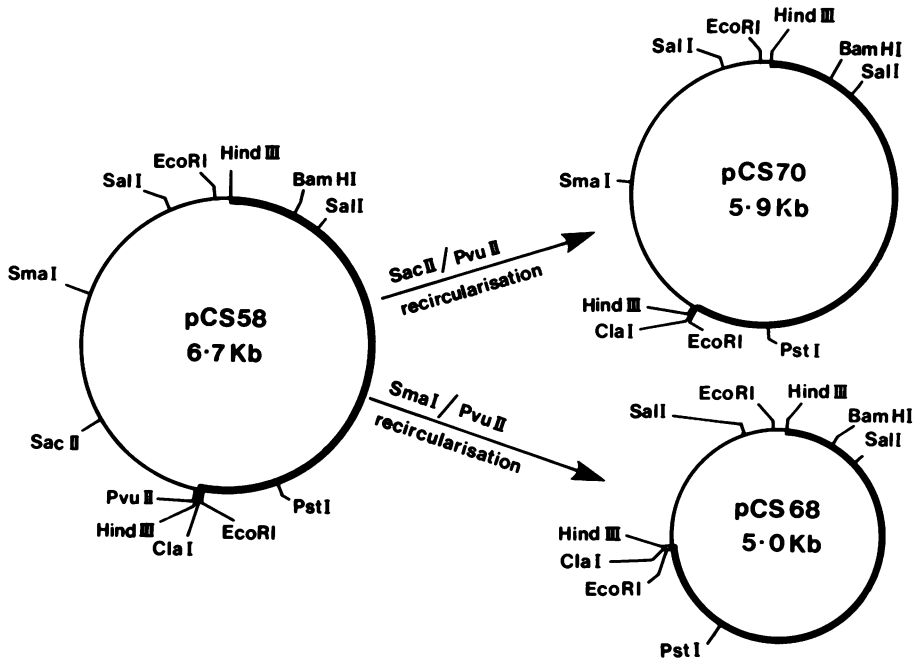

Fig. 3. Physical maps of hybrid plasmids. pCS70 and pCS68 were constructed by recircularisation of the *Sac*II/*Pvu*II and *Sma*I/*Pvu*II fragments of pCS58, respectively. Heavy lines indicate the vector pAT153 DNA and light lines the inserts of *E. coli* F26 chromosomal DNA.

ada$^+$ gene was previously cloned into the plasmid vector pAT153 to generate the recombinant plasmid pCS58 (Sedgwick, 1983). The cloned bacterial DNA encoded three different polypeptides, as analysed by the maxicell method of Sancar *et al.* (1981). Two of these, of 37 kd and 27 kd, apparently were products of a small operon and were both absent when the *ada*$^+$ gene was inactivated by transposon insertion. The third polypeptide of 42 kd was present in trace amounts only, and was not associated with the adaptive response. To investigate the roles of the *ada*$^+$ gene product(s) in further detail, subclones of the plasmid pCS58 were constructed as illustrated in Figure 3. Maxicell analysis (Figure 4) of one subclone, pCS70, showed synthesis of 37-kd and 27-kd polypeptides, but no 42-kd molecules. The smaller plasmid, pCS68, encoded only the 37-kd protein. These data show that the 37-kd and 27-kd proteins are encoded by two separate but adjacent genes. The subclones pCS70 and pCS68 were indistinguishable from the parent plasmid in their ability to confer resistance both to the killing and to the mutagenesis caused by MNNG in six different *ada* mutants (PJ1 to PJ6 of Jeggo, 1979). Furthermore, enzyme assays on cell extracts of BS24 (an *ada*-5 strain) carrying any one of the three *ada*$^+$ plasmids, showed elevated levels both of the O^6-methylguanine-DNA methyltransferase (25 ± 2 units/mg protein, compared with <1 unit/mg in control extracts of the same strain carrying pAT153) and the *alkA*$^+$-encoded DNA glycosylase (1.1 ± 0.2 units/mg, compared with 0.2 units/mg in control cell extracts). Thus, the regulatory gene of the adaptive response codes for a 37-kd polypeptide.

The size correspondence between the *ada*$^+$ gene product and the 37-kd protein recognized by the anti-transferase antiserum (Figure 1) suggests that this cross-reacting polypeptide might be the Ada protein. In agreement with this notion, the ^{35}S-labelled 37-kd protein encoded by pCS68 and pCS70 in maxicell preparations was found to be partly precipitated by the antiserum, whereas the 27-kd polypeptide encoded by pCS70 and the plasmid-encoded β-lactamase were not detectably recognized (Figure 5). Furthermore, the control plasmid pDR1453 (Sancar and Rupp, 1979), which encodes the RecA protein, yielded no polypeptide that could be precipitated by the antiserum (Figure 5). These data indicate that the 37-kd polypeptide which cross-reacts serologically with O^6-methylguanine-DNA methyltransferase is identical with the *ada*$^+$ gene product. A complicating factor, however, was that the anti-transferase antibodies had relatively inefficient immunocomplexing ability in solution and did not neutralize enzymatic activity in standard methyltransferase assays. For this reason, an independent confirmation of the main results seemed necessary.

Nucleic acid hybridizations

The sequences of the N-terminal region and the active site of the 18-kd O^6-methylguanine-DNA methyltransferase protein have been determined (Demple *et al.*, in preparation). These amino acid sequences allowed the synthesis of two different mixed oligonucleotides, corresponding to nucleic acid sequences within the structural gene for the transferase. The two oligonucleotide preparations were ^{32}P-labelled with polynucleotide kinase and employed as hybridization probes with nitrocellulose-bound DNA from a variety of plasmids. Under stringent conditions, the two probes hybridized efficiently both to the pCS68 plasmid, which encoded just the 37-kd Ada polypeptide and vector proteins (see Figure 4), and a much larger plasmid pCS33 (Sedgwick, 1983) containing *ada*$^+$ and several other adjacent bacterial genes (Figure 6). No detectable hybridization of the probes was observed with the vector pAT153, or with two plasmids containing *E. coli* chromosomal DNA in the region of the *recA*$^+$ gene (Figure 6). These concordant results, obtained with two independent synthetic oligonucleotide probes, show that the DNA sequences encoding the 18-kd transferase are present in the pCS68 plasmid.

Southern blot analysis of restriction enzyme digests of *E. coli* DNA showed only a single component hybridizing to

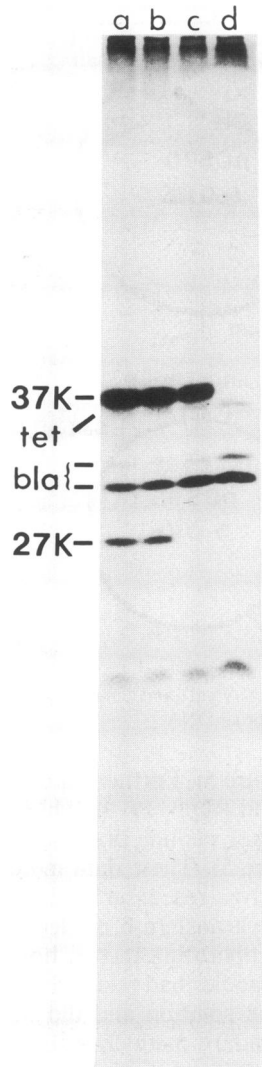

Fig. 4. Polypeptides encoded by pCS58 and its subclones were labelled with [^{35}S]methionine by the maxicell technique (Sancar *et al.*, 1981) and analysed on SDS-polyacrylamide gels. **Lane a**, pCS58; **b**, pCS70; **c**, pCS68; **d**, pAT153. **Lane a**, but not **lanes b–d**, contained a faint 42-kd band more easily detected at longer exposures (Sedgwick, 1983). The proteins labelled as tet and bla are products of the vector genes conferring tetracycline and ampicillin resistance, respectively. The mol. wt. markers were bovine serum albumin (mol. wt. 66 000), ovalbumin (45 000), carbonic anhydrase (29 000), trypsinogen (24 000), β-lactoglobulin (18 400) and lysozyme (14 300).

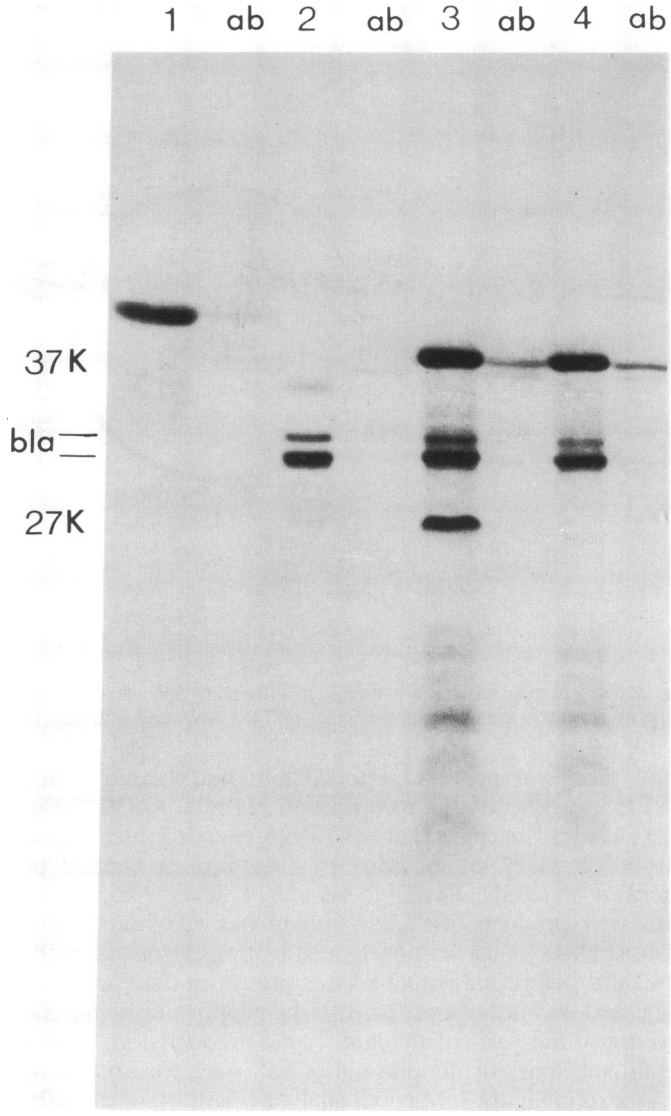

Fig. 5. Immunoprecipitation of ^{35}S-labelled proteins encoded by the various plasmids from diluted SDS lysates of maxicell preparations. **Lane 1:** pDR1453 (*recA*$^+$, this vector DNA confers tetracycline resistance but not ampicillin resistance); **lane 2:** pAT153 (vector); **lane 3:** pCS70 (*ada*$^+$, and a gene for a 27-kd protein); **lane 4:** pCS68 (*ada*$^+$). Proteins complexed in solution by the anti-transferase serum were run in the adjacent lanes. For immunocomplexing, maxicell lysates were incubated with 40 volumes of 100-fold diluted antibody in 0.2 M NaCl,50 mM Tris HCl, pH 7.2,0.05% Nonidet P-40 for 16 h at 4°C. An equal volume of a slurry of protein A-Sepharose beads (Pharmacia Ltd.) was added, and the mixture agitated gently for 6 h at 20°C. The beads were washed 5 times with the NaCl-Tris-Nonidet P-40 buffer, and bound proteins eluted by boiling in SDS- and 2-mercaptoethanol-containing electrophoresis buffer. The protein samples were analysed by SDS-polyacrylamide gel electrophoresis and autoradiography.

ada$^+$ sequences (Figure 7). The cleavage pattern was characteristic of the *ada*$^+$ gene (and the adjacent chromosomal sequence) in the pCS58 hybrid plasmid (Figure 3). Thus, cleavage of *E. coli* DNA with *Hind*III generated a single hybridizing DNA fragment of 3.1 kb, while double digestion with *Hind*III and *Sal*I generated two fragments of 2.7 kb and 0.4 kb, respectively. It is concluded that only one copy of *ada*$^+$ sequences is present in the *E. coli* chromosome.

Analysis of ada mutants

Several *E. coli ada* mutants, which are unable to express the adaptive response to alkylating agents, have been isolated (Jeggo, 1979; Sedgwick and Robins, 1980). Cell extracts of such mutants contain low or undetectable levels of O^6-methylguanine-DNA methyltransferase activity, both before and after exposure of the cells to MNNG (Mitra *et al.*, 1982; T.McCarthy, unpublished data). However, analysis of five different *ada* mutants by immunoblotting showed that all these strains produced an inducible *ada*-encoded polypeptide as cross-reacting material after MNNG treatment (Figure 8). The *ada*-1 mutant strain contained a trace amount of a 37-kd protein, detectable only after long autoradiographic exposure. In contrast, the four strains, *ada*-3, *ada*-5, *ada*-6 and BS23, all had substantial amounts of 37-kd protein, albeit less than the adapted F26 *ada*$^+$ strain used as control. We conclude that the mutant *ada* genes are, to a considerable extent, transcribed and translated.

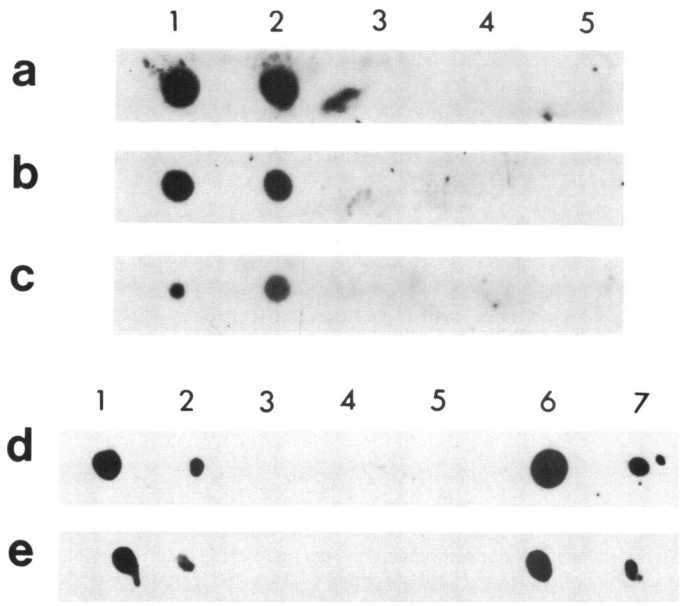

Fig. 6. Dot-blots of different recombinant DNA plasmids probed with ^{32}P-labelled synthetic oligonucleotides. **Panels a–c** were hybridized with the mixed oligonucleotide corresponding to the active site of the 18-kd methyltransferase, **panels d** and **e** with the probe corresponding to the N-terminal region of the same protein. The plasmids were **1** and **6:** pCS33 (containing *ada*$^+$ and adjacent genes), **2** and **7:** pCS68 (containing *ada*$^+$), **3:** pAT153 (vector), **4:** pDR1453 (containing *recA*$^+$), **5:** pMH21 (containing fragment from *recA*$^+$ region). Filters were washed at 40°C (**a** and **d**), 44°C (**b**), or 46°C (**c** and **e**). In **panels d** and **e**, the plasmid DNA in **lanes 1–5** had been linearized by *Bam*HI treatment before application to the nitrocellulose filter.

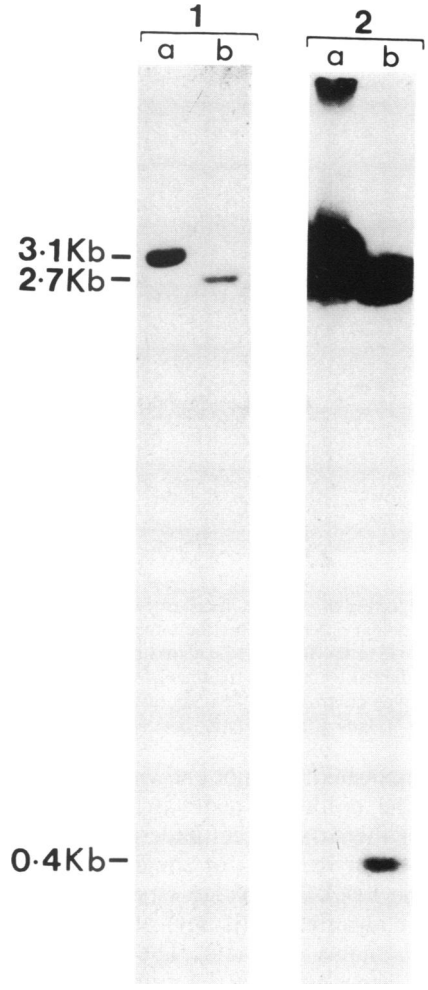

Fig. 7. Southern blot of restriction enzyme digests of *E. coli* F26 DNA probed with the nick-translated 3.1-kb *Hind*III fragment of pCS58. The digests of F26 DNA were: **lane a**, *Hind*III (1.5 units/μg DNA for 2 h at 37°C); **b**, *Hind*III (1.5 units/μg) and *Sal*I (1 unit/μg). Two exposure times are shown; 1 corresponds to 3 h, and 2 to 17 h.

Discussion

The adaptive response to alkylating agents is positively controlled by the *ada*$^+$ gene. Overproduction of the Ada protein by incorporation of the gene into a multicopy plasmid leads to elevated expression of the activities associated with the response, such as the *alkA*$^+$-encoded DNA glycosylase, even in the absence of alkylating agents (Sedgwick, 1983). The cloned *ada*$^+$ locus was previously found to produce two polypeptides of 37 kd and 27 kd, both of which were absent on transposon inactivation of *ada* (Sedgwick, 1983). By further subcloning, it has now been shown that these two polypeptides are products of two adjacent genes in a small operon. The first gene in the operon encodes a 37-kd protein, and this gene by itself suffices to complement *ada* mutations. Thus, it has been identified as the *ada*$^+$ gene. Similar results have also been obtained by LeMotte and Walker (personal communication).

Several *ada* mutants were found to produce an inducible, but presumably altered 37-kd protein. Since the *ada* strains were originally obtained by mutagenesis of *E. coli* with MNNG (Jeggo, 1979), which mainly causes transition mutations, this result might have been expected. It raises the prospect, however, that the *ada* mutants hitherto available may be 'leaky' with regard to some of the multiple fractions associated with the Ada gene product. Moreover, read-through of an altered *ada* gene after induction would presumably lead to an increased expression of the adjacent gene for a 27-kd protein that is part of the same operon. Our data do not define the role of that gene. However, Kataoka and Sekiguchi (personal communication) have recently identified it as *alkB*$^+$, another gene which contributes to the resistance of *E. coli* towards alkylating agents (Kataoka *et al.*, 1983).

The most unexpected result in our present study is that the O^6-methylguanine-DNA methyltransferase activity of *E. coli* is associated with the *ada*$^+$ gene product. Synthetic oligonucleotides corresponding to known partial amino acid sequences of the isolated enzyme were found to hybridize specifically to plasmid DNA containing the *ada*$^+$ gene. These results have recently been directly confirmed by DNA sequencing, in that 105 nucleotide residues within the *ada*$^+$ structural gene correspond to 35 amino acid residues of the N terminus of the purified 18-kd protein (Demple *et al.*, in preparation). Further, antibodies against the methyltransferase specifically recognize the *ada*$^+$ gene product.

A complicating factor in these experiments has been the extreme lability of part or all of the *ada*$^+$-encoded protein in cell extracts. The 37-kd gene product is processed to an 18-kd active domain, which corresponds to the previously isolated transferase enzyme. This is analogous to the presence of some peptide hormones as larger intracellular prohormones in mammalian cells, which are difficult to extract in intact form except by using denaturing solvents (Haritos *et al.*, 1984). The situation fortunately seems to be rare or unique in *E. coli*, and it makes the biochemical characterization of the Ada pro-

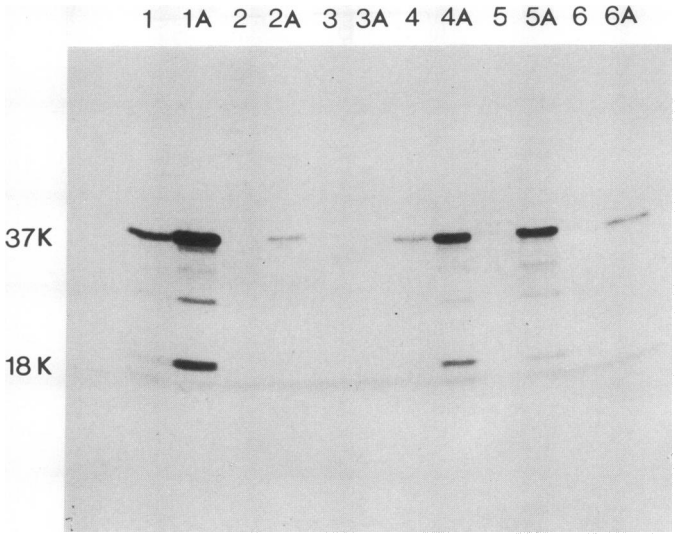

Fig. 8. Immunoblotting of proteins in cell extracts of various *E. coli ada* mutants. Experimental procedures are the same as those given in Figure 1. For each individual strain, the left lane shows unadapted cells, and the right lane cells adapted by MNNG treatment for 1 h. **Lanes 1** and **1A:** strain F26 (wild-type control); **lane 2:** BS23 (*ada*-7); **lane 3:** PJ1 (*ada*-1); **lane 4:** PJ3 (*ada*-3); **lane 5:** PJ5 (*ada*-5); **lane 6:** PJ6 (*ada*-6).

tein more complicated. It is not known at present if a separate protease in the crude cell extracts is responsible for the cleavage, or if the Ada protein undergoes self-processing, as has been observed recently for some other regulatory proteins, e.g., the LexA and phage λ repressors (Little, 1984). Moreover, the significance (if any) of this processing of the Ada protein remains uncertain. The rapid cleavage of the 37-kd protein generates an active fragment one-half the size of the *ada*$^+$ gene product with a new, unique N terminus. The 37-kd and 18-kd forms of the protein repair O^6-methylguanine in DNA in an indistinguishable fashion, with rapid transfer of the methyl group to a cysteine residue accompanied by suicide inactivation of the functional moiety (our unpublished data). O^4-Methylthymine is repaired in an analogous fashion (McCarthy *et al.*, 1984). Moreover, a methyltransferase activity for DNA phosphotriesters, induced as part of the adaptive response (McCarthy *et al.*, 1983), has also been shown to reside in the Ada protein (T.McCarthy, unpublished data).

The Ada protein plays a key role in the adaptive response to alkylating agents, apparently in a similar way to the RecA protein during the SOS response. Both proteins serve as regulatory effectors of inducible repair responses, control their own synthesis and, in addition, have associated activities needed for the cellular defence against environmental mutagenic agents. Further characterization of the structure and functions of the Ada protein may serve to elucidate the cellular mechanisms for induction of resistance to alkylating agents.

Materials and methods

Strains

The bacterial strains and the plasmid pCS58 containing the *ada*$^+$ region of the *E. coli* chromosome have been described previously (Jeggo, 1979; Sedgwick, 1983). The *recA*-related plasmids pDR1453 (Sancar and Rupp, 1979) and pMH21 (Yarranton and Sedgwick, 1982) were from S.G.Sedgwick.

Subcloning the ada$^+$ *gene and screening for the Ada phenotype*

Recombinant DNA techniques were as described by Maniatis *et al.* (1982). pCS58 DNA (Sedgwick, 1983) was digested with *Sma*I and *Pvu*II, or *Sac*II and *Pvu*II. The protruding ends of the *Sac*II digest were made blunt ended with T4 DNA polymerase. The fragments were recircularised by T4 DNA ligase and transformed into an *ada* mutant. Subcloned DNAs were screened for their ability to convey resistance to the toxicity and mutagenicity of MNNG to six different *ada* mutants by replica plating tests (Sedgwick, 1983). The proteins encoded by the plasmids were identified by the maxicell technique of Sancar *et al.* (1981).

Nucleic acid hybridizations

Mixed oligodeoxyribonucleotides, 14 residues long, were synthesized by the phosphotriester procedure (Gait *et al.*, 1982) and labelled radioactively (0.7−2 μCi/pmol) with [γ-^{32}P]ATP (Amersham, >5 μCi/pmol) and polynucleotide kinase. One set of oligonucleotides (employing all coding possibilities) corresponded to a peptide sequence at the active centre of the 18-kd O^6-methylguanine-DNA methyltransferase (Demple *et al.*, in preparation) and had the coding sequence:

```
          G
      C   C
5'-A T T C C A T G T C A T A G
      A   T   C   G C
```

The other oligonucleotide preparation was representative of the more probable subset of sequences (Konigsberg and Godson, 1983) expected to encode a peptide close to the N terminus of the 18-kd protein. It had the (anti-coding strand) sequence:

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5-T C G T C G C C C A G C A G
      A   A
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For dot-blots, purified plasmid DNA, 0.5 μg in 1 μl 1 M NaCl,10 mM Tris-HCl, pH 8,1 mM EDTA, was applied to nitrocellulose filters, air-dried, denatured by exposure to 0.5 M NaOH-1.5 M NaCl for 3 min, washed with 0.5 M Tris-HCl, pH 8.5,1.5 M NaCl and 0.3 M NaCl,0.03 M Na_3-citrate, dried, and baked at 80°C for 4 h in a vacuum oven. The filters were preincubated for 2 h in 0.9 M NaCl,0.09 M Tris-HCl, pH 7.5,6 mM EDTA, 0.5% Nonidet P40,0.2% SDS,2 x Denhardt's solution containing 100 μg/ml heat-denatured salmon sperm DNA and 35 μg/ml yeast tRNA. Hybridizations were conducted for 1 h at 32°C in the same solution supplemented with one of the radioactively labelled mixed oligonucleotides, at 1 pmol/ml for the N-terminal probe, or 10 pmol/ml for the active centre probe. After hybridizations, the filters were washed with 0.9 mM NaCl,0.09 M Na_3-citrate, twice at 32°C and then three times at a higher temperature (see legend to Figure 6) and finally analysed by autoradiography.

Southern blots were made according to standard procedures (Maniatis *et al.*, 1982).

Enzymes and cell extracts

O^6-Methylguanine-DNA methyltransferase was isolated in homogeneous form from *E. coli* BS21, a strain constitutive for the adaptive response, and assayed as described previously (Demple *et al.*, 1982). The *alkA*$^+$ gene product, 3-methyladenine-DNA glycosylase II, was assayed by its ability to release 3-methylguanine from alkylated DNA (Karran *et al.*, 1982). Restriction enzymes were purchased from New England Biolabs Inc. and T4 polynucleotide kinase from Boehringer.

Cell lysates and extracts were prepared from exponential cultures of *E. coli* growing in a glucose-minimal salts medium at 37°C. For adaptation, cells were treated with MNNG (1 μg/ml) for 1 h prior to collection. The cultures were chilled, and the bacteria harvested by low speed centrifugation at 4°C and washed with phosphate-buffered saline solution. In some cases, the bacterial pellets were frozen in a dry ice-acetone bath and stored at −80°C. Cell lysates were made by addition of 3 vol of 10% SDS of 70°C to the cold, freshly collected cell pellet or to frozen cells. The viscous lysates were diluted with 4 vol 5% 2-mercaptoethanol and heated at 100°C for 5 min prior to analysis by SDS-gel electrophoresis and immunoblotting. For extracts, cell pellets were suspended in 10 volumes of ice-cold extraction buffer containing 0.3 M KCl,50 mM Tris-HCl, pH 7.4,10 mM dithiothreitol,1 mM EDTA, 0.5 mM PMSF, and the cells were disrupted by three 5 s ultrasonic treatments. After centrifugation for 10 min at 12 000 *g* and 4°C to remove debris, and subsequent removal of an aliquot of the extract for protein and enzyme assays, SDS (final concentration 2%) and 2-mercaptoethanol (final concentration 5%) were added and the samples heated at 100°C for 5 min.

Antibody production

A New Zealand rabbit was injected intercutaneously and subcutaneously at several sites with 30 μg O^6-methylguanine-DNA methyltransferase emulsified with 3 volumes of complete Freund's adjuvant. Subsequent booster injections at 4 week intervals were given with 15 μg transferase emulsified in incomplete

Freund's adjuvant. Serum was collected 10 – 12 days after a booster injection.

Immunoblotting procedures

SDS-polyacrylamide slab gel electrophoresis was performed according to Laemmli (1970). Mol. wts. of polypeptides were estimated from their migration rates in comparison with a set of reference proteins (see legend to Figure 4). Immunoblotting was carried out according to Towbin *et al.* (1979). The incubation and washing buffers contained 200 mM NaCl, 50 mM Tris-HCl, pH 7.2, and 0.05% Nonidet P-40. ^{125}I-Labelled goat anti-rabbit antibodies were purchased from Amersham. Incubations with the primary and secondary antibody were conducted for 24 h each, at 8°C and 20°C, respectively.

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