AD-A192 002 ALKYLATION INDUCED DNA REPAIR AND MUTAGENESIS IN ESCHERICHIA COLI(U) NORWEGIAN DEFENCE RESEARCH ESTABLISHMENT KJELLER G EVENSEN 23 NOV 87 1/1

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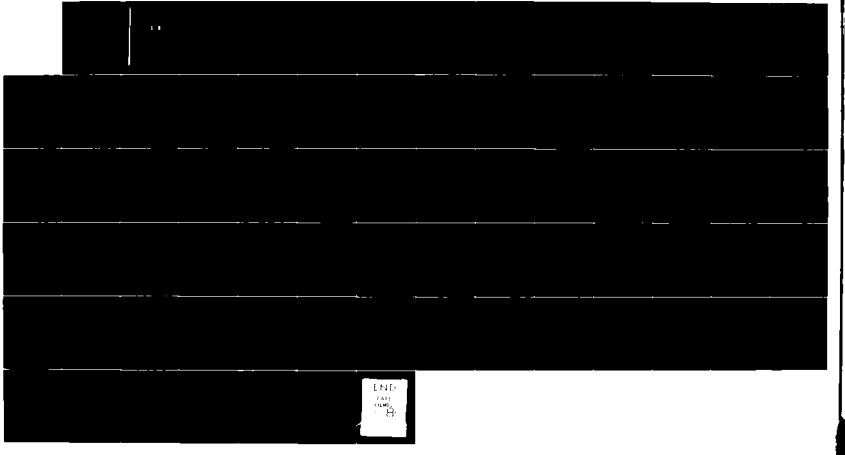

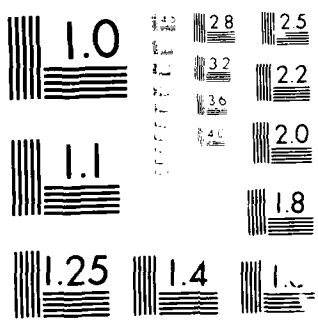
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BY

GRETHE EVENSEN

NDRE/PUBL-87/1001

ISSN 0800-4412

FORSVARETS FORSKNINGSINSTITUTT

NORWEGIAN DEFENCE RESEARCH ESTABLISHMENT

P O Box 25 – N-2007 Kjeller, Norway

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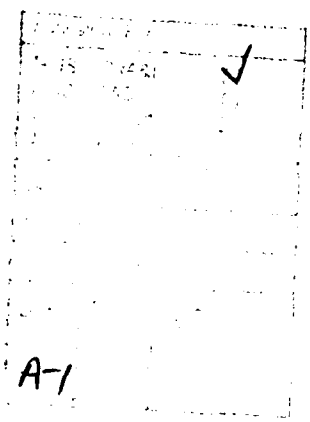

NDRE/PUBL-87/1001
ISSN 0800-4412

FORSVARETS FORSKNINGSINSTITUTT
NORWEGIAN DEFENCE RESEARCH ESTABLISHMENT
P O Box 25 - N-2007 Kjeller, Norway

November 1987

NORWEGIAN DEFENCE RESEARCH ESTABLISHMENT (NDRE)
FORSVARETS FORSKNINGSINSTITUTT (FFI)

POST OFFICE BOX 25
N-2007 KJELLER, NORWAY

UNCLASSIFIED

SECURITY CLASSIFICATION OF THIS PAGE
(when data entered)

REPORT DOCUMENTATION PAGE

1) PUBL/REPORT NUMBER	2) SECURITY CLASSIFICATION	3) NUMBER OF PAGES
NDRE/PUBL-87/1001	UNCLASSIFIED	74
1a) JOB REFERENCE	2a) DECLASSIFICATION/DOWNGRADING SCHEDULE	
FFITOX/485/141	-	

4) TITLE

ALKYLATION INDUCED DNA REPAIR AND MUTAGENESIS IN *ESCHERICHIA COLI*

5) NAMES OF AUTHOR(S) IN FULL (surname first)

EVENSEN Grethe

6) DISTRIBUTION STATEMENT

Approved for public release. Distribution unlimited.
(Offentlig tilgjengelig)

7) INDEXING TERMS

IN ENGLISH:	IN NORWEGIAN:
a) DNA repair	DNA reparasjon
b) Escherichia coli	b) Escherichia coli
c) Alkylating agents	c) Alkylerende agens
d) DNA glycosylases	d) DNA glykosylaser
e) 3-methyladenine	e) 3-metyladenin

THESAURUS REFERENCE: -

8) ABSTRACT (continue on reverse side if necessary)

This report summarizes studies on repair of methylmethane-sulfonate (MMS) alkylation lesions in DNA of the bacterium Escherichia coli. It shows that E.coli has two distinct 3-methyladenine (m^3A) DNA glycosylase activities; one is constitutively expressed and encoded by the tag gene (TagI), whereas the other is inducible and encoded by alkA (TagII). The tag glycosylase is identified radiochemically as a 21 kdal protein whereas the alkA product is a 30 kdal protein. It is induced upon exposure of the cells to low levels of alkylating agents, a treatment that induces the adaptive response. TagII is not under control of recA, necessary to induce the mutagenic SOS response. TagI appears responsible for rapid repair of m^3A alkylation products in unadapted cells. The inducible enzyme, TagII, is required for killing adaptation to alkylation resistance and for repair of potentially lethal lesions not recognized by the con-

9) DATE	AUTHORIZED BY (This page only)	POSITION
23 Nov 87	Erik Klippenberg	Director

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(when data entered)

ABSTRACT (continued)

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PREFACE

The present work was carried out at The Division for Environmental Toxicology of The Norwegian Defence Research Establishment in the period from 1982 to 1985, supported by The Norwegian Council for Scientific and Industrial Research (NTNF) and The Norwegian Defence Research Establishment.

It is a pleasure to express my gratitude to Professor dr. philos. Erling Seeberg for his continuous support and valuable advice throughout the present work. I am further grateful to my colleague and friend Inga Kaasen for her participation in the investigation which resulted in paper 2 and for all help.

Finally, I would thank Professor dr. philos. Wenche Blix Gundersen at the University of Oslo for her interest throughout the work.

OSLO, September 1987 Grethe Evensen

CONTENTS

RESEARCH REPORTS

Paper 1 Evensen, G. and E. Seeberg. 1982.
Adaptation to alkylation resistance involves the induction of a DNA glycosylase.
Nature 296:773-775.

Paper 2 Kaasen, I., G. Evensen and E. Seeberg. 1986.
Amplified expression of the *tag*$^+$ and *alkA*$^+$ genes in *Escherichia coli*: Identification of gene products and effects on alkylation resistance.
J. Bacteriol. 168:642-647.

Paper 3 Evensen, G. 1985.
Induction of 3-methyladenine DNA glycosylase II is *recA*$^+$ independent.
Mutation Res. 146:143-147.

Paper 4 Evensen, G. and E. Seeberg.
AP-sites as mutagenic intermediates. A direct role of DNA glycosylases in mutagenesis (submitted).

ALKYLATION INDUCED DNA REPAIR AND MUTAGENESIS IN ESCHERICHIA COLI

SUMMARY

This report summarizes studies on repair of methylmethane-sulfonate (MMS) alkylation lesions in DNA of the bacterium Escherichia coli. It shows that E.coli has two distinct 3-methyladenine (m^3A) DNA glycosylase activities; one is constitutively expressed and encoded by the tag gene (TagI), whereas the other is inducible and encoded by alkA (TagII). The tag glycosylase is identified radiochemically as a 21 kdal protein whereas the alkA product is a 30 kdal protein. It is induced upon exposure of the cells to low levels of alkylating agents, a treatment that induces the adaptive response. TagII is not under control of recA, necessary to induce the mutagenic SOS response. TagI appears responsible for rapid repair of m^3A alkylation products in unadapted cells. The inducible enzyme, TagII, is required for killing adaptation to alkylation resistance and for repair of potentially lethal lesions not recognized by the constitutive enzyme in unadapted cells. Persisting m^3A alkylation products in DNA are shown to be cytotoxic for cells but not mutagenic. It is indicated that DNA glycosylases have a direct role in mutagenesis by creating AP-sites as premutagenic lesions, processed by the SOS system. Increased mutations in tag or alkA mutants can be ascribed to more rapid induction of the SOS response by persisting 3-methylpurines.

1 INTRODUCTION

1.1 General introduction

The genetic material (DNA) is damaged by several agents present in the environment such as UV-irradiation, X-rays or chemical agents. DNA damage is also formed by hydrolytic decay at physiological pH and temperature. The various kinds of DNA damage have different effects on the biological function of the DNA. Certain lesions are misread by the polymerases and lead directly to mutations, whereas others block DNA replication and gene transcription.

All living organisms have mechanisms for repair of DNA. It has been calculated that in an average year the mammalian genome of $3 \cdot 10^9$ basepairs is subjected to only about 15 basepair changes (Alberts et al, 1985). Lindahl (1982) have calculated that spontaneous loss of bases due to

depurination and depyrimidination of DNA alone amounts to about 5 thousand residues per genome per day for a mammalian cell. This means that DNA repair mechanisms are extremely efficient and that nearly all DNA lesions in a cell are being repaired.

The importance of having mechanisms for repair of DNA is indicated by studies of human patients with certain types of rare hereditary diseases caused by defects in DNA repair (Setlow, 1978). The autosomal recessive human disease Xeroderma Pigmentosum (XP) is due to defective UV-excision repair. Patients suffering from this disease are hypersensitive to UV-light and will develope cancer at an early age. Further, it has been shown that mammary carcinoma in rats can be induced by a single exposure to the carcinogen N-methyl-N-nitrosourea (MNU) due to activation of the malignant Ha-ras-1 locus. Analysis of the induced oncogenes showed that they all carried the same activating $G^{35} \rightarrow A$ mutation (Zarbl et al, 1985). These findings strongly support the concept that damage to DNA can be carcinogenic.

1.2 Excision repair

Several mechanisms exist for repair of DNA. The most important DNA repair pathway involves the excision of an altered nucleotide or base residue. Two different types of excision repair are known, classified on the basis of the mechanism which initiates the repair activity. Nucleotide excision repair is initiated by DNA nucleases that incise DNA by the specific cleavage of a phosphodiester bond adjacent to damaged residues. Base excision repair is initiated by DNA glycosylases that remove damaged or modified bases in DNA by hydrolytic cleavage of the glycosylic bond between the phosphodiester backbone and the base (Lindahl, 1982). DNA nucleases introduce single strand breaks or gaps in DNA, while DNA glycosylases introduce AP(Apurinic/Apyrimidinic)-sites. AP-sites are removed by AP endonucleases that cleave the phosphodiester backbone specifically at these sites (see section 1.4). The resulting strand breaks are repaired by DNA polymerase I and DNA ligase as shown in figure 1.1.

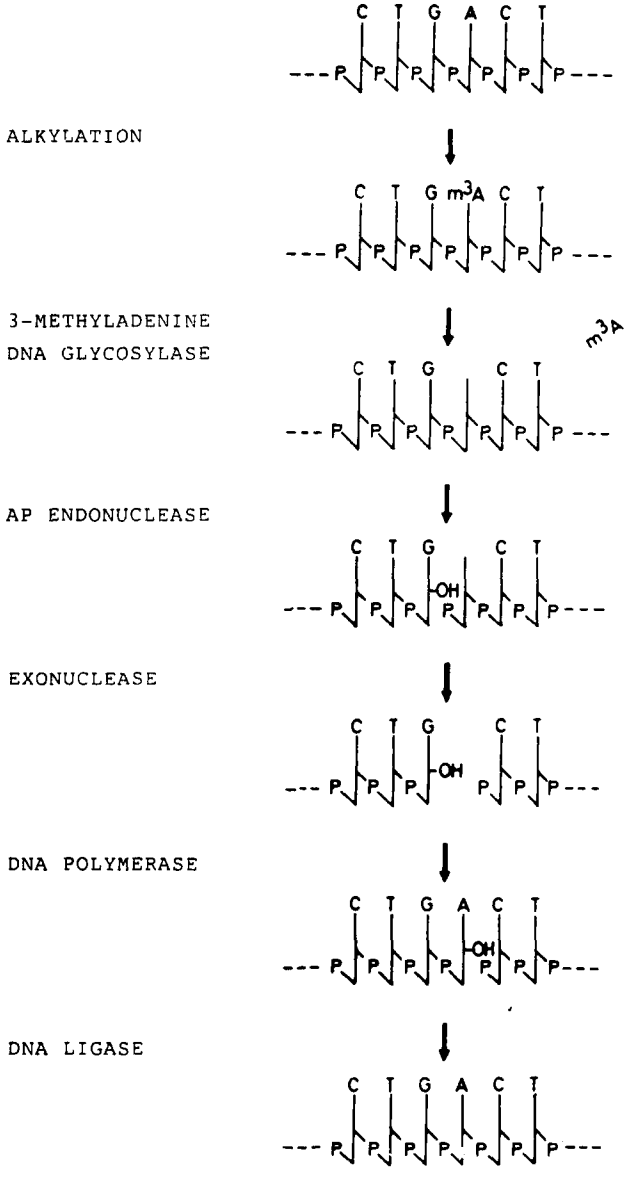

Figure 1.1 Excision repair of 3-methyladenine (m^3A) in DNA (From Lindahl, 1979).

1.3 DNA glycosylases

DNA glycosylases remove damaged or modified bases in DNA by hydrolysing the N-glycosylic bond (Lindahl, 1976, 1979). They act mainly on altered or damaged nucleotides which cause minor structural alterations of the DNA-helix structure, mostly lesions caused by radiation or simple alkylating agents. Most DNA glycosylases act on a single and specific type of modified base. The physical and biochemical properties of the different DNA glycosylases are similar; the enzymes are generally of low molecular weight (18,000-31,000) and do not require cofactors for enzyme activity. DNA glycosylases are identified in all organisms so far investigated from bacteria to mammalian cells.

1.4 Repair of AP-sites in DNA

AP-sites in DNA are formed by spontaneous hydrolysis or by the action of DNA glycosylases (Lindahl, 1979). They are repaired by an excision mechanism initiated by endonucleases which specifically act on AP sites. AP endonucleases are ubiquitous, and within one organism often more than one AP endonuclease activity is present. AP endonucleases can be divided into two classes, I and II, on the basis of their enzyme activity (Linn et al, 1981). Class I enzymes cleave the phosphodiester bond 3' to the AP-site, while class II enzymes cleave 5' to the AP-site.

The two major AP endonucleases in E.coli, endonuclease VI exonuclease III (Verly and Rassart, 1975) and endonuclease IV (Ljungquist, 1977) both incise DNA 5' to the AP-site and are class II enzymes. Likewise human placental AP endonuclease (Linsley et al, 1977) and human fibroblast AP endonuclease II (Linn et al, 1981) are class II enzymes. E.coli endonuclease III (Gates and Linn, 1977), Micrococcus luteus UV endonuclease (Grossman et al, 1978) and bacteriophage T$_4$UV endonuclease (Warner et al, 1980) have DNA glycosylase as well as AP endonuclease activity. These endonucleases hydrolyse the N-glycosylic bond creating AP-sites in DNA. The AP-sites are further acted upon by the AP endonuclease activity of the enzyme which introduces breakage 3' to the AP-site (class I enzymes).

Ap endonuclease class II enzymes generate 3'-hydroxyl (OH) termini in DNA which are good primers for DNA polymerase activity (Warner et al, 1980). However, the 5'-phosphate (P) termini generated by class II enzymes are not easily removed by the 5'-3' exonuclease activity of DNA polymerase I. It may be excised by a class I AP endonuclease or alternatively by a specific 5' deoxyribosephosphatase activity recently identified by Franklin et. al. (Lindahl, personal communication).

1.5 DNA damaged by monofunctional alkylating agents

Monofunctional alkylating agents like N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and methylmethanesulfonate (MMS) introduce methylgroups at specific positions in DNA nucleotides. Alkylation occurs both in the base residues and in the phosphodiester backbone. Different sites of the purines or pyrimidines are alkylated depending on the chemical nature of the alkylating agents.

Alkylating agents can be divided into two classes on the basis of the mechanism of alkylation. One class, to which MNNG and MNU belongs, introduces methyl groups by an S_N1 mechanism, and will produce 7-methylguanine (m^7G) and 3-methyladenine (m^3A) besides a great proportion of O^6-methylguanine (m^6G). Alkylating agents like MMS and DMS introduce methyl groups by a S_N2 mechanism producing predominantly m^7G and m^3A, and only a minor proportion of m^6G. Small amounts of 7-methyladenine (m^7A), 3-methylguanine (m^3G), O^2-methylcytosine (m^2C), O^2-methylthymine (m^2T), O^4-methylthymine (m^4T) and phosphotriesters occur in DNA upon alkylation (Figure 1.2, Lawley, 1974; Strauss et al, 1975; Swenson et al, 1976; Beranek et al, 1980).

In this report I will consider only the repair of DNA and repair of methyl lesions. It is however known that DNA in the same way as with methylating agents is ethylated by the corresponding ethylating agents. Further these ethyl lesions seem to be repaired by the same enzymes repairing methyl lesions in DNA.

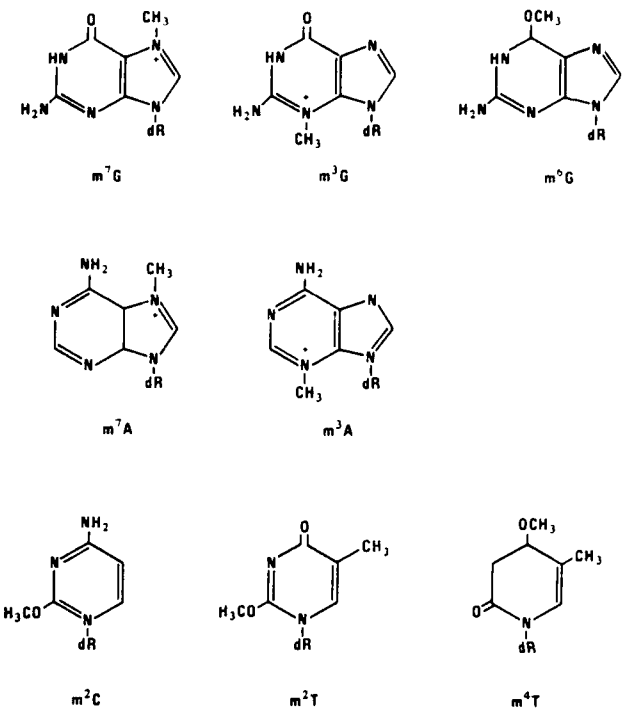

Figure 1.2. Alkylation products in DNA.

Alkylating agents are mutagens. The mutagenic effects have been ascribed largely to the ability of these compounds to alkylate DNA bases at oxygen atoms (Singer, 1976). The occurrence of the major O-alkylated base derivative (m^6G) in DNA *in vivo* has been directly correlated with the mutagenic and the carcinogenic effects of the agents (Lawley, 1980; Newbold et al, 1980; Doniger et al, 1985). m^6G appears to form base pairs with thymine to an equal extent as with cytosine during DNA replication, thus producing transition mutations (Abbott and Saffhill, 1979; Hall and Saffhill, 1979).

1.6 Repair of alkylated DNA in E.coli

1.6.1 Repair of 7-methylguanine (m^7G)

Quantitatively the major alkylation product in DNA, m^7G, appears relatively harmless to the cell. m^7G does not have miscoding properties and forms hydrogenbonds normally with cytosine. The methylgroup is located in the major groove and seems to be without influence on important cellular functions like replication and transcription. m^7G is removed from DNA very slowly in vivo. Years ago it was assumed that this lesion only was removed non- enzymatically through hydrolysis of the N-glycosylic bond (Prakash at al, 1970; Lawley and Orr, 1970). However, recent studies have shown that a DNA glycosylase exists that removes m^7G (Laval et al, 1981; McCarthey et al, 1984). However, the biological significance of m^7G repair is not obvious.

1.6.2 Repair of 3-methyladenine (m^3A)

Since 1970 it has been known that the major alkylation products m^3A and m^6G are rapidly removed from DNA in E.coli and mammalian cells through enzymatic processes (Lawley and Orr, 1970; Lawley and Warren, 1976; Margison and O'Connor, 1973). Strauss and collaborators (Strauss, 1962; Reiter et al, 1967; Strauss and Robbins, 1968) showed that cell free extracts of M.luteus and Bacillus subtilis introduced strandbreaks specifically into DNA treated with MMS. Partly purified enzyme fractions from E.coli were shown to contain a glycosylase which removed 3-methyladenine (Kirtikar and Goldwaith, 1974; Lindahl, 1976).

The essential role of 3-methyladenine DNA glycosylase in the cellular recovery from alkylation damage was demonstrated by studies of mutants defective in this enzyme (Karran et al, 1980). Cells defective in 3-methyladenine DNA glycosylase (tag) were deficient in removal of $m^3A^{\bullet}$ from DNA and were extremely sensitive to the lethal effects of alkylating agents, and also showed reduced ability to reactivate λ-phage treated with MMS.

1.6.3 Repair of O^6-methylguanine (m^6G), the adaptive response to alkylation damage in E.coli.

John Cairns and collaborators first observed that cells treated with a sublethal dose of alkylating agents were more resistant both to the killing and to the mutagenic effects of a challenging dose of the same agents. This phenomenon was called the adaptive response (Samson and Cairns, 1977; Jeggo et al, 1977).

Schendel and Robins (1978) showed that adapted cells were able to remove far more O^6-methylguanine residues upon a challenging dose of alkylating agents compared to non-adapted cells. Adapted cells were shown to remove as much as 3,000-10,000 m^6G residues soon after a challenging dose of MNNG. Remaining m^6G residues were removed slowly and inefficient as observed in unadapted cells (Robins and Cairns, 1979).

The mechanism for repair of m^6G was elucidated by Lindahl and coworkers (Karran et al, 1979). They showed that the methylgroups were not released as low-molecular weight material, but remained to an acid precipitable macromolecule. The macromolecule was purified as a 19,000 dalton protein, termed O^6-methylguanine DNA methyltransferase. Further, experiments showed that DNA methyltransferase transfers the methylgroup of m^6G to one of its own cystein residues (Olsson and Lindahl, 1980). The protein binds the methylgroup irreversibly and reacts only once. DNA methyltransferase has no catalytic activity and is strictly defined not an enzyme.

1.7 SOS repair in E.coli

Long before the adaptive response was discovered in E.coli, another inducible repair response called the SOS repair was described (Radman, 1974; Witkin, 1976). It is induced by several agents, like UV-radiation, cross-linking agents and alkylating agents that damage DNA or inhibit replication. The SOS response results in the induction of several unrelated functions, including prophage induction, mutagenesis, increased DNA repair and inhibition of cell division (Kenyon

and Walker, 1980; Kenyon et al, 1982). Genes coding for the DNA inducible functions, din-genes, are controlled by the recA$^+$ and the lexA$^+$ genes. In the normal state under non-induced conditions, these genes are repressed by the lexA$^+$ gene product (Little and Harper, 1979; Brent and Ptashne, 1980). Upon induction the repressor is proteolytically cleaved by an activated form of the recA$^+$ protein. This opens the operons for transcription (Little et al, 1980; Kenyon et al, 1982; Little and Mount, 1982). The molecular events leading to the sudden change in the RecA activity are not completely understood. In vitro the protease activity of RecA requires the presence of single-stranded DNA and ATP (Craig et al, 1980, 1981). The inducing signal could thus be the presence of a single-stranded region at the sites of DNA damage. Single-stranded regions are formed when DNA replication is blocked by DNA lesions (for a review see Walker, 1984).

Mutants defective in SOS processing are defective in recA, lexA and umuDC (Kato and Shinoura, 1977; Steinborn, 1978). The umuDC locus consists of two adjacent genes umuD and umuC (Elledge and Walker, 1983; Perry et al, 1985). Strains containing umuD or umuC mutations are nonmutable by a wide variety of agents. They can, however, still be mutated by agents like MNNG that induce direct mispairing lesions which do not require SOS processing for introduction of mutations (Walker, 1984). The roles of UmuD, UmuC and RecA gene-products in SOS mutagenesis are not known. UmuD and UmuC are likely involved in recA$^+$ repair pathway to polymerize bypass of lesions in the template (Walker, 1984, 1985).

1.8 Inducible responses to DNA damage in other organisms

Various organisms, both procaryotic and eucaryotic, seem to have inducible DNA repair responses. These responses to DNA damaging agents have similarities to the repair systems observed in E.coli.

1.8.1 SOS-type responses

A number of bacteria, both gram-negative and gram-positive, treated with agents like UV seem to have an inducible SOS repair response (for review see Walker, 1984). In some of these bacteria the SOS system is closely related to that of E.coli. By use of lac gene fusions it has been shown that Saccaromyces cerevisia has a set of genes which are induced in response to DNA damaging treatments. Furthermore, also mammalian cells appear to have a SOS like response to DNA damaging agents.

1.8.2 Adaptive-type responses

Adaption to higher levels of O^6-alkylguanine DNA alkyltransferase, have been observed in B.subtilis (Morohoshi and Munakata, 1983; Hadden et al, 1983) and M.luteus (Lindahl, 1982). Interestingly, the basic level of enzyme in nonadapted B.subtilis cells is significantly higher than that present in E.coli (Hadden et al, 1983). In contrast, methyltransferase activity and an adaptive response to mutagenesis by MNNG have not been detected in S.cerevisiae (Hadden et al, 1983), Haemophilus influenzae and Salmonella typhimurium (Ather et al, 1984). An enzyme activity that transfers O^6-alkyl groups to cystein in a protein acceptor molecule is present in a varity of mammalian cells, including human tissues (for review see Yarosh, 1985). The human enzyme has been extensively purified. The biochemical properties of the human and the bacterial enzymes are very similar. It is unclear whether the mammalian methyltransferases can be induced to high levels. Animal cells have much higher constitutive levels of the enzyme and an extensive induction activity as seen in E.coli does not seem to occur in mammalian cells.

3-methyladenine DNA glycosylase activities have been identified in various procaryotic and eucaryotic organisms, but it is not clear whether two distinct forms of this enzyme exist in all of these. A number of observations indicate that an inducible type II 3-methyladenine DNA glycosylase exists in other cells than E.coli (Laval et al, 1981; Cathcart and Goldwaith, 1981; Brent, 1979; Gombar et al, 1981).

1.9 Object of the present investigation

The object of the present investigation was to elucidate the biological effects of m^3A on DNA interactions. At the time this study was initiated the mechanisms for repair of alkylation lesions in DNA were known in some detail. The biological effects of m^7G and m^6G were at least partly understood; m^7G in DNA was shown to be innocuous, while m^6G was shown to be a miscoding lesion contributing to the mutagenic effects of alkylation treatment. However, much less was known about the biological effects of unrepaired m^3A. The existing data showed that this lesion was rapidly removed in every organism tested from bacteria to mammalian cells. The rapid removal of m^3A, however, made it difficult to say much about the biological effects of unrepaired m^3A in DNA; although the efficient repair indicated that m^3A had severe effect on the metabolic function of the DNA.

Elucidation of the biological effects of unrepaired m^3A was made possible by the isolation of an E.coli mutant defective in m^3A DNA glycosylase (tag), with reduced ability to remove m^3A from DNA in vivo (Karran et al, 1980). The mutant was isolated by Ingrid Øfsteng (1980) by MNNG-mutagenesis and selection for MMS sensitive mutants. MNNG is known to induce multiple mutations in DNA. To eliminate possible secondary mutations it was first necessary to isolate isogenic strains. When isogenic strains were constructed it was discovered that the original mutant had two mutations both contributing to the MMS sensitive phenotype. The initial part of this work was therefore assigned to characterize these single mutants genetically and elucidate the genetics behind the alkyl-DNA repair mechanisms. Subsequently, the biological effect of persisting m^3A in DNA was investigated.

2 RESULTS AND DISCUSSION

2.1 Two DNA glycosylases in E.coli for removal of m^3A in DNA

The original tag mutant of E.coli K-12, BK2106, was isolated on the criterium of being defective in host cell reactivation of alkylated phages and subsequently shown to be lacking 3-methyladenine DNA glycosylase activity. It was further shown to have normal ability to repair DNA damage caused by UV-light and X-rays, but to be sensitive to alkylating agents like MNNG and MMS (Karran et al, 1980). We have characterized the mutant further in this work and shown that the phenotypic properties are caused by to independent mutations, tag and ada, both contributing to the MMS sensitive phenotype (Paper 1).

When the ada mutation was eliminated by transduction the cells changed to become wild-type resistant to the lethal effects of MMS in agar medium. However, the single tag mutant thus constructed was still unable to reactivate alkylated λ-phages and showed considerable sensitivity to MMS exposure in buffer. These observations indicated the presence in E.coli of two independent pathways for repair of m^3A in DNA; one that was constitutively expressed and controlled by tag, the other inducible and controlled by ada (Paper 1). The inducible repair pathway would be turned on when the cells were exposed to alkylating agents in complete medium, but not when the cells were infected with alkylated phages. The cells would then be able to repair itself because of the inducible repair enzyme, but not infecting phage DNA since exposure of the phage would not cause cell induction. The results showed that the adaptive response used to induce m^6G DNA methyltransferase also induced high levels of m^3A DNA glycosylase activity in the tag mutant. During the course of this work other groups have also shown that E.coli has two separate enzymes for removal of m^3A residues in DNA (Karran et al, 1982; Thomas et al, 1982).

2.1.1 m^3A DNA glycosylase I (TagI)

The m^3A DNA glycosylase previously purified and characterized by Riazuddin and Lindahl (1978), termed TagI, was shown to be specific for removal of m^3A and 3-ethyladenine, and to be inhibited in a noncompetitive fashion by m^3A. This is the enzyme encoded by the tag gene.

We attempted to map the tag gene by conjugation (Evensen, unpublished). The tag mutant BK2114, which is a histidine auxotrophe (his$^-$) and resistant to streptomycin (StrR), was used as a recipient in crosses with various Hfr-strains (his$^+$, StrS) of E.coli. Recombinants were selected which were his$^+$, StrR, and tested for coconjugation of tag$^+$, as scored by proficient host cell reactivation (hcr$^+$) of alkylated phages. No hcr$^+$(tag$^+$) recombinants were found regardless of the origin of chromosome transfer. This indicated that tag$^+$ recombinants were selected against because of coinheritance of tag$^-$ and StrR and that tag was located very close to the gene for streptomycin, rpsL (72 min.). In further crosses his$^+$, nalidixine-acid resistant (NalR) recombinants were selected. The gene for nalidixine-acid resistance, gyrA, is located at about 47 min. on the E.coli map far from the rpsL gene. Recombinants which were his$^+$,NalR were tested for hcr$^+$ and StrR. The tag mutation was shown to coconjugate with rpsL at a frequency of 75%, indicating that tag is located between 70 and 74 min. on the standard E.coli K-12 map.

More recently, the tag gene has been cloned on a multicopy plasmid (Clarke et al, 1984). The resulting recombinant plasmid, pBK201, has the tag gene on a 6 kbp HindIII fragment. The presence of the tag plasmid in the cells resulted in 15-fold overproduction of TagI (Clarke et al, 1984). The position of tag on the restriction map of pBK201 was determined more exactly by mapping insertions that inactivate the glycosylase gene. The positions of the insertions showed that tag was located on a 0.9 kbp EcoRI fragment. This EcoRI fragment was subcloned into the EcoRI site of pBR322 (Paper 2). The resulting plasmid, pBK202, harbours the tag gene flanked by two 31 kbp HindIII/EcoRI fragments originating from pBR322

(Sutcliff, 1979). The presence of pBK202 resulted in much higher overproduction of TagI than the original multi-copy plasmid pBK201 (Paper 2). Increased TagI production seemed not only to be the result of reduction in plasmid size and higher copy number. Sequence data indicated that a fusion promoter had been formed which was stronger than the natural one (Steinum and Seeberg, 1986).

The tag gene product was identified radiochemically by the maxicell method (Sancar et al, 1979). SDS gel-electrophoresis of plasmid encoded proteins showed that the only gene product encoded by the cloned fragment was a 21,000 dalton protein (Paper 2). Tn1000(γδ)-insertions in tag were isolated by selecting ampicillin resistant(ApR), streptomycin resistant (StrR) transconjugants from a cross between AB2414F$^+$/ pBK202 (tag$^+$, StrS, ApR) and BK2118 (tag, alkA, StrR). The transconjugants were screened for MMS-sensitivity/resistance and plasmid DNA was isolated from sensitive clones. SDS gel-electrophoresis showed that plasmids with γδ-insertions inactivating tag did not encode the 21,000 dalton protein. TagI was thus positively identified as a 21,000 dalton protein (Paper 2). From previous data it has been shown by gelfiltration that TagI has a molecular weight of about 20,000, in agreement with our identification. The TagI enzyme was recently purified (Sakumi et al, 1986; Bjelland and Seeberg, 1987). The molecular weight of the purified protein on SDS/polyacrylamide gel was found to be 21,000 - 22,500 dalton, in agreement with our result and with the molecular weight deduced from the nucleotide sequence of the tag gene (Steinum and Seeberg, 1986).

2.1.2 m^{3}A DNA glycosylase II (TagII)

Pretreatment of tag mutant cells with MNNG (adaptation) induces high levels of m^{3}A DNA glycosylase activity. The inducible m^{3}A DNA glycosylase activity was shown to be unaffected by free m^{3}A (Paper 1), and to be considerably more heat stable than TagI (Karran et al, 1980). These results indicated that the inducible enzyme in BK2114 was not a mutated form of normal tag gene product, but a separate enzyme (termed TagII).

TagII was shown to be controlled by the *ada*$^+$ gene required for induction of m^{6}G DNA methyltransferase and the adaptive response (Paper 1). Adaptive treatment of *ada*,*tag*$^+$(BK2110) did not cause the induction of TagII activity. The observation that the *ada* mutation prevented induction of both TagII and DNA methyltransferase suggested that *ada* could be a control gene for the adaptive response and that structural genes for one or both of the enzymes could be found elsewhere on the chromosome. We therefore tested other MMS sensitive mutants to search for structural gene mutants. Previously, Sekiguchi and collaborators had isolated and characterized one mutant, *alkA*, (Yamamoto et al, 1978 and Yamamoto and Sekiguchi 1979) which was shown to have reduced survival and increased mutation frequency to MMS. However, no enzyme deficiency had been linked to the mutation. Enzyme assay of extracts from this mutant showed that the *alkA* mutant was deficient in induction of TagII but proficient in induction of m^{6}G DNA methyltransferase. Similar results were obtained with an *alkA* deletion mutant isolated by B. Duncan (Paper 1). The results indicated that *alkA* is the structural gene for TagII. This has afterwards been confirmed by other studies (McCharthey et al, 1984; Nakabeppu et al, 1984).

Karran et. al. (1980) had previously reported the existence of a minor m^{3}A DNA glycosylase activity both in BK2106 and in wild type cells. The enzyme was reported to be heat stable and can now be identified as TagII. As *alkA* cells are mutated in the structural gene for TagII, they have less residual TagII than the *ada* mutant defective in induction of TagII. This explains the greater sensitivity of the former mutant compared with the latter (Paper 1).

The *alkA* gene has recently been cloned by two different groups (Clarke et al, 1984; Nakabeppu et al, 1984a). In the plasmid from our laboratory, pBK101, the *alkA* is located on a 9.5 kbp BamH1 fragment (Clarke et al, 1984). Plasmid pBK101 restores TagII deficiency in *alkA* mutant cells, and results in 3-fold overproduction of this enzyme after alkylation induction. The *alkA* bearing fragment in pBK101 was subcloned (Paper 2).

Specific labeling of the plasmid encoded proteins by the maxicell method revealed that the alkA gene encoded a polypeptide with a molecular weight of about 30,000 (Paper 2). Nakabeppu et. al. (1984b) have sequenced the entire alkA gene. A molecular weight of 30,000 in agreement with the radiochemical identification was deduced from the sequence.

Studies of the general properties and substrate specificity of TagII have been performed recently in several laboratories (Thomas et al, 1982, McCarthey et al, 1984). It is shown to have a broad substrate specificity, unlike most other DNA glycosylases. It catalyses the release of the N-alkylated purines m^3A, m^7A, m^3G, m^7G and the O-alkylated pyrimidines m^2C and m^2T. The methylgroups of m^3A, m^3G, m^2C and m^2T all protrude into the minor groove of the double helix (Figure 2.1). McCarthey et. al. (1984) have proposed that the role of TagII is to patroll the minor groove of the DNA helix and to remove potentially cytotoxic and mutagenic methylgroups.

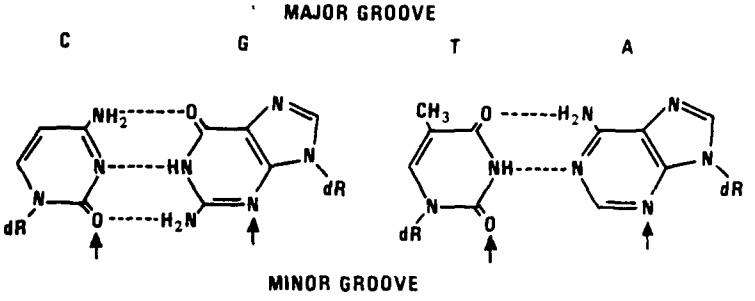

Figure 2.1 Schematic diagram of recognition sites (arrows) of TagII (From McCarthey et al,1984).

2.1.3 Biological effects of amplified TagI and TagII

When cells mutated in tag were transformed with the tag$^+$ plasmid, pBK202, they showed w.t. resistance to MMS (Clarke et al, 1984). Surprisingly, when the tag$^+$ plasmid was introduced into the alkA mutant, the alkylation sensitive phenotype of the alkA mutant was almost completely suppressed (Paper 2). The alkA suppressing effect of the tag$^+$ plasmid must be due to overproduction of TagI by the multicopy plasmid since the alkA mutant has intact tag gene and w.t. expression of TagI. The results indicate that TagI and TagII have overlapping roles in repair of alkylated DNA and that the existence of one of the two enzymes will complement the deficiency of the other.

When alkA mutant cells were transformed by the alkA$^+$ plasmid, pBK101, the alkylation sensitive phenotype of alkA was not fully suppressed (Clarke et al, 1984). This is not caused by incomplete expression of TagII activity from the plasmid since TagII activity was shown to be fully restored, even overproduced, by the alkA plasmid in the mutant (Paper 2). To minimize the size of chromosomal insert, the alkA$^+$ gene was subcloned. The resulting smaller alkA$^+$ plasmids, however, gave even less suppression of alkA sensitivity. When w.t. cells were transformed with alkA$^+$ plasmids it appeared that the alkA$^+$ plasmids sensitized cells to MMS treatment. The sensitizing effect appears to be correlated to overproduction of TagII, since the most effective overproducers of TagII were the most effective in sensitizing w.t. cells to MMS.

At present we do not have a satisfactory explanation for the sensitizing effect of amplified TagII activity. Since TagI at high levels does not have a similar effect it cannot be due to glycosylase activity per se. TagII has a broader substrate specificity compared to TagI. This implies that TagII can initiate more repair events at the same time. Too many on-going repair events can be detrimental for the cell since this will increase the possibility of double strand breaks and DNA degradation. However, at "physiological" doses of alkylation each lesion is in average several kbp apart (Karran et al, 1980). About 60-70 % of these are m^{7}G

which are poor, if at all, a substrate for TagII (Karran et al, 1980). It thus seems unlikely that too many repair events should be the reason for the sensitizing effect of TagII amplification. It could be that the alkA gene or the TagII enzyme has some other property or function in the cell which causes the negative effects upon amplification. It has been shown that m^3A in DNA will induce the SOS response (see section 2.4.2). Due to high m^3A DNA glycosylase activity and effective repair of m^3A it is possible that the SOS repair may not be induced. As a consequence, other MMS-induced lesions, that are lethal without SOS processing, could cause increased MMS-sensitivity.

2.2 Adaptation to higher resistance to alkylating agents

Mutants defective in the adaptive response were first described by Jeggo (1979). They were selected on the basis of being deficient in mutagenic adaptation, but most of them were also defective in killing adaptation. One mutant, however, was defective only in mutagenic adaptation. Jeggo et. al. (1978) have further reported that polA mutants lacking DNA polymerase I are defective in killing adaptation but that mutagenic adaptation is normal in these strains. These results indicated that mutagenic adaptation and killing adaptation was due to induction of different repair pathways.

2.2.1 Mutagenic adaptation

The first biochemical basis for adaptation were described by Schendel and Robins (1978) who showed that m^6G were removed more rapidly in adapted than in unadapted cells. As m^6G can be misread as adenine during replication making transition mutations, it thus appeared to be a good correlation between mutagenic adaptation and removal of m^7G. Moreover, normal mutagenic adaptation in polA mutants supported the view that m^6G DNA methyltransferase would be responsible for mutagenic adaptation since transferase repair of m^7G does not require DNA polymerase I.

2.2.2 Killing adaptation

Defective killing adaptation in polA mutants suggested that killing adaptation was due to the induction of an excision repair pathway requiring DNA polymerase I for repair synthesis. Since DNA polymerase I would be required to complete repair after the initial activity of TagII, we tested whether TagII defective ada and alkA mutants were shown to be defective in killing adaptation (Paper 1). Both tag mutant cells and wild type cells showed increased alkylation resistance by the adaptive treatment wheras the ada mutant was deficient and the alkA mutant was even sensitized. These results strongly supported the view that TagII may be responsible for killing adaptation to alkylation resistance.

2.2.3 Genetic control of the adaptive response

The ada locus was first identified by isolation of mutants which block the induction of the adaptive response (Jeggo, 1979). More recently, another type of ada mutant was isolated, adc which cause constitutive expression of the adaptive response (Sedgwick and Robins, 1980). The ada locus maps at 47.3 min. on the E.coli map (Sedgwick, 1982).

The ada gene was cloned and shown to code for a 39,000 dalton protein (Sedgwick, 1983). Previous attempts to separate the structural gene for m^{7}G DNA methyltransferase and the ada gene using standard genetic techniques were not successful. No mutant deficient only in expression of the transferase activity was found. Recently, Teo et. al. (1984) reported that antibodies raised against homogeneous 19,000 dalton m^{7}G DNA methyltransferase were found to cross-react with the 39,000 dalton Ada protein. This indicated that the Ada protein harboured the DNA methyltranferase activity, and that the 19,000 dalton methyltransferase was a fragment of the Ada protein arising as a result of degradation or processing of the 39,000 dalton protein at purification.

Further, the active center for the m⁷G DNA methyltransferase has been shown to be located close to the polypeptide C-terminus and to have the unusual sequence Pro-Cys-His, proceeded by a very hydrofobic region (Demple et al, 1985). The methyltransferase catalyses removal of ethylgroups from O⁶-ethylguanine at a slower rate than the removal of methylgroups from m⁷G (Sedgwick and Lindahl, 1982). In addition, it has been shown that the methyltransferase can repair O⁴-methylthymine and one of the two stereoisomers of methylphosphotriesters in DNA (McCarthey et al, 1984; McCarthey and Lindahl, 1985). The active site for repair of methylphosphotriesters is, however, different from the active site for repair of O⁶-methylguanine.

The molecular mechanism for adaptive response has now been elucidated in some detail. Previously it was known that increased dosage of *ada*$^+$ gene on a multicopy plasmid led to an increase in the levels of both m⁷G DNA methyltransferase and TagII even in the absence of DNA damaging agents (Sedgwick, 1983). However, cells containing multiple copies of the *ada* gene could be further induced by exposure to methylating agents (LeMotte and Walker, 1985). It thus seemed likely that alkylating agents could generate an inducing signal leading to the induction of *ada*. Teo et. al. (1986) have recently used the cloned *ada* and *alkA* genes and purified Ada protein in cell-free systems to identify the inducing signal. They showed that self-methylation of Ada protein by transfer of a methylgroup from a phosphotriester in alkylated DNA to a cystein residue in the protein converted it to an activator of transcription. The covalently modified Ada protein was shown to bind specifically to promoter regions containing the sequence d(AAANNAAAGCGCA) immediately upstream of the RNA polymerase binding sites. In addition a weak stimulatory effect of the unmethylated Ada protein on *in vitro* transcription was observed in good agreement with the results of Sedgwick (1983).

The results of Teo et. al. show that transfer of a methyl-group from methylphosphotriesters rather than from O⁶-methylguanine generates the signal for induction of the adaptive response. This is consistent with previous observations that some effective inducers of the adaptive response produce little O⁶-methylguanine in DNA, whereas others that produce more m⁶G, are ineffective (Jeggo, 1979; Lawley, 1974, 1976). Several recent studies have reported that deletions or sequence alterations in the C-terminal part of the Ada protein modulate its ability to serve as a regulatory factor (LeMotte and Walker, 1985; Sekiguchi et al, 1986). Interestingly, an Ada protein deleted in the C-terminal end including the cystein acceptor for the methylgroup of O^6-methylguanine was shown to be a stronger inducer of the adaptive response than the Ada protein (LeMotte and Walker, 1985 ; Teo et al, 1986).

A third gene under *ada* control, *aidB*, has been identified which alters cellular resistance to alkylating agents (Volkert and Ngyen, 1984). In addition, *ada* forms a small operon together with *alkB* (Kataoka et al, 1983; Kataoka and Sekiguci, 1985) encoding a 27,000 dalton protein that counteract lethal alkylation damage independent of the adaptive response. The *alkB* gene has been cloned (Kataoka and Sekiguchi, 1985) and the *alkB* protein purified (Kondo et al, 1986). However, the precise role of the *alkB* gene in DNA repair remains obscure. A model of the adaptive response is shown in figure 2.2.

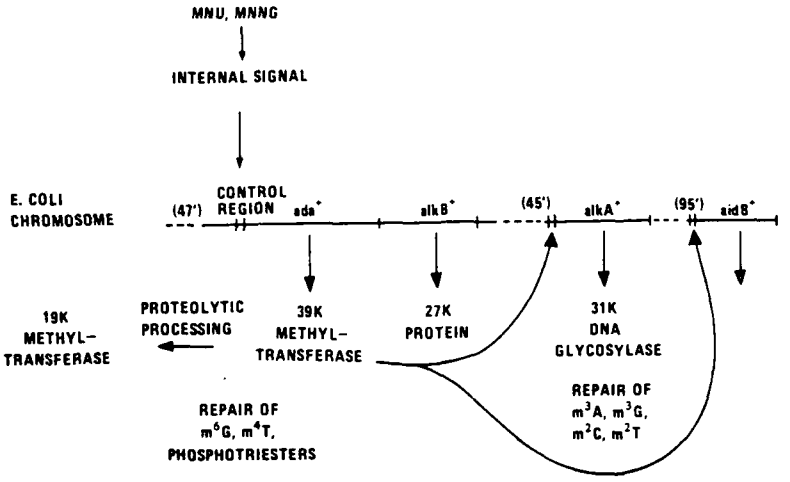

Figure 2.2 Adaptive response to alkylation damage (From Demple et al, 1985).

2.3 Comparison of the adaptive response and the SOS regulatory systems

E.coli has two different pathways for inducible repair, the SOS response and the adaptive response (Little and Mount, 1982; Walker, 1984). The two repair pathways are supposed to have their own sets of regulatory control elements. However, MNNG which induce the adaptive response can also induce the SOS response (Bagg et al, 1981), although the in vivo signals that induce SOS and adaptive systems are likely to be quite different. Both systems have a positively acting regulatory element; RecA and Ada. Further, the ada and the recA genes are both strongly activated by inducing treatments, and their own function is required for their own induction.

Recently, a third inducible repair response have been described for E.coli. It is induced by oxidative treatment of DNA, and is positively regulated by the oxyR gene (Demple and Hallbrook, 1983; Christman et al, 1985).

2.3.1 *recA*$^+$ independent induction of TagII

It has previously been shown that *recA* mutant cells can be adapted to higher survival and lower mutation frequency by alkylation treatment (Jeggo et al, 1977; Schendel et al, 1978; Yamamoto and Sekiguchi, 1979). However, the effect of SOS induction on the synthesis of the enzymes known to be controlled by the adaptive response has not been investigated. A double mutant carrying *recA*,*tag* was constructed and shown to be much more sensitive to MMS exposure than either single mutant (Paper 3) consistent with the notion that *recA* and *tag* mutations represent blocks in separate repair pathways. However, the low survival could also be a result of *recA* preventing induction of TagII. However, the *recA*,*tag* double mutant could be adapted to higher survival by alkylation induction (Paper 3). Further, adapted mutant cells were able to reactivate alkylated λ-phages. These adaptive responses were correlated to the induction of TagII activity, as measured directly in cell extracts from adapted cells (Paper 3). In conclusion, the *in vivo* and the *in vitro* tests consistently showed that the induction of TagII did not require a functional *recA* gene, and that the SOS and the adaptive responses are independent processes. The *alkA* gene is not a *recA*/*lexA* controlled operon.

2.4 Biological effects of unrepaired 3-methyladenine in DNA

3-methyladenine is formed in similar amounts by weakly mutagenic and carcinogenic agents such as MMS and more strongly mutagenic compounds such as MNNG (Beranek et al, 1980). This suggests that m^3A is a potentially lethal or inactivating lesion rather than a strongly mutagenic one. However, since m^3A normally is rapidly removed by DNA glycosylase it is difficult to assess the mutagenic potential of persisting m^3A in DNA. To address the question of the biological effects of unrepaired m^3A in DNA, we investigated MMS induced mutagenesis in mutants with reduced ability to repair m^3A.

2.4.1 Cytotoxic effects of persisting m^3A in DNA

Cells mutated in tag or ada were more sensitive to and showed higher mutation frequency after MMS treatment than wild type cells (Paper 4). Mutant cells defective in both ada and tag showed additive mutagenic and killing effects relative to the single tag and ada mutants, in agreement with the observation that ada and tag block separate repair pathways. The additive effect of the tag mutation indicated that m^3A was both a premutagenic and a cytotoxic lesion.

When alkA cells, defective in TagII, were treated with MMS they showed reduced survival and increased mutation frequency compared to the tag mutant. This is probably due to absence of TagII and inability to repair the biological significant alkylation products m^3G, m^2C and m^2T. A mutant defective both in tag and alkA, unable to remove m^3A enzymatically, was extremely sensitive to MMS. This shows that persisting m^3A in DNA is lethal to the cell. The lethal effect of m^3A in DNA can be explained by the results of Botieux et. al. (1984) which show that m^3A inhibits the DNA replication complex.

The MMS sensitivity of the tag,alkA double mutant is more than additive compared to the single mutants as could be expected since TagI and TagII have overlapping roles in repair of alkylated DNA. We also expected that the double tag,alkA mutant would be more mutable than the single tag or alkA mutant. Upon MMS exposure, however, the tag,alkA mutant, surprisingly, showed only a very low mutation frequency, much lower than for the single mutants (Paper 4). This indicates that unrepaired m^3A in DNA has very limited mutagenic effect.

2.4.2 Mutagenic bypass synthesis of depurinated DNA

Plasmids having cloned genes for $TagI^+$ or $TagII^+$ were introduced into tag cells. Due to complementation of the TagI deficiency, the transformed cells showed decreased level of MMS induced mutations and increased cell survival compared to untransformed cells. However, mutant tag,alkA cells, transformed by $TagI^+$ or $TagII^+$ plasmids, however, showed increased

MMS induced mutation frequency (Paper 4). This experiment shows unambiguously that low mutation frequency is due to TagI and TagII deficiency and not to any undetected gene mutation which could be present in the double mutant strain. Moreover, these results indicate that removal of m^3A, spontaneously or by the action of TagI or TagII, is prerequisite for the introduction of mutations in DNA by MMS alkylation.

TagI and TagII initiate the errorfree repair of m^3A by hydrolysing the glycosylic bond between sugar and base leaving AP(apurinic)-sites in DNA (Lindahl, 1976). It is, thus, reasonable to believe that AP-sites are the true premutagenic lesions induced by MMS treatment. Previous reports have presented data indicating that AP-sites may play a role in the mutagenicity of a number of chemical carcinogens. AP-sites are known to have non-coding function and cannot be copied under normal conditions thus blocking DNA synthesis (Schaaper et al, 1982; Foster et al, 1982; Boiteux and Laval, 1982). Expression of mutations induced by AP-sites in E.coli seem to require the induction of the error-prone SOS repair system (Strauss et al, 1982; Loeb, 1985). It is believed that umuDC encodes functions directly involved in mutagenesis and that the increased mutation rate results from a relaxed replication fidelity required to allow bypass replication of blocking lesions (Walker, 1984). Further, AP-sites are reported to give rise to transversions due to preferential insertions of adenines across from AP-sites during bypass synthesis (Kunkel, 1984; Miller and Low, 1984). Our results indicate that mutations induced by MMS treatment are formed at AP-sites by the action of the SOS repair response. This model is supported by the observation that the SOS induction in tag, tag,ada, alkA, tag,alkA mutants was shown to be enhanced by MMS exposure (Paper 4). Further, MMS induced mutations seen in tag cells were dependent on intact $umuC^+$ (Paper 4, Foster and Eisenstadt, 1985).

AP-sites are believed not to induce the SOS response themselves (Miller and Low, 1984). From our results it is likely that persisting m^3A induces the SOS response. This is supported by the observation that tag, alkA mutant cells, deficient in TagI and TagII, show immediate induction of the SOS response by MMS treatment. Similarly, other groups have shown that the induction of SOS response is enhanced in tagA and alkA strains (Eisenstadt et al, 1982; Boiteux et al, 1984; Foster and Eisenstadt, 1985).

3 CONCLUDING REMARKS

Recent studies of DNA repair have shown that mechanisms for repair of alkylation lesions are widespread and found in both procaryotic and eucaryotic organisms, including humans. Cells have evolved the ability to repair alkylation damage rapidly and faithfully by means of specific alkylation repair enzymes. The importance of removing alkylation damage is stressed by the existence of inducible repair enzymes which increase the capacity for alkylation repair. The reason why cells have evolved extensive repair capacity for DNA alkylation damage is not obvious, as simple methylating agent are not normally abundant in the environment. It has been suggested that non-enzymatic intracellular methylation of DNA by S-adenosylmethionine could be a reason for the requirement of alkylation repair systems. However, it is not clear from existing data that this kind of DNA methylation is frequent enough to account for the existing high repair capacity. Further work is needed to elucidate the role of elaborate mechanism for alkylation repair in the cellular metabolism.

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Reprinted from Nature, Vol. 296, No. 5859, pp 773-775, 22 April 1982

Adaptation to alkylation resistance involves the induction of a DNA glycosylase

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Cells exposed to low doses of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) or methyl methanesulphonate (MMS) acquire resistance to both the mutagenic and lethal effects of a challenging dose of the same agents (ref. 1, and ref. 2 for a review). This response, termed adaptation, has been ascribed to the induced synthesis and accumulation of *O*-6-methylguanine (m^6G) DNA methyltransferase[3,4] which rapidly demethylates the m^6G residues induced by the challenging dose[3-5]. Mutant studies, however, indicate that mutagenic adaptation and killing adaptation are at least partly under different genetic control and may therefore involve the induction of different repair enzymes[4,5]. Whereas mutagenic adaptation correlates with the induction of the transferase, the data presented here show that killing adaptation can be ascribed to the induction of a DNA glycosylase. This inducible glycosylase releases the alkylation product 3-methyladenine (m^3A) from DNA *in vitro* as does the constitutive m^3A DNA glycosylase previously characterized by Riazuddin and Lindahl[6]. However, the enzymes are encoded by different genes and appear to have different roles in DNA repair *in vivo*.

We have previously isolated from MNNG-mutagenized clones of *Escherichia coli* a mutant that is extremely sensitive to MMS, unable to reactivate alkylated phages and deficient in m^3A DNA glycosylase activity[9]. Further characterization of this strain (BK2106) has revealed that it carries two mutations, *tag* and *ada*, and not only lacks the glycosylase, but also is deficient in the induction of m^6G DNA methyltransferase. Elimination of the *ada* mutation in this strain by P1 transduction renders the cell wild-type resistant to the lethal effects of MMS in agar medium (Table 1). However, the *ada*⁻ *tag* transductant (BK2114) is still unable to reactivate alkylated phages (Fig. 1) and shows considerable sensitivity to MMS exposure in buffer (Fig. 2). These observations suggested that *E. coli* may have two pathways for the repair of m^3A residues in DNA, one that is constitutively expressed and controlled by *tag*, and another that is inducible and controlled by *ada*. The inducible repair pathway would be turned on when the cells were exposed to alkylating agents in complete medium, but not when the cells were infected with alkylated phages. Table 1 shows that the adaptive treatment used to induce m^6G DNA methyltransferase also induces high levels of m^3A DNA glycosylase activity in the *tag* mutant (BK2114). Such induction is not observed in the *tag ada* double mutant (BK2106). Adapted BK2114 has also regained essentially normal ability to reactivate alkylated phages which implies that the inducible enzyme can replace the constitutive enzyme also in repair of phage DNA (Fig. 1). These results show that adaptation induces two different enzymes for repair of alkylated DNA, m^6G DNA methyltransferase and m^3A DNA glycosylase, and that both enzymes are under *ada* control.

Whereas constitutive m^3A DNA glycosylase (TagI) in wild-type cells is inhibited by m^3A (ref. 8), the inducible glycosylase (TagII) in BK2114 is unaffected by m^3A (Table 1). Adaptation of wild-type cells also results in the induction of m^3A DNA

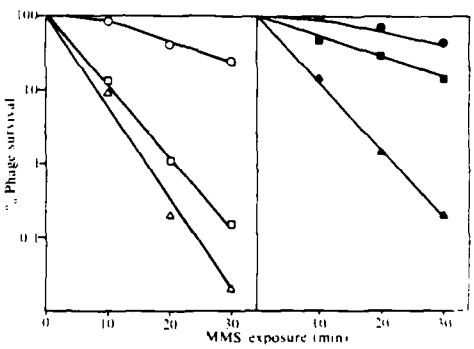

Fig. 1 Host cell reactivation of alkylated phages in non-treated and adapted cells. Phage λcI857 were exposed to 0.05 M MMS for the times indicated and plated on exponentially growing untreated (open symbols) or adapted (closed symbols) cells of AB1157 (wild type, circles), BK2106 (*tag ada*, triangles) or BK2114 (*tag*, squares). Cells were adapted as described in Table 1 legend and conditions for the plating procedure were as published previously[9].

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Table 1 Properties of *E. coli* mutants defective in alkylation repair

Bacterial strains	Relevant genotype	Plating efficiency on nutrient agar containing 3 mM MMS* (%)	m^3A DNA glycosylose activity in cell extracts (pmol base released per mg protein) Noninduced (TagI)	Induced	Induced + 3 mM m^3A (TagII)	m^6G DNA methyltransferase activity in extracts from induced cells (% m^6G remaining in substrate)
AB1157	Wild type	97	26.5	41.2	36.3	9.5
BK2106	*tag ada*	5×10^{-4}	6.4	0.7	1.8	94.5
BK2114	*tag*	67	3.8	26.3	34.0	6.5
BK2110	*ada*	1.4	24.3	23.1	5.3	94.0
MS23	*alk-1*	0.06	25.7	23.1	1.8	6.7
BD793	Δ*alk*(*mgl-ant*P2H)	0.16	27.0	26.2	0.5	2.9

Strain BK2106 is a MNNG-mutagenized derivative of AB1157 (ref. 9) and carries two mutations which both contribute to its MMS sensitivity. The mutation which maps at 47 min and co-transduces with *gyrA* is shown here to be in the *ada* gene and not in the *tag* gene as was previously assumed[9]. Other *ada* mutations have also recently been mapped to the same position on the map[10]. The *tag* mutation responsible for m^3A DNA glycosylase (TagI) deficiency in BK2106 has now been mapped to a region between 70 and 74 min on the chromosome (G.E. and E.S., in preparation). Strains BK2106 and BK2114 (BK2106 *gyrA ada*⁺); BK2110 (AB1157 *gyrA ada*) and AB1157; and MS23 and AB1157 (refs 10, 11) are all isogeneic pairs constructed by P1 transduction. BD793 was isolated by B. Duncan and is a *his*⁺ eductant of phage P2-lysogenized W1485F⁻. Cell extracts were prepared by a combination of plasmolysis and lysozyme digestion as previously described[14]. m^3A DNA glycosylase activity was assayed by the method of Riazuddin and Lindahl[6]. The numbers presented are calculated on the basis of ethanol-soluble radioactivity released from ^3H-dimethylsulphate-treated DNA using various amounts of extracts ranging from 0 to 20 μg protein. In this range a linear response was obtained between glycosylase activity and extract added. Parallel assays were also run where the reaction mixtures were subjected to polyethenelmmine TLC together with authentic 7-methylguanine and 3-methyladenine. In all cases where significant glycosylase activity was observed, both in induced and noninduced cells, >95% of the radioactivity released was identified as m^3A. m^6G DNA methyltransferase activity was assayed by the method of Karran *et al.*[3] and 20 μg protein were added to each assay. Cells were induced by growth in M9 salts supplemented with 1% glucose and 1% casaminoacids in the presence of 0.5 μg MNNG for 90 min.

* Stationary phase cells calculated relative to plating on nutrient agar without MMS.

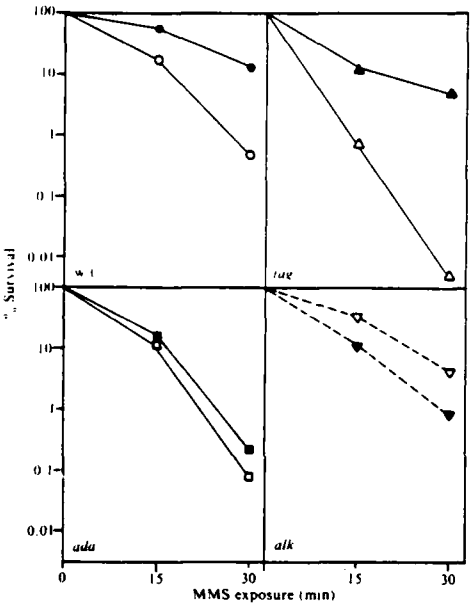

Fig. 2 Killing adaptation to alkylation resistance in wild-type and TagII-deficient mutants. Fresh overnight cultures were diluted 10-fold in K medium (M9 salts + 1% glucose and 1% casaminoacids). To one-half of each culture was added MNNG at a final concentration of 0.5 μg ml^{-1} while the other half served as an untreated control. The cells were grown for 90 min at 37 °C and then washed once by centrifugation and resuspended in 1/10 volume of phosphate buffer. MMS was added at a final concentration of 0.06 M for strains AB1157 (wild type; ○, ●), BK2114 (*tag*; △, ▲) and BK2110 (*ada*; □, ■), and of 0.02 M for MS23 (*alk-1*; ▽, ▼). At intervals, samples were plated for survival after appropriate dilution. Closed symbols refer to adapted cell cultures (MNNG-treated) while open symbols represent untreated controls

glycosylase activity not inhibited by m^{3}A, indicating that the inducible enzyme in BK2114 is not a mutated form of the normal *tag* gene product, but is indeed a separate enzyme. Karran *et al.*[6] previously observed a minor m^{3}A-noninhibited m^{3}A DNA glycosylase in both wild-type cells and strain BK2106. This enzyme is more heat-stable than TagI and constitutes ~5% of the total m^{3}A DNA glycosylase activity in wild-type cells. It is probably the same as that which is induced to high levels in BK2114 by the adaptive treatment. In fact, Lindahl and co-workers[10] have recently observed that the heat-stable activity of m^{3}A DNA glycosylase increases several-fold in wild-type cells during adaptation.

The observation that the *ada* mutation prevents induction of two different repair enzymes suggests that the *ada* gene is a control gene for the adaptive response and that structural genes for one or both of the enzymes may be found elsewhere on the chromosome. While searching for structural gene mutants we observed that the *alk-1* mutant[11,12] was deficient in the induction of TagII, but proficient in induction of the transferase (Table 1). Similar results were obtained with another *alk* mutant (isolated by B. Duncan). No other enzyme defect has been linked to mutations in the *alk* gene. *alk* mutants are very sensitive to MMS exposure and it seems likely that the MMS sensitivity is caused by TagII deficiency, in which case the low levels of TagII activity present in wild-type cells must be essential for some repair not effected by TagI. It is possible that TagII has a broader substrate specificity than TagI and is responsible for repair of a quantitatively minor but biologically important alkylation product other than m^{1}A. TagI has a narrow substrate specificity and a very low K_m value for its substrate[13], suggesting that it will quickly bind and remove m^{1}A from alkylated DNA. Other minor products, however, could perhaps be removed by TagII. The *alk* mutants seem to have less residual TagII activity than the *ada* mutant, which would explain the greater sensitivity of the former compared with the latter (Table 1).

Mutants defective in the adaptive response were first described by Jeggo[7]. They were selected on the basis of their deficiency in mutagenic adaptation but most of them also appeared to be defective in killing adaptation. One mutant, however, was only defective in mutagenic adaptation. Jeggo *et al.*[8] have further reported that *polA* mutants defective in DNA polymerase I are defective in killing adaptation, but show normal mutagenic adaptation. These studies are consistent with the view that mutagenic adaptation and killing adaptation are due to the induction of separate repair pathways. They support the view that m^{6}G DNA methyltransferase is responsible for mutagenic adaptation since transferase repair of m^{6}G will not require DNA polymerase I. The *ada*$^+$-dependent inducible glycosylase may well be responsible for killing adaptation because DNA polymerase I is needed to complete repair of the apurinic site formed by the glycosylase action. A critical test for this interpretation is that *alk* as well as *ada* mutants should be defective in killing adaptation. Figure 2 shows that this is indeed the case and, furthermore, that the *alk-1* mutant is even sensitized by the adaptive treatment. These results therefore strongly support the view that TagII, not the transferase, is responsible for killing adaptation to alkylation resistance.

Thus *E. coli* seems to have two distinct m^{3}A DNA glycosylase activities: one that is constitutively expressed and controlled by *tag*, and a second that is inducible and controlled by *ada* and *alk*. The constitutive enzyme appears responsible for rapid repair of m^{3}A alkylation products in unadapted cells. The inducible enzyme seems to be required for killing adaptation to alkylation resistance and probably also for repair of some potentially lethal lesions not recognized by the constitutive enzyme in unadapted cells.

We thank Tomas Lindahl for communicating that strain BK2106 is defective in m^{6}G DNA methyltransferase and for the gift of transferase substrate, Bruce Duncan and Mutsuo Sekiguchi for the gift of bacterial strains, Neil Clarke for valuable discussions, and Peter Strike for a critical reading of the manuscript. This research was supported by the Norwegian Council for Scientific and Industrial Research.

Received 22 December 1981; accepted 24 February 1982.

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Printed in Great Britain by Macmillan Production Ltd., Basingstoke, Hampshire

JOURNAL OF BACTERIOLOGY, Nov. 1986, p. 642–647
0021-9193/86/110642-06$02.00/0

Vol. 168, No. 2

Amplified Expression of the *tag*$^+$ and *alkA*$^+$ Genes in *Escherichia coli*: Identification of Gene Products and Effects on Alkylation Resistance

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Received 26 March 1986/Accepted 8 July 1986

We have constructed plasmids which overproduce the *tag* and *alkA* gene products of *Escherichia coli*, i.e., 3-methyladenine DNA glycosylases I and II. The *tag* and *alkA* gene products were identified radiochemically in maxi- or minicells as polypeptides of 21 and 30 kilodaltons, respectively, which are consistent with the gel filtration molecular weights of the enzyme activities, thus confirming the identity of the cloned genes. High expression of the *tag*$^+$-coded glycosylase almost completely suppressed the alkylation sensitivity of *alkA* mutants, indicating that high levels of 3-methyladenine DNA glycosylase I will eliminate the need for 3-methyladenine DNA glycosylase II in repair of alkylated DNA. Furthermore, overproduction of the *alkA*$^+$-coded glycosylase greatly sensitizes wild-type cells to alkylation, suggesting that only a limited expression of this enzyme will allow efficient DNA repair.

N^3-methylated adenine is quantitatively one of the major products formed in DNA exposed to simple alkylating agents such as methyl methanesulfonate (MMS) and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) (1, 15). This modified base is rapidly excised in both procaryotic and eucaryotic cells by 3-methyladenine DNA glycosylases, which release the alkylated base in a free form (14, 17, 18, 29). The widespread existence from bacteria to human cells of such DNA repair enzymes suggests that alkyl groups in the N-3 position of adenines severely impair the metabolic function of the DNA.

Direct evidence for the importance of 3-methyladenine removal has been obtained from studies of *Escherichia coli* mutants lacking 3-methyladenine DNA glycosylase activity (11). *E. coli* has two enzymes of this type, one that is constitutively expressed, TagI (17, 25, 32), and another that is induced during exposure of the cells to alkylating agents, TagII (3, 7, 10, 23). Mutants lacking one or the other of these enzymes are slightly to moderately sensitive to alkylation, whereas double mutants show extreme sensitivity (3, 7). These results are direct evidence for the cytotoxic effects of persisting 3-methyladenines in DNA.

TagI and TagII have different substrate specificities. TagI appears to be specific for the removal of 3-methyladenines, whereas TagII can release 3-methylguanine, O^2-methylcytosine, O^2-methylthymine, and 7-methylguanine in addition to 3-methyladenines (10, 20, 32). It has therefore been suggested that TagI functions to remove 3-methyladenines from DNA, whereas the primary role of TagII is to remove other quantitatively minor lesions. Nevertheless, TagII can, once induced, effectively replace TagI in 3-methyladenine repair (6, 7).

The cloning of genes encoding TagI and TagII, *tag* and *alkA*, was recently reported (3, 23, 33). We selected these genes on the basis of their ability to complement the alkylation-sensitive phenotype of a *tag ada* double mutant (3). This strain is mutated in the structural gene of TagI (*tag*) and in the *ada* gene, which positively regulates the induction of TagII (4, 7, 11, 16, 22). The mutant therefore phenotypically appears as TagI$^-$ TagII$^-$, and its alkylation sensitivity provides a strong selection for recombinant plasmids expressing one or the other of the glycosylase activities. In this work the *tag* and *alkA* genes have been subcloned, yielding smaller plasmids, which results in elevated expression of the glycosylase genes. These plasmids have been used for the radiochemical identification of the *tag* and *alkA* gene products and to investigate the effect of glycosylase overproduction on alkylation survival.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this work are listed in Table 1.

Enzymes. Restriction endonucleases, T4 DNA polymerase, and DNA ligase were purchased from Boehringer Mannheim Biochemicals or New England Biolabs, Inc., and were used according to the manufacturers' instructions.

Construction of plasmids. Plasmid pBK121 *alkA*$^+$ was derived from pBK111 (3) carrying a γδ insertion about 2 kilobase pairs away from the *alkA* gene. Plasmid pBK111 has a *Bam*HI-*Hin*dIII chromosomal insert of 6.2 kilobase pairs. The γδ insert has a *Bam*HI site close to the end toward the *alkA* gene, thus allowing recloning of a *Bam*HI fragment carrying the *alkA* gene into pBR322. The other plasmids used in this study were constructed as described in the legends to Fig. 1 and 3.

Labeling of plasmid-coded proteins. Polypeptides encoded by pBK202 and derivatives were labeled with [^{35}S]methionine by the maxicell method of Sancar et al. (26), and those encoded by pBK161 and derivatives were labeled by the minicell method of Meagher et al. (21). Insertional inactivation of the glycosylase genes was carried out as previously described (8, 26).

SDS-polyacrylamide gel electrophoresis. Labeled proteins were analyzed on sodium dodecyl sulfate (SDS)–15% polyacrylamide slab gels by the method of Laemmli (13). The gels were treated with En^{3}Hance (New England Nuclear Corp.), dried, and subjected to fluorography at −70°C. ^{14}C-labeled protein markers were purchased from Amersham Corp.

Alkylation survival. Alkylation survival was measured

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TABLE 1. Strains and plasmids used in this work

Strain or plasmid	Relevant genotype or phenotype	Source or reference
AB1157	Wild type	P. Howard-Flanders
BK2106	*tag-2 ada*	Karran et al. (11)
BK2118	*tag-2 alkA1*	Evensen (6)
MS23	*alkA1*	Yamamoto et al. (34)
CSR603	*uvrA recA phr*	Sancar et al. (26)
DS410	*minA minB*	Dougan and Sherratt (5)
AB4014	F⁻	B. Low
pBR322	Ap, Tc^r	Bolivar et al. (2)
pBK201	*tag*⁺ Mp^r	Clarke et al. (3)
pBK202	*tag*⁺ Ap^r Tc^r	This work (Fig. 1)
pBK111	*alkA*⁺ Ap^r	Clarke et al. (3)
pBK121	*alkA*⁺ Ap^r	This work (Fig. 3)
pBK132	*alkA*⁺ Ap^r	This work (Fig. 3)
pBK133	Δ*alkA* Ap^r	This work (Fig. 3)
pBK141	*alkA*⁺ Ap^r	This work (Fig. 3)
pBK151	*alkA*⁺ Ap^r	This work (Fig. 3)
pBK161	*alkA*⁺ Tc^r	This work (Fig. 3)

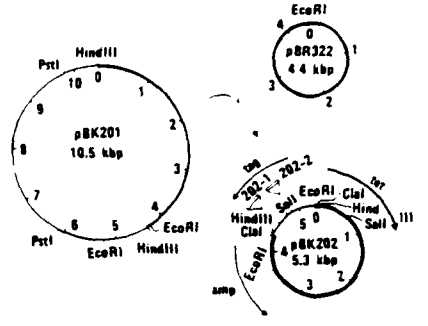

FIG. 1. Subcloning of *tag*. Plasmid pBK202 was constructed by recloning the *Eco*RI fragment from pBK201 (3) into the *Eco*RI site of pBR322. kbp, Kilobase pairs.

either by exposing exponentially growing cells to MMS (20 mM) or MNNG (0.33 mM) in phosphate buffer (pH 6.0) for various periods of time at 37°C or by plating the cells on nutrient agar containing various amounts of alkylating agent (7). The buffer exposure measures the survival of unadapted cells, whereas the plate exposure is an indirect measure of survival of adapted cells, since the low concentration of MMS used in the plates will not immediately kill the cells and hence allows for some degree of induction. This is indicated by the response of *tag* mutant cells, which are essentially wild-type MMS resistant when scored by the plate method presumably because TagII is induced and can replace TagI in repair (3, 7). Upon buffer exposure, *tag* mutant cells are significantly more sensitive than wild-type cells.

Glycosylase assays. Cell extracts for enzyme analysis were prepared by sucrose plasmolysis and lysozyme treatment as previously described (28). 3-Methyladenine DNA glycosylase activity was measured essentially as described by Riazuddin and Lindahl (25). The cells were adapted by growing the cells in K medium for 90 min in the presence of 0.5 μg of MNNG per ml (7).

RESULTS

Identification of the *tag* gene product. Mapping of γδ insertions in pBK201 (*tag*⁺) indicated that the entire *tag* gene was located in between the *Eco*RI and the *Hin*dIII sites near the *Eco*RI site of pBR322. The *tag* gene was subcloned by cutting with *Eco*RI and reinsertion into the *Eco*RI site of pBR322 (Fig. 1). The resulting plasmid, pBK202, harbors the *tag* gene flanked by two 31-base-pair *Hin*dIII-*Eco*RI fragments originating from pBR322 (31). In this plasmid the *tag* gene is present as a cartridge which can be moved to other vectors by cuts with either one of three different enzymes, *Hin*dIII, *Cla*I, and *Eco*RI. Much more TagI enzyme is produced from pBK202 than from the original multicopy plasmid, pBK201 (Table 2). This appears to be not only the result of reduction in plasmid size and higher copy number; sequence data indicate that a fusion promoter has been formed which is stronger than the natural one (30).

Polypeptides encoded by pBK202 and derivatives having three different γδ insertions within the *tag* gene were examined by the maxicell method (26). Only three major protein species appeared on the autoradiogram, the *tet* and the *amp* gene products and a third polypeptide of approximately 20 kilodaltons (Fig. 2). This band was absent from extracts isolated from cells carrying plasmids with γδ insertions in the *tag* gene. We conclude that this protein is the *tag* gene product. A new faint band appears on the autoradiogram expressed from one of the insertion plasmid (pBK202-1), and this seems likely to represent a truncated polypeptide from the *tag* gene. Of the three insertion plasmids, pBK202-1 had the insertion mapped closest to the *amp* side on pBR322. Since larger truncated peptides were not seen in lanes from the other insertion plasmids, it may suggest that transcription is counterclockwise on the map in Fig. 2. This agrees with the nucleotide sequence analysis of the *tag* gene (30). From the insertion plasmid pBK202-3 the *tet* gene product is not expressed (Fig. 2). F-mediated transfer of nonconjugative plasmids is known to generate deletions with high frequency (8), and restriction enzyme analysis confirmed that pBK202-3 was deleted for the *tet* gene (data not shown).

In the autoradiogram shown in Fig. 2, which is heavily

TABLE 2. 3-Methyladenine DNA glycosylase activity in *E. coli* transformed with *tag* and *alkA* plasmids

Strain (plasmid)	Enzyme activity[a]	
	Noninduced cells	Adapted cells
BK2118 (*tag alkA1*)	<0.1	<0.1
AB1157 (wild type)	21	25[b]
BK2118(pBK201) (*tag*)	300	
BK2118(pBK202)	1,400	
BK2118(pBK121)	80	500
BK2118(pBK131)	140	580
BK2118(pBK132)	300	1,200
BK2118(pBK133)	<0.1	<0.1
BK2118(pBK141)	70	500
BK2118(pBK151)	54	270
BK2118(pBK161)	44	240

[a] Activity is expressed as picomoles of alkylated base released per milligram of protein in extract.

[b] Adaptation of wild-type cells does not always result in a substantial total increase in 3-methyladenine DNA glycosylase activity. However, the activity in the adapted cells is refractory to inhibition by free 3-methyladenine and hence represents TagII activity (10). Therefore, it seems that TagI activity sometimes is being reduced or partly consumed during adaptation.

overexposed to show any truncated polypeptides, there is a faint remaining band of 20 kilodaltons present in the lanes from cells with the insertion plasmids. We believe that this is caused by a small fraction of the cell population carrying plasmids where the insertion has been lost and hence represents normal *tag* gene product. This view is supported by survival experiments, which show that cultures of *tag alkA* mutant cells with the insertion plasmids contain a small fraction (less than 0.1%) of apparently MMS-resistant clones.

Identification of the *alkA* gene product. We were unable to identify the *alkA* gene product in maxicells carrying the original plasmid, pBK101. Initially, we thought this could be due to insufficient expression of the *alkA* gene product from this plasmid, which has a large chromosomal insert of more than 10 kilobase pairs. To improve expression, smaller plasmids were constructed (pBK132 and pBK151; Fig. 3) which yielded elevated levels of TagII, both in uninduced and in alkylation-induced (adapted) cells (Table 2). Still, we could not identify a polypeptide band on autoradiograms of SDS gels from minicells which was absent from cells carrying plasmids with insertions in the *alkA* gene.

All of the plasmids examined thus far carried inserts within the *tet* gene of pBR322 and expressed high levels of β-lactamase from the *amp* gene. Since the M_r of TagII from the biochemical characterization had been characterized to about 30,000 (28), the possibility remained that the *alkA* gene product was masked by the strong 30-kilodalton β-lactamase band on the gels. Therefore, another plasmid was constructed with the *alkA* gene fragment within the *amp* gene (pBK161; Fig. 3). Specific radioactive labeling of polypeptides expressed in minicells carrying this plasmid demonstrated the synthesis of a protein of 30 kilodaltons which was absent from minicells carrying plasmids with γδ insertions within the *alkA* gene (Fig. 4). Polypeptides synthesized in minicells carrying the *tag*$^+$ plasmid were examined on the same SDS gels. Plasmid pBK202 *tag*$^+$ produces β-lactamase, which comigrates with the *alkA* gene product. Also the *tag* gene product is expressed, which comigrates with the 21-kilodalton marker on the gels. This marker was not included in the gels from the maxicell experiments (Fig. 2), and the comigration of *tag* and the marker more precisely

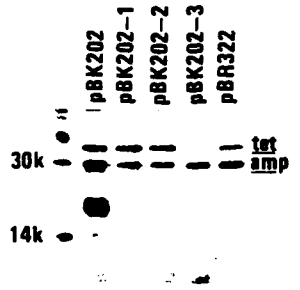

FIG. 2. SDS-polyacrylamide gel electrophoresis of polypeptides expressed in maxicells carrying pBK202 (*tag*$^+$) and derivative plasmids. The left lane contains marker proteins (30-kilodalton carbonic anhydrase, 14-kilodalton lysozyme). pBK202 derivatives pBK202-1 through -3 contain γδ insertions within the *tag* gene. Note that the third insertion also eliminates the *tet* gene product and has generated a deletion of the *tet* gene.

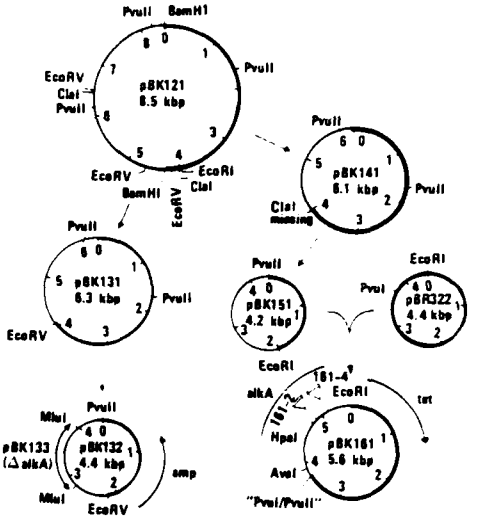

FIG. 3. Subcloning of *alkA*. Plasmid pBK121 was derived from plasmid pBK111 (3) as described in Materials and Methods. Plasmids pBK131 and pBK141 were made from pBK121 by cutting with *Eco*RV and *Cla*I, respectively, followed by recircularization. The *Cla*I site was lost in pBK141, suggesting that a mutation had been formed at the ligation site. Further cutting of the plasmids with *Pvu*II followed by recircularization yielded pBK132 and pBK151, respectively. Plasmid pBK161 was constructed by inserting the *Pvu*II-*Eco*RI fragment of pBK151 between the *Eco*RI and *Pvu*I sites of pBR322. Plasmid pBR322 was first cut with *Pvu*I, and protruding 3′ ends were removed with the 3′ → 5′ exonuclease activity of T4 DNA polymerase to make blunt ends. The DNA was extracted and then cut with *Eco*RI. Subsequent cutting of pBK151 with *Eco*RI and *Pvu*II allowed the insertion of the *alkA* gene fragment into the *amp* gene of pBR322 by normal ligation at the *Eco*RI site and blunt end ligation of the *Pvu*I-*Pvu*II ends. The ligation mixture was introduced into BK2118 (*tag alkA*), and pBK161 was isolated from tetracycline- and MMS-resistant cells. Only the relevant restriction sites are shown. kbp, Kilobase pairs.

determines 21 kilodaltons as the molecular mass of the *tag* gene product. This agrees very well with the calculated molecular mass of 21,104 daltons as determined by nucleotide sequence analysis.

While this work was in progress, Nakabeppu et al. (23, 24) reported the cloning of the *alkA* gene and the identification of the *alkA* gene product as a 30-kdalton protein; our results agree with their data.

Overproduction of TagII sensitizes wild-type cells to alkylation. We were initially puzzled by the observation that the *alkA*$^+$ plasmid first isolated did not fully suppress the alkylation-sensitive phenotype of *alkA* mutant cells (3). This was not caused by incomplete expression of TagII activity from the plasmid, since TagII activity was fully restored, even overproduced, by the *alkA*$^+$ plasmid in the mutant. After subcloning, we again tested the ability of the various *alkA*$^+$ plasmids to complement *alkA* mutants with respect to alkylation survival (Fig. 5). There appeared to be large variability between the different plasmids in the capability to restore alkylation resistance of *alkA* mutant cells. When wild-type

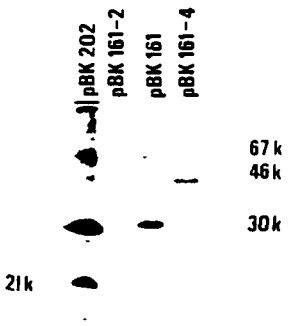

FIG. 4. SDS-polyacrylamide gel electrophoresis of polypeptides expressed in minicells carrying pBK161 (*alkA*$^+$) and derivative plasmids. The left lane contains a marker (21-kilodalton soybean trypsin inhibitor). pBK161 derivatives pBK161-2 and -4 contain γδ insertions in the *alkA* gene. The right lane contains protein markers (69-kilodalton bovine serum albumin, 46-kilodalton ovalbumin, 30-kilodalton carbonic anhydrase, 14-kilodalton lysozyme).

cells were transformed by the same plasmids, cells became much more sensitive to alkylation (Fig. 5B). Survival of the various transformed bacteria depended not on the genotype of the host (*alkA*$^+$ or *alkA*$^-$), but on the type of plasmid present (Fig. 5).

TagII activity is overproduced from all the different *alkA*$^+$ plasmids investigated, some more than others, as judged from glycosylase assays of crude extracts from transformed cells (Table 2). The plasmids which were most effective in sensitizing wild-type cells to MMS also yielded the highest enzyme levels. We have plotted the level of enzyme activity in cell extracts from noninduced or in alkylation induced (adapted) cells versus cell survival of transformed *alkA1* mutant cell (Fig. 6). There appears to be a correlation between the extent of overproduction and cell survival, with an optimum level of *alkA*$^+$ expression for maximum survival. We hence conclude that overproduction of TagII enzyme sensitizes wild-type cells to MMS exposure. In control experiments, the plasmid pBK132 was deleted for the *alkA* gene, and the resulting plasmid (pBK133) did not sensitize wild-type cells (Fig. 5), nor did plasmids with γδ insertions in the *alkA* gene (data not shown).

Overproduction of TagI suppresses the alkylation sensitivity of *alkA* mutants. We also transformed the *tag*$^+$ plasmid into *alkA* mutant cells and measured survival to alkylation. Surprisingly, in contrast to the *alkA*$^+$ plasmids, the *tag*$^+$ plasmid pBK202 completely suppressed the alkylation-sensitive phenotype of the *alkA1* mutant (Fig. 7). This was true both when survival was measured by the MMS-agar plate method (data not shown) and when survival was measured after exposing the cells for various periods of time in buffer to either MMS or MNNG (Fig. 7). With this method we also tested the sensitizing effect of the *alkA*$^+$ plasmid pBK131 on wild-type survival, with results similar to those obtained with the MMS-agar plate method.

The complementation by the *tag*$^+$ plasmid of *alkA1* sensitivity must be due to overproduction of TagI by the multicopy plasmid, since *alkA* mutant cells have a *tag*$^+$ gene and express TagI at normal levels (34). The original *tag*$^+$ plasmid pBK201 yields less TagI than pBK202 and also suppresses the alkylation sensitivity of *alkA1* to a lesser extent. Plasmid pBK202 also suppresses the MMS sensitivity of an *alkA* deletion mutant, implying that the complementing effects are not allele dependent (data not shown).

DISCUSSION

The *tag* and *alkA* genes have been subcloned, and their gene products have been identified radiochemically as polypeptides of 21 and 30 kilodaltons, respectively. These molecular masses correspond to those determined previously for TagI and TagII enzyme activities by gel filtration (25, 32) and confirm that the *tag* and *alkA* genes as cloned indeed are the structural genes for these enzymes. The size correlation between the identification and the activity determination also indicates that both enzymes are active as monomers.

Overproducing TagI and TagII plasmids has the opposite effect of what could be expected on alkylation survival of *alkA* mutant cells. Whereas some of the *alkA*$^+$ plasmids only marginally complement the alkylation-sensitive phenotype of *alkA* mutant cells, the *tag*$^+$ plasmid fully suppresses the alkylation sensitivity. The lack of effect by the TagII-overproducing *alkA*$^+$ plasmids is accounted for by the sensitizing effect of TagII overproduction on wild-type survival. Maples and Kushner (19) have previously described a similar effect for the *uvrD*$^+$ gene product. Wild-type cells carrying the *uvrD*$^+$ gene on a multicopy plasmid are much more sensitive to UV irradiation and MMS exposure than cells without the plasmids. However, since the *uvrD*$^+$ gene codes for a DNA helicase and an ATPase, one can easily imagine that excessive enzyme will cause excessive DNA unwinding, ATP consumption, and DNA degradation during repair and thus have a negative effect on survival. There is not such an obvious explanation for why overproduction of TagII should have a sensitizing effect. Because TagI does not have a similar effect, one cannot simply ascribe this to glycosylase action per se, but will have to consider the characteristics of TagII compared with TagI. TagII has a broader substrate specificity than TagI, which implies that TagII can initiate more repair events at the same time. Too many ongoing

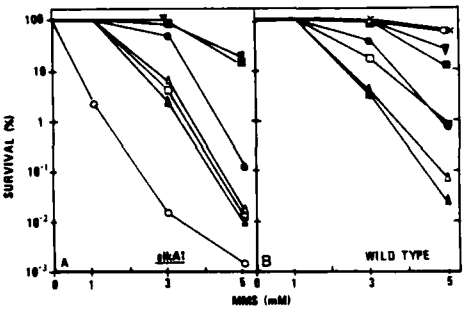

FIG. 5. Survival of wild-type (A) and *alkA1* mutant (B) cells transformed with *alkA*$^+$ plasmids on nutrient agar containing MMS. AB1157 (wild type) and MS23 (*alkA1*) transformed with various plasmids as indicated were grown to the stationary phase, and various dilutions were plated on nutrient agar containing different amounts of MMS. Plating efficiencies were calculated relative to CFU on plates without MMS. Symbols: (○) no plasmid, (▼) pBK161, (■) pBK151, (●) pBK141, (□) pBK121, (△) pBK131, (▲) pBK132, (×) pBK133.

repair events may be detrimental for the cells, since it will increase the possibility for creating double-strand breaks and causing DNA degradation. At doses of alkylation which are biologically relevant, the number of total alkylations is still only in the size order of thousands per chromosome, which means that each lesion on the average is several kilobases apart (11). About 60 to 70% of these are 7-methylguanines, which are very poor, if at all, substrates for TagII (10). It seems unlikely therefore that too many repair events should be the reason for the sensitizing effect of TagII amplification. More likely to us seems the possibility that the *alkA* gene or TagII enzyme has some other hitherto unknown property or function in the cell which causes the negative effects upon amplification.

From the sequence data published for the *ada* and *alkA* genes (4, 22, 24) we have calculated that both of these genes, in particular *alkA*, have an unusually high proportion of rare codons (30). The codon usage in *E. coli* is thought to be part of translational regulation of gene expression (9, 12). Genes with a high proportion of rare codons are limited in expression because of limiting amounts of tRNA available for rare codons. In cases with multicopy plasmids under adaptive conditions, it is plausible that too much of the tRNA for rare codons will be tied up in the expression of nonuseful amounts of these gene functions, thus limiting expression of other genes essential for repair. In other words, under such conditions other steps in repair might be deficient due to the lack of production of other enzymes. This could also explain the difficulty in the isolation of *adc* (*ada* constitutive) mutants from *E. coli* K-12, but not from strain B (27), since *E. coli* B may well have a different codon usage than strain K-12.

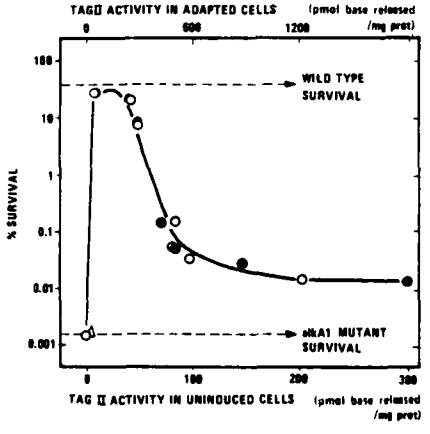

FIG. 6. Survival of *alkA1* mutant cells transformed with *alkA*$^+$ plasmids on nutrient agar containing MMS as a function of TagII activity in cell extracts. The data from Table 2 on the expression of TagII activity in cell extracts were plotted against survival of MS23 (*alkA1*) mutant cells on agar containing 5 mM MMS (Fig. 5). The ratio of enzyme activity for adapted (○) and nonadapted (●) cells in the figure axis was chosen as 1:6. This value corresponds to an average of that observed for cells containing the various *alkA*$^+$ plasmids (except for pBK131 and pBK132, for reasons as given in Discussion).

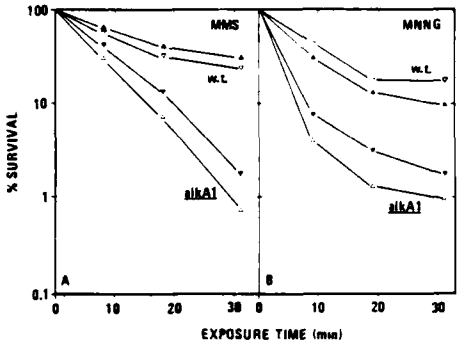

FIG. 7. Survival of wild-type and *alkA1* mutant cells transformed with pBK131 (*alkA*$^+$) and pBK202 (*tag*$^+$) after exposure to MMS (A) or MNNG (B) as described in Materials and Methods. The exposure was stopped by diluting the cells at least 100-fold in buffer. Symbols: (▲) MS23 (*alkA1*) transformed with pBK202, (▽) AB1157 (wild type), (▼) AB1157 transformed with pBK131, (△) MS23 (*alkA1*).

Table 2 shows that the different *alkA*$^+$ plasmids express TagII at different levels, even though all of them appear to respond to the *ada*$^+$-dependent induction. Judging from the maps of the plasmids, sequences both upstream and downstream for the *alkA* gene are of importance for this variation. Nakabeppu et al. (24) have determined the direction of transcription for the *alkA* gene, which is clockwise on the maps drawn in Fig. 2 (except for pBK161). In the case of plasmids pBK131 and pBK132, which show high constitutive expression of *alkA*, it is plausible that the *alkA* gene can be transcribed from the *tet* promoter. *Eco*RV, used to make PBK131, cuts just behind the *tet* promoter in pBR322, whereas *Cla*I, used to make pBK141, cuts in the middle of the promoter (31). Therefore, the *alkA* gene cannot be transcribed from the *tet* promoter in pBK141 and pBK151, and these plasmids also show much less constitutive expression of *alkA*$^+$ than pBK131 and pBK132.

The complementation of the *alkA* mutant sensitivity by *tag*$^+$-overproducing plasmids emphasizes the overlapping roles of TagI and TagII in the repair of alkylated DNA. In the original cloning experiments, we selected for plasmids which would restore alkylation resistance of a *tag ada* double mutant and picked up the *alkA* gene as well as the *tag* gene. From the data presented here, one would expect that an *alkA* mutant could be used to select for recombinant plasmids carrying the *tag* gene. In fact, Yamamoto et al. (33) did isolate the *tag* gene in their cloning of the *alkA*$^+$ gene using the *alkA1* mutant for the selection.

ACKNOWLEDGMENTS

G.E. was supported by a fellowship from the Norwegian Council for Scientific and Industrial Research. This research was supported by a grant (D.68.90-018) to E.S. from the Norwegian Research Council for Science and the Humanities.

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Mutation Research, 146 (1985) 143–147
DNA Repair Reports
Elsevier

MTR 06106

Induction of 3-methyladenine DNA glycosylase II is *recA*$^+$-independent

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(Received 30 November 1984)
(Revision received 1 March 1985)
(Accepted 23 March 1985)

Summary

The *recA*1 mutation was transduced into the *tag*-2 mutant of *E. coli*, thus making a strain deficient in the induction of SOS repair as well as in the constitutive repair of 3-alkylated adenines in DNA. The double mutant *recA tag* is more sensitive to methyl methanesulfonate exposure than either single mutant, indicating that *recA* and *tag* mutations block different pathways in repair of alkylation damage. The double mutant is more deficient in host cell reactivation of alkylated phages than the *tag* single mutant. However, alkylation induction of the double mutant with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine resulted in killing adaptation of the cells to methyl methanesulfonate and restored the host cell reactivation capacity for alkylated λ phage to wild-type levels. These adaptive responses can be ascribed to the induction of 3-methyladenine DNA glycosylase II which is shown by enzyme analysis to proceed normally in the *recA* mutant background. The results imply that the induction of the *alkA* gene encoding 3-methyladenine DNA glycosylase II is independent of SOS induction.

E. coli cells exposed to low levels of alkylating agents like *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) and methyl methanesulfonate (MMS) show increased survival and are less mutated by a challenging dose of the same agents (Samson and Cairns, 1977; Jeggo et al., 1977). This response, termed adaptation, is positively regulated by the *ada*$^+$ gene (Sedgwick, 1983) and is associated with the induction of two separate DNA repair enzymes; O^6-methylguanine (m^6G) DNA methyltransferase and 3-methyladenine (m^3A) DNA glycosylase II (Schendel and Robbins, 1978; Karran et al., 1979, 1982; Evensen and Seeberg, 1982). The m^6G DNA methyltransferase rapidly demethylates the m^6G residues introduced by the challenging dose (Olsson and Lindahl, 1980; Foote et al., 1980; Demple et al., 1982), while m^3A DNA glycosylase II (TagII) releases the alkylating products 3-methyladenine (m^3A), 3-methylguanine (m^3G) and 7-methylguanine (m^7G) from DNA (Karran et al., 1982; Thomas et al., 1982; McCarthy et al., 1984). TagII appears to be the product of the *alkA* gene (Evensen and Seeberg, 1982; Clarke et al., 1984) while the transferase is the *ada* gene product (Teo et al., 1984). The transferase thus regulates both its own synthesis and that of TagII.

E. coli cells also have another inducible response to DNA damage. This response, termed SOS repair, results in the induction of several unrelated functions, including prophage induction, mutagenesis, increased DNA repair and inhibition of cell division (Radman, 1974; Witkin, 1976; Kenyon and Walker, 1980; Kenyon et al., 1982). The SOS response is controlled by the *recA*$^+$ and the *lexA*$^+$ genes. The *lexA*$^+$ gene product is a

repressor which, upon induction, becomes cleaved by activated proteolytic activity of the *recA* protein. The *lexA*-controlled operons are thus opened for transcription (Kenyon et al., 1982; Little and Mount, 1982).

It has previously been shown that *recA* mutant cells can be adapted to higher survival and lower mutation by alkylation induction (Jeggo et al., 1977; Schendel et al., 1978; Yamamoto and Sekiguchi 1979). This has suggested that the adaptive response and the SOS response are separate functions. However, the effect of SOS induction on the synthesis of the enzymes known to be controlled by the adaptive response has not been investigated. We have looked at the effect of the *recA*1 mutation on the induction of TagII in a strain carrying *tag*, deficient in TagI. The *tag* mutation accentuates the need for TagII in repair of alkylated DNA and facilitates the detection of TagII activity both in in vivo and in vitro assays. The results show that the *recA* mutation has no effect on TagII induction implying that the *alkA* gene which codes for TagII is not under *recA*/*lexA* control.

Materials and methods

Alkylating agents

Methyl methanesulfonate (MMS) was from Janssen Chimica, and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) was from Aldrich Chemical Co. Radioactive [*Me*-^{3}H]dimethylsulfate (DMS) (1.4 Ci/mmole) was obtained from New England Nuclear. 3-Methyladenine was purchased from Fluka AG.

Strains

Bacterial strains are derivatives of *E. coli* K-12 and listed in Table 1.

Media

Cells were grown at 37°C in L-broth (1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl, 0.1% (w/v) glucose (pH 7.3)). Bacteria suspended in 2 ml top agar were plated on the same medium containing 1.5% (w/v) agar (L-agar plates). Minimal salt medium (M9) contains: 0.018 M NH_4Cl, 0.022 M KH_2PO_4 and 0.042 M $Na_2PO_4 \times 2H_2O$.

TABLE 1

STRAINS OF *Escherichia coli* USED IN THIS WORK

Strain	Relevant genotype	Source and or reference
AB1157	Wild type	Howard-Flanders et al. (1968)
BK2106	*tag*-2, *ada*	Karran et al. (1980)
BK2114	*tag*-2	Evensen and Seeberg (1982)
JC10236	*srlC* 300::Tn10	D.W. Mount
KL16-99	*recA*1	Low (1968) via B. Bachmann
BK2124	*tag*-2, *recA*1.	This work

K medium is M9 salts + 1% glucose, 1% casamino acids, 0.001 M $MgSO_4 \times 7H_2O$, 0.0005 M $CaCl_2$ and 0.0001% vitamin B_1.

Phage λ buffer is 0.06 M Tris–HCl, pH 7.2, 0.01 M $MgSO_4 \times 7H_2O$, and 0.005% (w/v) gelatin.

BS buffer is 0.01 M $Na_2HPO_4 \times 2H_2O$, 0.01 M KH_2PO_4 and 0.0086 M NaCl.

Isolation of strain BK2124

BK2124 (*recA tag*) was constructed by transducing *recA*1 into the TagI-deficient strain BK2114. P1 transductions were performed as described by Miller (1972). A tetracycline-resistant (TetR) derivative of BK2114 was made by transducing BK2114 with a P1 lysate of JC10236 (*srlC* 300::Tn10) selecting for tetracycline-resistant colonies on agar plates containing 10 μg/ml of tetracycline. TetR colonies unable to grow on sorbitol as sole carbon source were selected and were transduced to *srl*$^+$ with P1 lysate from KL16-99 (*recA*1). The *srl*$^+$ recombinants were streaked on nutrient agar UV-irradiated (10 J/m^2) and scored for UV-sensitivity. Introduction of the *recA*1 mutation was detected as a change from a UV-resistant to a UV-sensitive phenotype.

MNNG adaptation

Stock solutions of MNNG (4 mg/ml) were made in dimethyl sulfoxide and stored frozen.

Unless otherwise indicated MNNG was added at a final concentration of 0.5 μg/ml to fresh overnight cultures diluted 1/10 in K medium. Cells were incubated 90 min, at 37°C.

Survival measurements

Adapted and non-adapted cultures in log phase ($1-2 \times 10^8$ cells/ml) were washed once by centri-

fugation and resuspended in 1/10 volume of BS buffer. MMS was added at a final concentration of 0.06 M for the wild-type and *tag* strains and of 0.02 M for the *recA tag* strain. At intervals, the reaction was stopped by diluting the cells 100-fold in BS buffer. Samples were plated for survival on nutrient agar after appropriate dilutions.

Reactivation of MMS-treated λ phage

Phage λ*cI*857 was exposed to 0.05 M MMS for various times, diluted at least 100-fold in λ buffer, and plated on exponentially growing untreated or adapted cells (Evensen and Seeberg, 1982).

Assay of TagII activity in extracts

Cell extracts of untreated, MNNG-adapted or UV-induced cells were prepared by a combination of plasmolysis and lysozyme digestions as previously described (Seeberg et al., 1976).

m^3A DNA glycosylase activity was assayed by the method of Riazuddin and Lindahl (1978), modified by Evensen and Seeberg (1982).

Results and discussion

The *recA*1 mutation was introduced into the TagI-deficient strain (*tag*-2) by P1 transduction (see Materials and Methods). Cells carrying the *recA* mutation are by themselves sensitive to alkylating agents presumably due to defective post-replication repair (Howard-Flanders, 1968). TagI-deficient mutant cells are moderately sensitive to MMS exposure in buffer because of a deficiency in the excision repair of 3-methyladenine residues in DNA (Karran et al., 1980; Evensen and Seeberg, 1982). The double mutant *recA tag* is much more sensitive to MMS exposure than the single *tag* mutant (Fig. 1) consistent with the fact that *recA* and *tag* mutations represent blocks in separate repair pathways. However, the low survival of the *recA tag* double mutant could also be taken to suggest that *recA* would prevent induction of TagII which serves as a back-up enzyme in the repair of 3-methyladenine residues (Evensen and Seeberg, 1982). This is inconsistent, however, with the observation that the *recA tag* mutant can be adapted to higher survival by alkylation induction (Fig. 1). Mutants defective in TagII induction, i.e. *ada* and *alkA*, are also defective in killing adaptation (Evensen and Seeberg, 1982).

Further evidence for the induction of TagII in the *recA* mutant was obtained by measuring host-cell reactivation of MMS-treated λ phages in adapted and non-adapted cells. Cells carrying *tag*

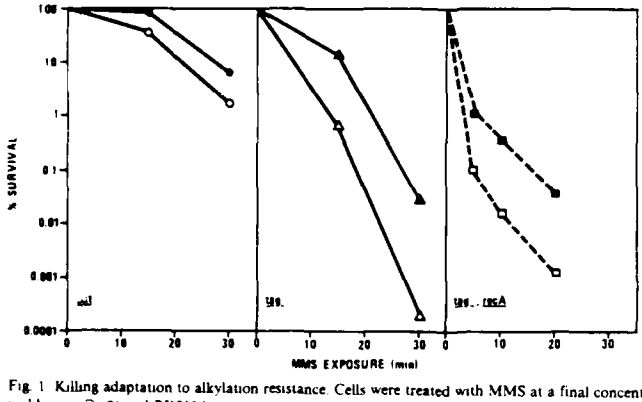

Fig. 1. Killing adaptation to alkylation resistance. Cells were treated with MMS at a final concentration of 0.06 M for strain AB1157 (wild type, ○, ●) and BK2114 (*tag*, △, ▲) and of 0.02 M for BK2124 (*tag*, *recA*, □, ■). Closed symbols refer to adapted cell cultures (MNNG-treated with MNNG at a final concentration of 0.1 μg/ml), while open symbols represent untreated controls.

are deficient in host-cell reactivation of MMS-treated phages, but become proficient by adaptation since TagII is then induced and can replace TagI in repair of the alkylated phage DNA (Evensen and Seeberg, 1982). The double mutant *recA tag* has even lower capacity for host-cell reactivation than *tag* cells without adaptation, but can equally well by adapted by prior growth in the presence of sublethal concentrations of MNNG (Fig. 2). This is an in vivo assay for the induction of TagII in the *recA* mutant background.

We have also measured TagII activity directly in cell extracts from adapted and non-adapted cells (Fig. 3). The results support the in vivo assay in that TagII is induced to normal levels in *recA* mutant cells by adaptation. A control was included where *tag* (*recA*$^+$) cells were induced for the SOS response by UV-irradiation. The UV-irradiation did not result in any induction of TagII. UV-irradiation of a mutant containing the *aidA*::Mu-dI(Ap^R, *lac*) fusion has been shown not to induce β-galactosidase activity (Volkert and Nguyen, 1984). The *aidA* fusion has been mapped to the *alkA* region and likely is within the *alkA* operon.

In conclusion, both in vivo and in vitro tests show clearly that induction of TagII does not require a functional *recA* gene. This supports the view that the SOS response and the adaptive response are independent processes and that the *alkA* gene which is the structural gene for TagII is not a *lexA* / *recA*-controlled operon.

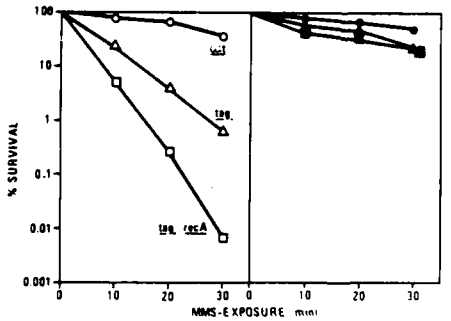

Fig. 2. Host-cell reactivation of alkylated λ phages in non-treated (open symbols) and adapted cells (closed symbols). AB1157 (wild type, ○, ●), BK2114 (*tag*, △, ▲), BK2124 (*tag, recA*, □, ■).

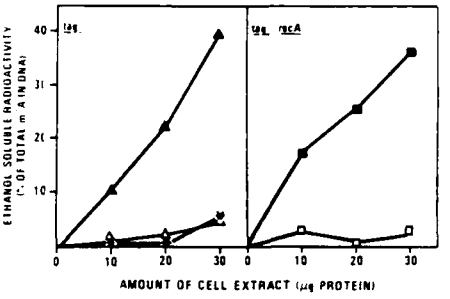

Fig. 3. Assay of 3-methyladenine DNA glycosylase II (TagII) in crude extracts of uninduced cells (open symbols), MNNG-induced cells (closed symbols) and UV-induced cells (asterisks). BK2114 (*tag*, △, ▲, *), BK2124 (*tag, recA*, □, ■).

After this work was completed we learned that Nakabeppu et al. (1984) have constructed a plasmid with the *alkA* operator coupled to *lacZ*. They show that expression of β-galactosidase from the hybrid gene is not affected by the *recA* or *lexA* phenotype of the host cell, consistent with our results.

Acknowledgements

I am grateful to Dr. Erling Seeberg for a critical reading of the manuscript.

This research was supported by the Royal Norwegian Council for Scientific and Industrial Research (NTNF).

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AP-SITES AS MUTAGENIC INTERMEDIATES. A DIRECT ROLE OF DNA GLYCOSYLASES IN MUTAGENESIS.

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SUMMARY

We have investigated the mutagenic effects of persistant 3-methyladenine (m^3A) in DNA by measuring methylmethanesulfonate (MMS) induced mutagenesis in m^3A repair deficient mutants. In *tag*$^-$ mutant cells, lacking constitutive m^3A DNA glycosylase, and in *alkA* mutant cells, lacking inducible m^3A DNA glycosylase, the MMS induced mutations were increased several-fold compared to wild type cells. In a double mutant, *tag*,*alkA*, lacking both constitutive and inducible DNA glycosylase the rate of mutations was extremely low. By complementation of the repair deficiency with plasmid coded *tag*$^+$ or *alkA*$^+$ enzymes, the rate of mutations in the *tag* mutant was reduced, but however increased in the *tag*,*alkA* double mutant. From our results it is concluded that m^3A enhance mutagenesis by promoting SOS induction but is not itself a premutagenic lesion. It is indicated that DNA glycosylases are directly involved in mutagenesis by generating AP(Apurinic)-sites as premutational lesions.

INTRODUCTION

Alkylating agents like N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and methylmethanesulfonate (MMS) are strong mutagens. The mutagenic effects are partly due to their ability to alkylate bases at O-atoms (Singer 1976). The major O-alkylated lesion, O^6-methylguanine (m^6G), causes mutations directly by mispairing with thymine during replication resulting in GC-AT transition mutations (Abbot and Saffhill 1979; Hall and Saffhill 1983).

Another pathway of alkylation mutagenesis requires SOS processing. Mutations induced via the SOS response are associated with the induction of the umuDC operon and regulated by the recA and lexA genes (Witkin 1976; Little and Mount 1982; Walker 1984 1985). So far, uncertainty exists as to which alkylation product is the primary target for the SOS induced alkylation mutagenesis. One candidate is 3-methyladenine (m^3A) which is one of the major products formed in DNA by alkylation (Beranek et al. 1980). However, in all systems investigated m^3A is rapidly excised by repair enzymes and it has therefore been difficult to correlate the occurrence of m^3A with the rate of mutation (Karran et al. 1980; Lindahl 1982). In Escherichia coli excision of 3-methyladenine is mediated by two separate DNA glycosylases encoded by the tag and alkA genes (Evensen and Seeberg 1982; Karran et al. 1982; Thomas et al. 1982). The tag enzyme is specific for 3-methyladenine removal (Riazuddin and Lindahl 1978), whereas the alkA enzyme also excises a number of minor lesions including 7-methylguanine (m^7G), 3-methylguanine (m^3G), O^2-methylthymine (m^2T) and O^2-methylcytosine (m^2C) (Thomas et al. 1982; McCharteY et al. 1984). The tag enzyme is constitutively expressed, while the alkA enzyme is a part of the adaptive response and is induced when cells are exposed to low levels of alkylating agents (Samson and Cairns 1977; Evensen and Seeberg 1982; Karran et al. 1982). The alkA enzyme constitutes 5-10 % of the total m^3A DNA glycosylase activity in non-induced cells, but is increased 10-20 fold by adaptation requiring a functional ada^+ gene (Karran et al. 1980; Sedgwick 1983).

We have previously characterized mutants defective in excision of 3-methyladenine, lacking the tag coded DNA glycosylase (Karran et al. 1980; Evensen and Seeberg 1982). In this communication we have exploited these mutants to investigate wether m^3A is a premutagenic lesion by measuring the rate of alkylation induced mutagenesis in tag^- versus tag^+ cells. MMS rather than MNNG was chosen for alkylation to minimize the contribution of mutations due to O^6-methylguanine. The results showed that MMS induced mutations increased several-fold in a tag^- mutant compared to wild type, which we initially interpreted as m^3A being a premutagenic lesion. However, a low rate of mutation observed in a double mutant strain, tag,alkA, lacking both glycosylases, indicated that increased mutagenesis in $tag,alkA^+$ was due to increased SOS induction by persisting m^3A, but that the AP(Apurinic)-sites generated by the alkA coded glycosylase was the primary target for the SOS-dependent alkylation mutagenesis.

MATERIALS AND METHODS

Chemicals. Methylmethanesulfonate (MMS) was from Janssen Chimica. Rifampicin was from Sigma.

Bacterial strains. Bacterial strains are derivatives of E.coliK-12 and listed in Table 1. Strains BK2118, BK2134 and BK2138 were constructed by P1 transductions performed as described by Miller (1972). BK2118(tag,alkA) was constructed by transducing the alkA mutation in MS23 into the BK2114 mutant selecting for his^+ and screening for MMS sensitivity. The umuC deriviatives (BK2134, BK2138) were constructed by transducing BK2118 and BK2114 with a P1 lysate of GW2110(umuC122::Tn5) selecting for kanamycin (KanR) reistant colonies on agar plates containing 25 μg/ml of kanamycin (Glazebrook et al. 1983). Strains used for analysis of β-galactosidase induction were isolated by conjugation of TP110 col::Mud(ApR,lacZ) plasmid into Mu^+ lysogens of various mutants selecting for ApR on the Mud phage and KanR of the plasmid TP110. Recipient cultures must be Mu^+ lysogens to prevent zygotic induction. Mu^+ lysogens were obtained by spotting 1 drop of a supernatant from fresh overnight culture of strain N1260 onto a lawn of recipient cells and afterwards picking growing colonies (Glazebrook et al. 1983).

Survival measurements. Cell cultures in log phase were washed once by centrifugation, resuspended in phosphate-buffer and treated with the alkylating agent MMS (see figure legends for specification of dose). At intervals, the reaction was stopped by diluting the cells 100-folds in phosphate-buffer (Evensen. 1985). Samples were plated for survival on nutrient agar after appropriate dilutions.

MMS induced mutagenesis. Cell cultures in log phase resuspended in phosphate-buffer were treated with various doses of MMS. At intervals, the reaction was stopped by diluting the cells 100-fold in L-broth. Cells were incubated routinely over-night at 37 ^{o}C. Samples were plated for total number of viable cells on nutrient agar and for rifampicin resistant(RifR) mutants on rifampicin(rif)-agar (100 μg/ml).

MMS induced SOS response; quantitativ β-galactosidaseassay. The Colicinlb function of TP110 (group I1 plasmid, Glazebrook et al. 1983) is under control of the lexA/recA genes and belongs to the SOS response. In the TP110col::Mud(ApR,lacZ) plasmid (which is a low copy number plasmid) the colicin(col) promoter is fused to the structural gene for β-galactosidase. SOS-treatments of TP110 harbouring cells will turn on the col gene and β-galactosidase production. β-galactosidase production is thus a measure of SOS induction. Cells were grown in L-broth, resuspended in phosphate-buffer and treated with MMS at a final consentration of 0.01M for induction of the SOS response. Samples were assayed for β-galactosidase expression as described by Miller (1972).

Table 1. Strains of E.coli and plasmids used in this work

Strain or plasmid	Relevant geno- or phenotype	Source and/or reference
AB1157	w.t.	Howard-Flanders
BK2114	tag	Evensen and Seeberg 1982
BK2110	ada	Evensen and Seeberg 1982
BK2106	tag,ada	Karran et al. 1980
MS23	alkA	Yamamoto et al. 1978
BK2118	tag,alkA	This work
BK2124	tag,recA	Evensen 1985
GW2100	umuC122::Tn5,KanR	LaMotte
BK2134	tag,umuC	This work
BK2138	tag,alkA,umuC	This work
MH927#122	NalR(StrS) TP110col::Mud(ApR,lacZ)	Glazebrook et al. 1983
N1260	(Muc^+)	Glazebrook et al. 1983
pBK101	$alkA^+$,ApR	Clarke et al. 1984
pBK201	tag^+,ApR	Clarke et al. 1984
TP110col::Mud (ApR,lacZ)	KanR,ApR	Glazebrook et al. 1983

RESULTS

Increased alkylation mutagenesis in tag mutant strains. The formation of rifampicin resistant (RifR) colonies as a function of MMS exposure was measured in tag and tag,ada mutant cells as compared to the corresponding wild type or ada mutant cell (Fig. 1). In tag cells the number of mutants reaches a maximum after a brief treatment; no further increase is caused by longer incubation with MMS. This is in contrast to what is observed in both wild type and ada cells where the number of mutants increases gradually during the entire exposure period. The response of the tag,ada double mutant appears to be additive with respect to that observed for either single tag or ada mutant strain. This is as could be expected since ada prevents the repair of O^6-methylguanine residues and tag and ada block two separate repair pathways. However, in both ada^+ and ada^- background introduction of the tag mutation increases the rate of MMS induced mutation, consistent with a premutagenic role of 3-methyladenine.

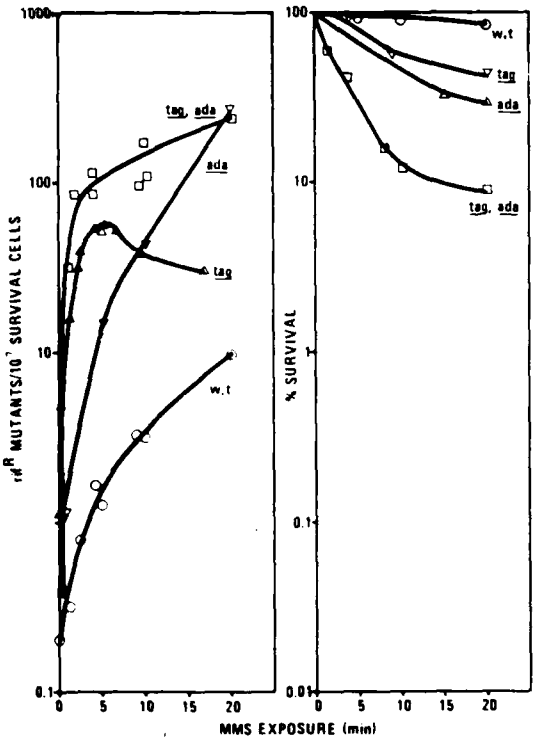


Fig. 1. MMS induced RifR mutants and cell survival of tag, ada and tag,ada mutant cells. Cells were treated with MMS at a final concentration of 0.01M. (O-O AB1157 w.t., Δ-Δ BK2114 tag, ▽ - ▽ BK2110 ada, □-□ BK2106 tag,ada)

Reduced alkylation mutagenesis in tag,alkA double mutant cells. It has been shown previously that the alkA mutation leads to increased alkylation mutagenesis (Yamamoto and Sekiguchi 1979). Since alkA mutant cells are defective in 3-methyladenine DNA glycosylase II, increased mutagenesis could also be ascribed to premutagenic effect of m^3A. If so, one would expect that a double mutant strain carrying both tag and alkA would be even more mutable by MMS. Surprisingly, it was found that RifR mutations were undetectable in the surviving cell fraction of the double mutant strain after 10 to 20 min. MMS exposure as normally used for these experiments (Fig. 2, Table 2). Survival was very low, consistent with the cytotoxic effects of persisting m^3A (Karran et al. 1980; Clarke et al. 1984).

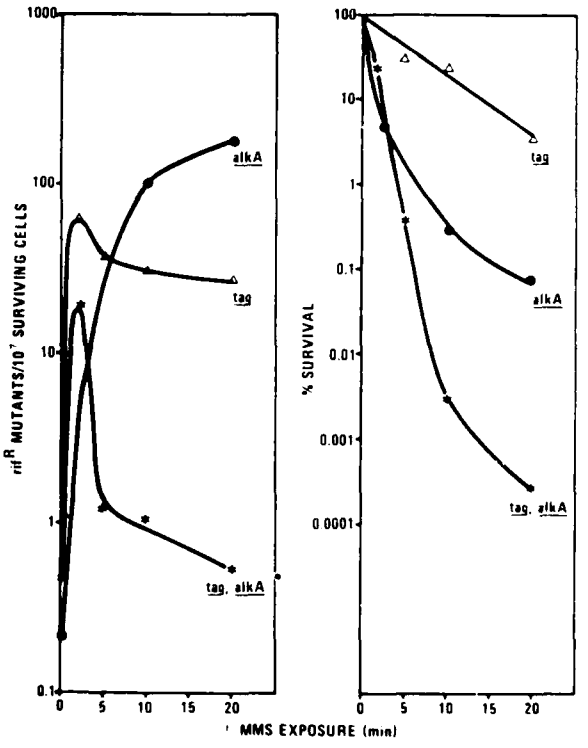


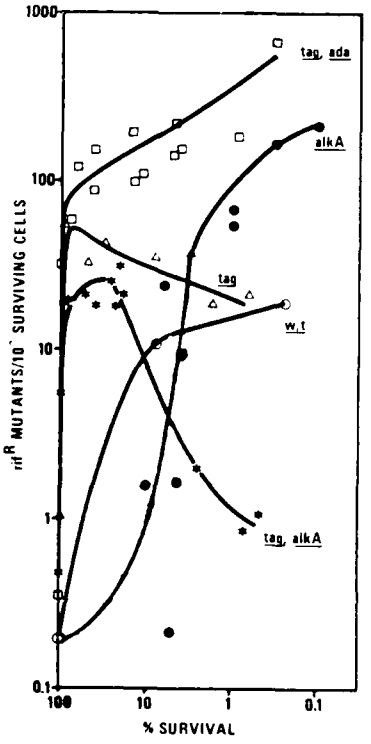
Fig. 2. MMS induced RifR mutants and cell survival of tag, alkA and tag,alkA mutant cells. Cells were treated with MMS at a final concentration of 0.01M. (Δ - Δ BK2114 tag, ●-● MS23 alkA, *-* BK2118 tag,alkA)

Table 2. Alkylation (MMS) induced mutation frequencies in E.coli mutants deficient in tag or alkA coded 3-methyladenine DNA glycosylase. Cells treated with MMS at a final consentration of 0.02M, 20 min. at 37 ^{0}C.

Strain	Genotype	Phenotype	Mutation frequency
AB1157	w.t.		6
BK2114	tag	TagI$^-$	27
BK2114/pBK201	tag/tag$^+$		3
BK2124	tag,recA	TagI$^-$,SOS$^-$	0.1
BK2106	tag,ada	TagI$^-$,TagII$^-$,Ada$^-$	231
BK2106/pBK101	tag,ada/alkA$^+$	TagI$^-$,TagII$^-$,Ada$^-$/TagII$^+$	25
BK2106/pBK201	tag,ada/tag$^+$	TagI$^-$,TagII$^-$,Ada$^-$/TagI$^+$	17
BK2118	tag,alkA	TagI$^-$,TagII$^-$	0.6
BK2118/pBK101	tag,alkA/alkA$^+$	TagI$^-$,TagII$^-$/TagII$^+$	5
BK2118/pBK201	tag,alkA/tag$^+$	TagI$^-$,TagII$^-$/TagI$^+$	31

In Fig. 3 the number of mutants have been plotted against the degree of survival after MMS treatment. In this graph mutagenesis is related to the cytotoxic effects of MMS and not to the initial number of alkylations. It appears that MMS mutagenesis in the tag,alkA double mutant is detectable at doses which give 90 to about 20% survival but at 1% survival is not significant. The other tag mutant strains (i.e. tag and tag,ada) also show a high number of mutations at doses which have only a small effect on survival but the effect sustains for lower survival and is much increased in tag,ada. In contrast, MMS mutagenesis in tag$^+$ strains (i.e. wild type and alkA mutant cells) becomes significant first after doses which reduce survival to about 10% and then increases gradually (see also Fig.s 1 and 2).

Fig. 3. Mutation frequency plotted as a function of survival. MMS dose and time of incubation were adjusted as to give about 0.01% cell survival. (O-O AB1157 w.t., Δ - Δ BK2114 tag, ●-● MS23 alkA, □-□ BK2106 tag,ada, *-* BK2118 tag,alkA)



Taken together, the response of the double mutant strain does not support the notion that m^3A is premutagenic. However, it appears that persisting m^3A promotes mutation but that some DNA glycosylase action is prerequisite for mutagenesis. This is further supported by experiments with the double mutant strain being transformed by plasmids carrying either tag$^+$ or alkA$^+$ (Table 2). In the tag$^+$ or alkA$^+$ transformed cells where glycosylase activity is restored, MMS mutagenesis is enhanced. It is thus confirmed that reduced mutagenesis is due to the double glycosylase deficiency and not to any unknown secondary mutation of the tag,alkA double mutant. Introduction of the tag$^+$ or alkA$^+$ plasmid in the tag and tag,ada mutant has the adverse effect, both reducing mutagenesis. However, both these strains already possess the alkA coded glycosylase activity which may be sufficient for mutagenesis to occur.

Enhanced induction of the SOS response in 3-methyladenine repair defective strains. A plausible explanation for the results described above is that the actual premutagenic lesions of the MMS mutagenesis are the AP-sites generated by the glycosylase action. Even the low level of mutagenesis observed at low doses for the double mutant strain may be explained in this way since slow spontaneous loss of methylated bases may allow both survival and mutagenesis when alkylation is limited. Previous reports have indicated that AP-sites are mutagenic through SOS processing (for a review see Loeb, 1985). This agrees with our observation that the SOS defective recA,tag mutant cells were not mutated by MMS (Table 2).

To evaluate the contribution of SOS response to MMS mutagenesis in our strains, SOS induction was measured as a function of MMS treatment. Cells were transformed with plasmid TP110col::Mud (ApR,lacZ) which carries a colicinIb-lacZ fusion. The col gene of the group I1 plasmids belongs to the SOS regulatory network and is activated by SOS induction (Glazebrook et al. 1983). In cells carrying the fusion plasmid induction of β-galactosidase activity is a measure of the SOS response. Fig. 4 shows a dose response of β-galactosidase activity induction following MMS treatment. It appears that all the mutants with tag and/or alkA or tag,ada mutations have more extensive β-galactosidase induction than wild type, although the dose response is different. In the tag,alkA double mutant maximum induction is observed for very low doses. It thus appears to be a good correlation between the induction of the SOS response and persistence of m^3A after alkylation. Boiteux et al. (1984) have also published results similar to those presented in Fig. 4, and concluded that m^3A is an SOS inducing lesion.

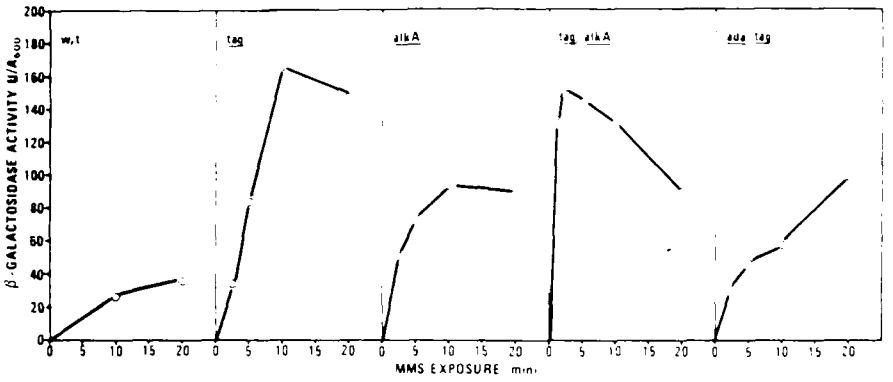


Fig. 4. SOS induction in various E.coliK-12 strains lysogenic for Mud^{+} and containing TP110col::Mud(ApR,lacZ). Cells were assayed for enzyme activity as described. Cells were incubated 90 min. for expression of β-galactosidase activity. From β-galactosidase activity plotted is subtracted background activity observed in untreated cells.

To verify that MMS induced mutations in the tag mutant were indeed SOS dependent we introduced a umuC mutation both in tag and in tag,alkA (Fig. 5). The presence of umuC reduced mutation rate by more than 90% after 5 min. MMS exposure in tag. The MMS mutagenesis observed in tag,alkA at very low doses was also reduced by introducing umuC.

In the course of the survival measurements of the various strains for low doses of MMS exposure we consistently observed that the tag,alkA double mutant was more resistant to MMS at low doses than the alkA mutant (Fig. 6). In view of the dose dependence of the SOS response we believe that this survival effect can be ascribed to more extensive SOS induction in tag,alkA than alkA at low doses, for instance related to more effective induction of postreplication repair.

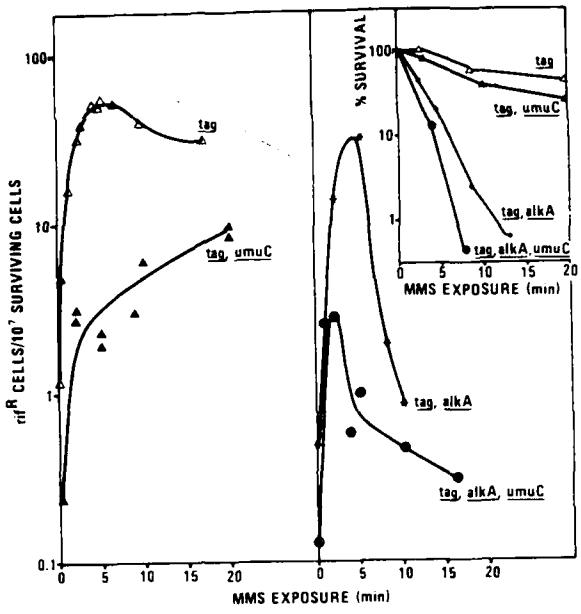


Fig.5. MMS induced RifR mutants and cell survival of tag,alkA, tag,umuC and tag,alkA,umuC mutant cells. Cells were treated with MMS at a final concentration of 0.01M. (Δ-Δ BK2114 tag, ✱-✱ BK2118 tag,alkA, ▲-▲ BK2134 tag,umuC, ●-● BK2138 tag,alkA,umuC).

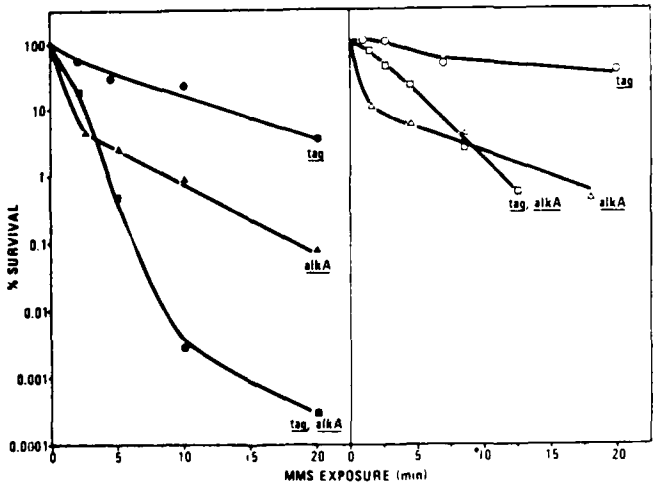


Fig.6. Cell survival as a function of MMS exposure. Cells were treated with MMS at a final concentration of 0.01M (0-0 BK2114 tag, Δ-Δ MS23 alkA, - BK2118 tag,alkA) or 0.02M (●-●- BK2114 tag, ▲-▲ MS23 alkA, ■-■ BK2118 tag,alkA).

DISCUSSION

We have investigated the mutagenic effects of persistent m^3A in DNA by measuring MMS induced mutagenesis in m^3A repair deficient tag mutants as compared to the corresponding tag^+ strains. It is concluded that m^3A enhances mutagenesis by promoting SOS induction but is not by itself a premutagenic lesion. It is indicated that DNA glycosylases are directly involved in mutagenesis by generating AP-sites as premutational lesions. On the basis of these results we propose a model for MMS mutagenesis as indicated in Fig. 7.

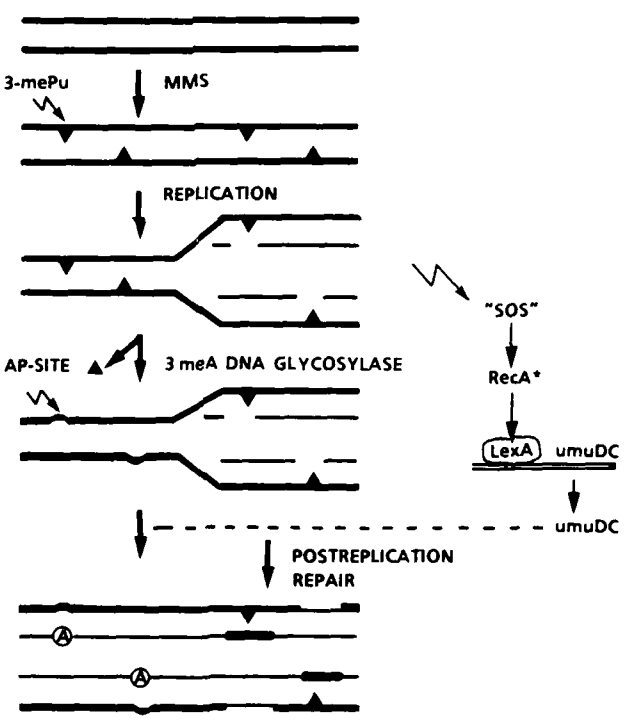


Fig. 7. A schematic model for MMS mutagenesis.

Persistence of m^3A stalls the replication complex and leaves gaps in the DNA that serve as signals for SOS induction. Once induced, the umuDC functions will allow mutation to occur by replication at AP-sites newly generated by DNA glycosylase action but not acted upon by AP endonucleases. Alternatively, mutation might be formed at the original m^3A stalling replication if removed by the glycosylase. The tag and alkA glycosylases have both a preference for m^3A in double stranded DNA but activity at a low rate in single stranded DNA has also been detected (Thomas et al. 1982).

AP-sites are not believed to indcue the SOS response themselves (Miller and Low 1984). It appears that persisting m^3A induces the SOS response (Boiteux et al. 1984). Absence of tag and alkA glycosylases lead to much more efficient induction of the SOS response by MMS treatment (Fig. 4 ; Boiteux at al. 1984). Similarly, Foster and Eisenstadt (1985) have shown that the induction of SOS response is enhanced in the alkA strain.

AP-sites seem to target mutations once the SOS system is induced. The predominant mutations observed are transversions, resulting from insertion af an adenine across from depurinated bases (Boiteux and Laval 1982; Miller and Low 1984; Kunkel 1984). Bypass polymerization of depurinated m^3A will primarily lead to AT-TA transversions. Foster and Eisenstadt (1985) have reported that the most frequent mutations in alkA cells treated with MMS appear to be AT transversions, the base substitution that would be expected if the premutagenic lesions are AP-sites left by the removal of damaged adenines. In our study we have selected for the RifR phenotype to measure mutation frequency. Previous reports describe that only base substitutions can generate RifR mutants (Miller and Low 1984). In tif-1 induced RifR mutants, exclusively GC-TA and AT-TA transversions occur. Thus, by selection of RifR mutants we are able to pick up mutations occuring at AP-sites.

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