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Repair-deficient 3-methyladenine DNA glycosylase homozygous mutant mouse cells have increased sensitivity to alkylation-induced chromosome damage and cell killing

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In *Escherichia coli*, the repair of 3-methyladenine (3MeA) DNA lesions prevents alkylation-induced cell death because unrepaired 3MeA blocks DNA replication. Whether this lesion is cytotoxic to mammalian cells has been difficult to establish in the absence of 3MeA repair-deficient cell lines. We previously isolated and characterized a mouse 3MeA DNA glycosylase cDNA (Aag) that provides resistance to killing by alkylating agents in *E.coli*. To determine the *in vivo* role of Aag, we cloned a large fragment of the *Aag* gene and used it to create Aag-deficient mouse cells by targeted homologous recombination. *Aag* null cells have no detectable *Aag* transcripts or 3MeA DNA glycosylase activity. The loss of Aag renders cells significantly more sensitive to methyl methanesulfonate-induced chromosome damage, and to cell killing induced by two methylating agents, one of which produces almost exclusively 3MeAs. *Aag* null embryonic stem cells become sensitive to two cancer chemotherapeutic alkylating agents, namely 1,3-bis(2-chloroethyl)-1-nitrosourea and mitomycin C, indicating that Aag status is an important determinant of cellular resistance to these agents. We conclude that this mammalian 3MeA DNA glycosylase plays a pivotal role in preventing alkylation-induced chromosome damage and cytotoxicity.

Keywords: chemotherapy/DNA alkylation repair/gene targeting/3-methyladenine/null embryonic stem cells

Introduction

Cells are continuously exposed to alkylating agents that can cause mutation and cell death (reviewed by Singer and Grunberger, 1983). These agents produce many types of DNA lesions whose biological effects depend upon the nature of the alkyl group and its position on DNA. For example, in *Escherichia coli*, 3-methyladenine (3MeA) lesions block DNA replication and are lethal, and O^6-methylguanine (O^6MeG) lesions mispair during DNA replication and can cause mutations (Coulondre and Miller, 1977; Boiteux *et al.*, 1984; Loechler *et al.*, 1984; Snow *et al.*, 1984; Larson *et al.*, 1985). Many enzymes have evolved to repair the DNA damage produced by endogenous and exogenous alkylating agents (reviewed in Friedberg *et al.*, 1995).

The repair of DNA alkylation damage by 3MeA DNA glycosylases has been studied extensively in microbial organisms. *Escherichia coli* has two DNA glycosylases, Tag and AlkA, that repair the potentially lethal 3MeA lesions. Tag is constitutively expressed while AlkA, which has a somewhat broader substrate range, is induced when cells are exposed to low levels of DNA alkylation damage (reviewed by Lindahl *et al.*, 1988). 3MeA DNA glycosylase homologs have now been cloned from many eukaryotic organisms and their biochemical characterization has dramatically changed our view of the putative biological functions of this class of enzymes (Chen *et al.*, 1989; Berdal *et al.*, 1990; O'Connor and Laval, 1990, 1991; Chakravarti *et al.*, 1991; Samson *et al.*, 1991; Engelward *et al.*, 1993; Santerre and Britt, 1994). Far from specifically releasing 3MeA from DNA, the 3MeA DNA glycosylases can repair a stunning variety of damaged DNA bases, some of which are not alkylated. These substrates include numerous different N^3- and N^7-alkylated purines, O^2-alkylated pyrimidines, deaminated adenine (hypoxanthine), oxidized guanine (8-oxoguanine), oxidized derivatives of thymine, bases with cyclic lesions, cross-linked bases and haloethylated bases; it should be noted that no one glycosylase has been assayed for the repair of all of these lesions (Thomas *et al.*, 1982; McCarthy *et al.*, 1984; Habraken and Ludlum, 1989; Habraken *et al.*, 1991a,b; Singer *et al.*, 1992; Matijasevic, 1992, 1993; Bessho *et al.*, 1993; Bjelland *et al.*, 1993, 1994; Dosanjh *et al.*, 1994a,b; Roy *et al.*, 1994; Saparbaev and Laval, 1994). Thus, while all the 3MeA DNA glycosylases are able to repair simple methylated DNA lesions (indeed it was on this basis that their genes were cloned), some may also play important roles in protecting cells against a variety of other cytotoxic and mutagenic DNA lesions.

We cloned the mouse 3MeA DNA glycosylase cDNA (*Aag*) in order to explore the role of this enzyme in mammalian cells, and ultimately in a whole animal. The mouse Aag enzyme has been shown to release 3MeA, 3-methylguanine (3MeG), 7-methylguanine (7MeG), 8-oxoguanine and hypoxanthine (Bessho *et al.*, 1993; Engelward *et al.*, 1993; Roy *et al.*, 1994; Engelward and Samson, unpublished). Since both 8-oxoguanine and hypoxanthine are endogenously produced mutagenic lesions, Aag may act to limit spontaneous mutation. To our knowledge, there are no known mammalian cells that are specifically deficient in 3MeA DNA glycosylase activity. We therefore created *Aag* null mouse embryonic stem (ES) cells by homologous recombination. Null *Aag* cells display no detectable *in vitro* release of 3MeA, suggesting that there is only one 3MeA DNA glycosylase

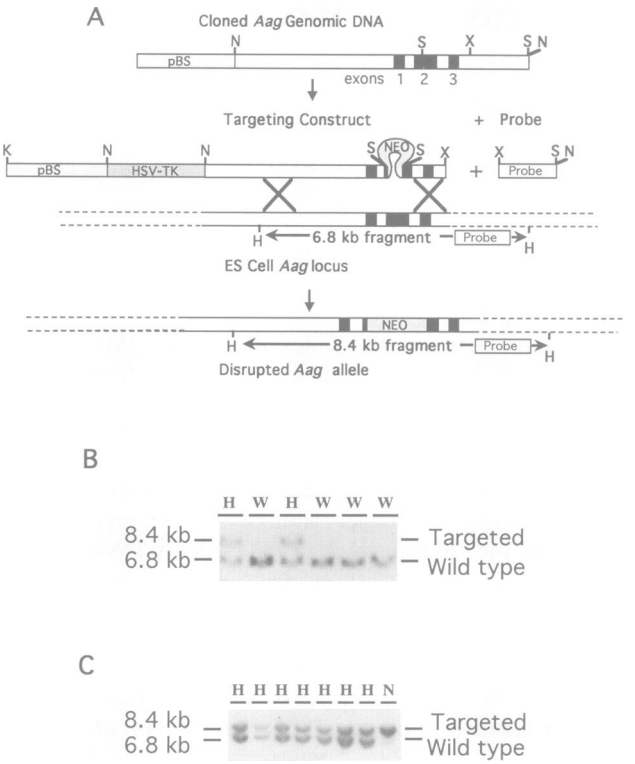

Fig. 1. (**A**) Targeting strategy for the *Aag* locus. The restriction sites are: N, *Not*I; S, *Sal*I; X, *Xba*I; K, *Kpn*I; H, *Hin*dIII.
(**B** and **C**) Southern analysis of genomic DNA from *Aag* wild-type (W), heterozygous (H) and null (N) clones probed with the *Xba*I–*Sal*I fragment. (B) Six of 432 clones screened for insertion of *neo*.
(C) Eight of 234 clones screened for insertion of *hyg*. The wild-type allele visible in the *Aag* null clone 38, far right, is due to the presence of mouse STO feeder cells.

expressed in mouse ES cells. Characterization of the *Aag* null cells reveals that Aag not only plays an important role in protecting mammalian cells against the toxic effects of simple alkylating agents, but it also protects against the toxic effects of two agents used for cancer chemotherapy, namely 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) and mitomycin C.

Results

Creation of Aag null cells by targeted homologous recombination

Our goal was to create *Aag* null cells by targeted disruption of both *Aag* alleles. The *Aag* alleles were targeted consecutively with two positive/negative selection vectors, each containing different positive drug selection marker genes, namely neomycin (*neo*) and hygromycin (*hyg*) (Mansour *et al.*, 1988; te Riele *et al.*, 1990).

We used the previously isolated *Aag* cDNA (Engelward *et al.*, 1993) to clone, by hybridization, a 15 kb fragment of the *Aag* gene from a λ-FIX mouse genomic library. An 8.5 kb *Not*I fragment of the genomic clone was subcloned into pBluescript and characterized further to establish the position and orientation of introns and exons, using a combination of restriction enzyme analysis, polymerase chain reaction and DNA sequencing (data not shown).

Figure 1A illustrates the design of the *Aag–neo* targeting

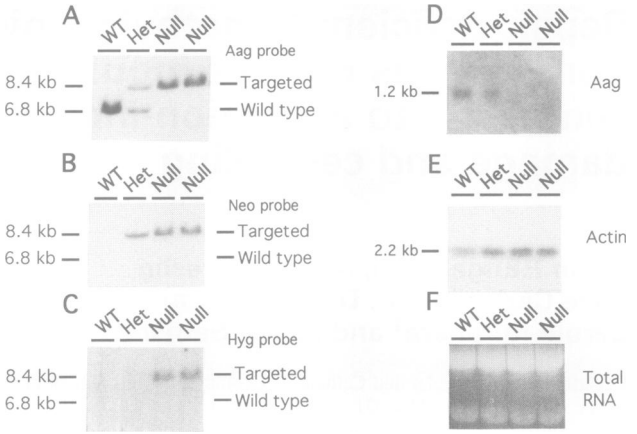

Fig. 2. (**A–C**) Southern analysis of DNA from wild-type (WT), *Aag* heterozygous clone 96 (Het) and *Aag* null clones 29 and 38 (Null) ES cells grown in the absence of feeder cells. Genomic DNA was digested with *Hin*dIII, probed with (A) the *Xba*I–*Sal*I fragment from the original *Aag* genomic clone (flanking the targeting vector); (B) a *Pst*I fragment of the coding region of *neo*; or (C) an *Ava*II fragment of the coding region of *hyg*. (**D–F**) Analysis of total RNA from *Aag* wild-type clone 33, *Aag* heterozygote clone 34 and *Aag* null clones 29 and 38 probed with the *Aag* cDNA (D), actin cDNA (E) or stained with ethidium bromide (F).

vector. The *neo* gene, under the control of the phosphoglycerate kinase (PGK) promotor, was inserted into the *Sal*I site located at amino acid 86 in the beginning of exon 2. The *neo* insertion is expected to stop normal transcription upstream of a putative active site for *Aag* located between amino acids 114 and 130 (Chakravarti *et al.*, 1991), which span exons 2 and 3. The herpes simplex virus thymidine kinase gene (HSV-*tk*) under the PGK promotor was inserted adjacent to the 5′ end of the *Aag* fragment to allow for selection against random integration events.

The linearized *Aag–neo* targeting vector was electroporated into mouse AB1 ES cells. Selection for *neo*$^+$ (G418^R) and HSV-*tk*$^-$ (FIAU^R) resulted in a 5-fold reduction in surviving colonies over G418 selection alone, thus enriching for homologous recombinants. DNA was prepared from 432 doubly G418- and FIAU-resistant clones and analyzed by Southern hybridization using as a probe the 1.7 kb genomic fragment flanking the 3′ end of the targeting vector (Figure 1A). Twenty-eight clones (6.5%) contained the predicted genomic structure resulting from insertion of *neo* into one *Aag* allele (8.4 kb *Hin*dIII fragment) in addition to the wild-type allele (6.8 kb *Hin*dIII fragment) (Figure 1B).

One of these heterozygous clones was made null by inactivating the remaining wild-type allele (te Riele *et al.*, 1990). An *Aag* heterozygote was expanded and electroporated a second time with a vector identical to *Aag–neo*, except with the *hyg* gene in place of *neo*. Doubly hygromycin- and FIAU-resistant colonies were isolated, expanded, and similarly analyzed (Figure 1C). Five of 324 hyg^R/FIAU^R clones were identified as containing two disrupted *Aag* alleles. The targeting efficiency for the *neo*-containing allele was significantly greater than for the remaining wild-type *Aag* allele (~4-fold, data not shown).

*Hin*dIII-digested genomic DNA from the original wild-type AB1 ES line, an *Aag* heterozygote, and two of the *Aag* null clones was probed with the 1.7 kb *Xba*I–*Sal*I

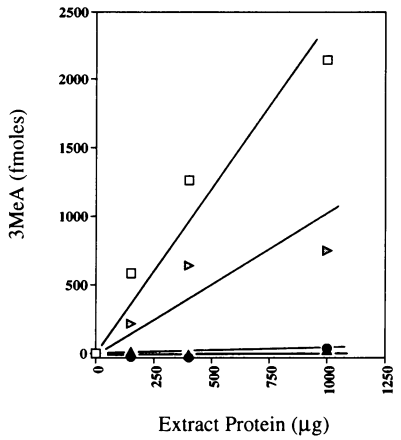

Fig. 3. DNA glycosylase activity in cell-free extracts. Various amounts of ES extract protein were incubated with 44 μg of [^{3}H]dimethyl sulfate-treated calf thymus DNA (1228 c.p.m./μg; 1.8 Ci/mmol methyl group) for 1 h at 37°C. 3MeA released by wild-type clone 33 (open square), *Aag* heterozygote clone 34 (shaded triangle), *Aag* null clone 29 (filled circle) and *Aag* null clone 38 (filled triangle) is shown.

Aag genomic fragment and fragments of *neo* and *hyg* to confirm insertion of the marker genes (Figure 2A–C). Since the inserted *neo* and *hyg* expression cassettes were almost identical in size, the *Aag* null clones have two mutant alleles that co-migrate at 8.4 kb (Figures 1C and 2A). As expected, the *neo* probe hybridizes to DNA from the *Aag* heterozygote and the *Aag* nulls, while the *hyg* probe only hybridizes to DNA from the *Aag* null clones (Figure 2B and C).

To confirm that the targeted disruptions stopped normal transcription of the *Aag* gene, we assayed the *Aag* null cells for *Aag* mRNA. Northern analysis of total RNA from ES clones showed that *Aag* transcripts are not detectable in null cells but are detected in wild-type and heterozygous cells (Figure 2D). Reprobing with an actin gene fragment and ethidium bromide staining of total RNA shows that all lanes contained roughly equal amounts of total RNA (Figure 2E and F).

Glycosylase activity

The genomic structure of the targeted *Aag* locus and the lack of *Aag* mRNA transcripts suggested that the null *Aag* cells would be deficient in Aag enzyme activity. However, we did not know whether other mammalian enzymes also function to release 3MeA from alkylated DNA or whether extremely low levels of functional *Aag* mRNA might be produced by alternative splicing. Figure 3 clearly shows that extracts from *Aag* null cells are apparently devoid of 3MeA DNA glycosylase activity, that wild-type cells express fairly high levels of Aag activity and that heterozygous cells express intermediate levels. We infer from these results that disruption of the *Aag* locus by *neo* and *hyg* successfully arrests *Aag* expression. Interestingly, the heterozygous cells show no evidence of increased *Aag* expression (from the remaining wild-type allele) to compensate for the insertional mutation.

Growth rates and cellular morphology

We examined the *Aag* null cells for altered cellular morphology and found that there are no observable differences in the morphology of wild-type, *Aag* heterozygote

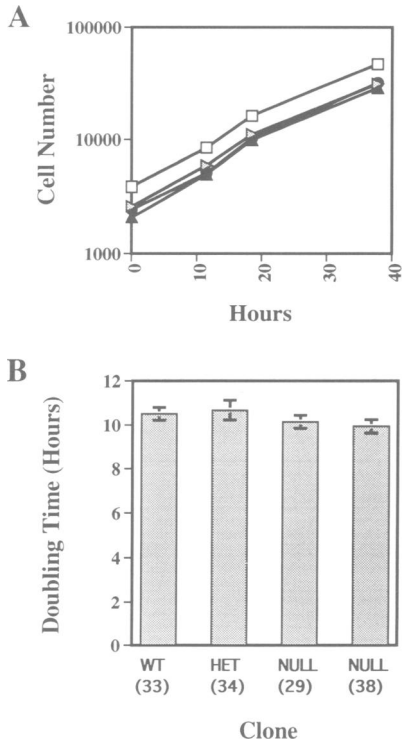

Fig. 4. (**A**) Growth curves of four ES clones: wild-type clone 33 (open square); *Aag* heterozygote clone 34 (shaded triangle); *Aag* null clone 29 (filled circle); *Aag* null clone 38 (filled triangle). (**B**) Doubling time in hours for the same four clones (clone numbers in parentheses) with 95% confidence intervals.

and *Aag* null cells when grown in the presence of leukocyte inhibitory factor (LIF) (data not shown). ES cells with normal cellular morphology are thought to be more likely to contribute to all tissues of a whole organism than morphologically altered cells.

Alkylated bases have been found in normal (i.e. untreated) mammalian cells and it is believed that some normal cellular metabolites can alkylate DNA (reviewed by Vaca *et al.*, 1988; Marnett and Burcham, 1993). It was therefore important to determine whether the *Aag* null cells divide more slowly due to endogenously produced unrepaired DNA lesions that block replication. Doubling times were determined for wild-type, heterozygous and null *Aag* ES cells by following the cell density of log phase cultures. Figure 4 shows that the *Aag* mutations had no effect on growth rate. Thus, if there is growth-inhibiting endogenous DNA damage repaired by Aag, these cells can tolerate higher than normal levels of this damage, perhaps because other repair pathways compensate for the Aag deficiency.

Sensitivity to DNA-damaging agents

To address the *in vivo* role of Aag in protecting mammalian cells against killing by various DNA-damaging agents, we tested the effect of simple methylating agents, more complex alkylating agents used for cancer chemotherapy, and UV light on glycosylase-deficient cells.

Null *Aag* cells are significantly more sensitive than heterozygous and wild-type cells to killing by the simple alkylating agent methyl methanesulfonate (MMS) and by $MeOSO_2(CH_2)_2$-lexitropsin ($MeOSO_2$Et-Lex) a methylat-

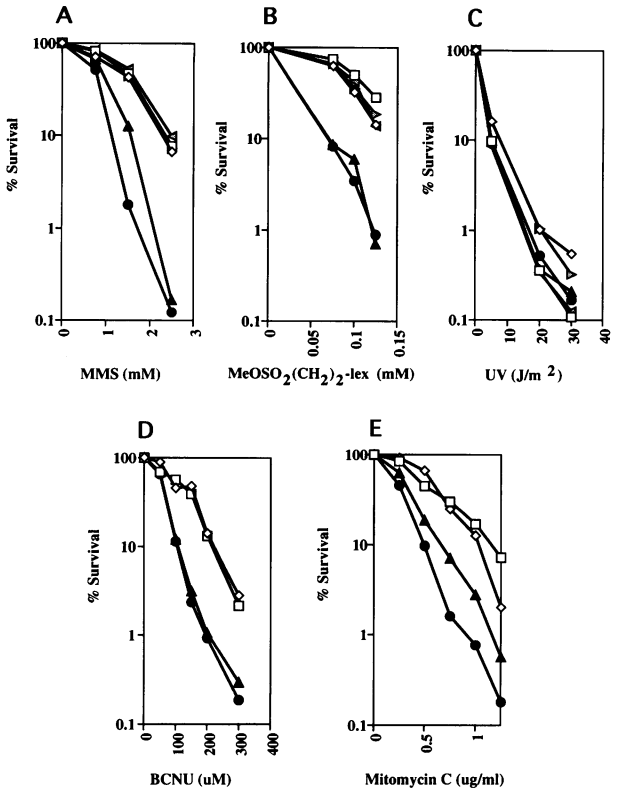

Fig. 5. Alkylation- and UV-induced cell killing of ES clones. The colony-forming ability of wild-type untransfected AB1 line (open diamond), wild-type clone 33 (open square), *Aag* heterozygote clone 34 (shaded triangle point right), *Aag* heterozygote clone 101 (shaded triangle point left), *Aag* null clone 29 (filled circle) and *Aag* null clone 38 (filled triangle) was measured after treatment with MMS (**A**), $MeOSO_2Et$-Lex (**B**), UV (**C**), BCNU (**D**) and mitomycin C (**E**). (Heterozygotes are only shown in panels A, B and C.)

ing agent that targets the minor groove of AT-rich DNA sequences (Zhang *et al.*, 1993) (Figure 5A and B). The increased sensitivity of the null *Aag* cells correlates with their inability to repair 3MeA *in vitro*. The heterozygous cells only express about half the wild-type level of Aag, but nevertheless display wild-type resistance to MMS and $MeOSO_2Et$-Lex. Since Aag heterozygous cells are no more sensitive than wild-type cells to these methylating agents, we infer that, for 3MeA, the Aag enzyme is not rate limiting as the first step in the base excision repair pathway.

We tested the ES clones for sensitivity to UV irradiation. At present, there is no evidence that any 3MeA DNA glycosylase repairs UV-induced cytotoxic DNA damage. In addition, *Saccharomyces cerevisiae* lacking 3MeA DNA glycosylase activity are not more susceptible to killing by UV (Chen *et al.*, 1990) and, likewise, altered levels of *Aag* expression in mammalian cells do not appear to affect UV sensitivity of these cells (Figure 5C).

The yeast 3MeA DNA glycosylase protects cells against killing by chloroethylnitrosoureas used for cancer chemotherapy (Matijasevic *et al.*, 1993). We therefore asked whether a mammalian glycosylase might provide similar protection against two types of agents widely used for cancer chemotherapy, namely the chloroethylnitrosourea, BCNU (reviewed in Ludlum, 1995), and mitomycin C (reviewed in Verweij and Pinedo, 1990). *Aag* null cells

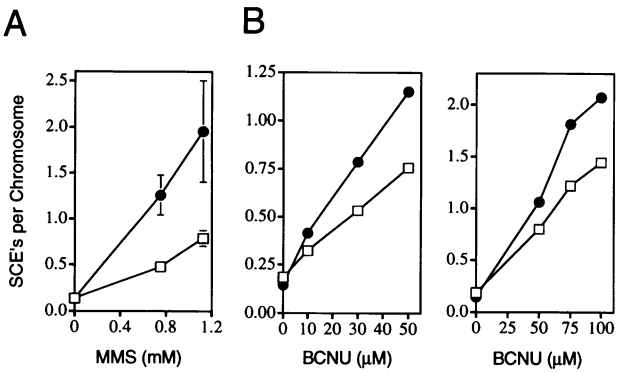

Fig. 6. Sister chromatid exchange in wild-type clone 33 (open square) and *Aag* null clone 29 (filled circle) ES cells induced by MMS (**A**) and BCNU (**B**). The frequency of SCEs induced was measured as described in Materials and methods.

are significantly more sensitive than wild-type cells to both chemotherapeutic agents (Figure 5D and E). We do not know of any evidence that 3MeA DNA glycosylases repair the large multicyclic DNA adducts induced by mitomycin C and were therefore surprised that *Aag* null cells are sensitive to mitomycin C. Thus, mammalian 3MeA DNA glycosylases may have an even broader substrate range than was previously believed.

Chromosome damage

Susceptibility to the induction of chromosome damage often correlates with susceptibility to cell killing and mutation (Carrano *et al.*, 1978; Natarajan *et al.*, 1984). We therefore determined whether the Aag DNA repair enzyme protects cells against alkylation-induced sister chromatid exchange (SCE). Untreated *Aag* null and wild-type cells do not have significantly different spontaneous SCE levels. However, compared with wild-type, *Aag* null cells are more susceptible to SCEs induced by both MMS and BCNU (Figure 6A and B). To our knowledge, this is the first evidence that a mammalian 3MeA DNA glycosylase protects against chromosome damage.

Discussion

3MeA DNA glycosylases are small single subunit DNA repair enzymes that initiate base excision repair by removing damaged DNA bases via cleavage of the *N*-glycosylic bond. Null mutant studies show that these enzymes protect *S.cerevisiae* and *E.coli* from alkylation-induced cell killing. Here we describe the generation of *Aag* null mouse cells by gene targeting. To our knowledge, these cells represent the only known mammalian 3MeA DNA glycosylase-deficient mutants, and, as such, provide a valuable model system for determining the *in vivo* role of this enzyme in mammalian cells and the *in vivo* consequences of the reduced repair of a variety of damaged DNA bases.

3MeA lesions are somehow able to block *E.coli* and yeast DNA replication, and thus unrepaired 3MeAs kill these microbial cells (Karran *et al.*, 1980; Evensen and Seeberg, 1982; Chen *et al.*, 1989). MMS produces many different DNA lesions, including 7MeG, 3MeG and O^6MeG, in addition to 3MeA. Here we show that a mouse 3MeA DNA glycosylase protects cells against MMS-induced cytotoxicity. However, given that the Aag

glycosylase has a broad substrate specificity and that MMS produces a variety of DNA lesions, this experiment does not indicate directly whether 3MeA itself is cytotoxic to mammalian cells. In order to ask specifically about the biological consequences of 3MeA, we used $MeOSO_2Et$-Lex, a minor groove-targeting S_N2-type alkylating agent that forms almost exclusively 3MeA lesions (Zhang *et al.*, 1993). The *Aag* null ES cells become very sensitive to killing by $MeOSO_2Et$-Lex (Figure 5B), and thus we conclude that 3MeA itself is cytotoxic to mammalian cells. The observation that the *E.coli* 3MeA/3MeG Tag glycosylase protects Chinese hamster V79 cells from MMS killing supports this conclusion (Klungland *et al.*, 1992). It is interesting to note that the Aag enzyme must be limiting for 3MeA base excision repair in V79 cells, unlike in mouse ES cells. We presume that 3MeA kills mammalian cells by blocking DNA replication, but how such a small lesion can block replication remains to be determined.

The *Aag* locus lies adjacent to the α-globin gene on mouse chromosome 11 (Engelward *et al.*, 1993). Mice with homozygous chromosomal deletions spanning this region have long been known to be developmentally lethal, and it was recently shown that these deletions include the *Aag* gene (Popp *et al.*, 1979; Hendrey *et al.*, 1995). Since many other genes may also be deleted in this mouse strain, it is not yet clear whether lethality stems from a deficiency in Aag, but it is a formal possibility that *Aag* mutations might affect mouse development. Interestingly, the *Aag* null ES cells are morphologically normal and grow at the same rate as wild-type ES cells *in vitro* (Figure 4).

We know that 3MeA lesions are cytotoxic and that these lesions are thought to be present in untreated cells due to endogenous sources of methylation (reviewed in Marnett and Burcham, 1993). Yet *Aag* null cells, which show no detectable repair of 3MeA by glycosylases *in vitro*, divide normally. This can be interpreted in at least three ways: (i) 3MeA levels are too low to cause a detectable change in growth rate; (ii) the mammalian replication machinery can bypass endogenous levels of 3MeA lesions; or (iii) other repair pathways not detected by our *in vitro* assay remove endogenously produced 3MeAs. Determining the *in vivo* level of spontaneous 3MeA lesions in *Aag* null cells will clarify this issue.

In the interest of developing safer and more effective treatments for cancer, we must identify the cytotoxic and mutagenic lesions induced by chemotherapeutic agents and determine which repair pathways modulate these biological outcomes. For example, a clear correlation exists between chloroethylnitrosourea sensitivity and a cell's ability to repair O^6-alkylguanine lesions via the MGMT DNA methyltransferase repair protein (Erickson *et al.*, 1980). While MGMT appears to prevent the formation of cross-links (Erickson *et al.*, 1980; Bodell *et al.*, 1988; Gonzaga and Brent, 1989), other chloroethylnitrosourea-induced lethal lesions are apparently removed by the nucleotide excision repair pathway (Wu *et al.*, 1992). That the yeast (Matijasevic *et al.*, 1993) and human (Samson *et al.*, unpublished) 3MeA DNA glycosylases protect *E.coli* from killing by CNU led us to question whether Aag protects mammalian cells against chemotherapeutics. Strikingly, the absence of Aag renders cells more sensitive to both the cytotoxic and the chromosome-damaging effects of BCNU, providing *in vivo* evidence that a mammalian 3MeA DNA glycosylase ameliorates the toxicity of the chloroethylnitrosourea compounds. It will be important to determine the relative contributions of MGMT, nucleotide excision repair and Aag in protecting cells against chloroethylnitrosoureas.

To our surprise, *Aag* null cells are very sensitive to another kind of cancer chemotherapeutic agent, mitomycin C. Mitomycin C produces four major adducts *in vivo* (two monoadducts and two cross-links) all of which include the large multicyclic core of mitomycin C adducted to DNA (Bizanek *et al.*, 1993). The anti-tumor quality of mitomycin C has been associated with its ability to form cross-links in DNA, which presumably block replication (Iyer and Szybalski, 1964). However, recent studies show that mitomycin–DNA guanine-N^2-linked monoadducts are not only precursors of all the observed adducts but are themselves potent inhibitors of DNA replication (Basu *et al.*, 1993). It is not yet clear exactly which mitomycin C-induced adduct Aag repairs, but it seems likely to be significantly different from any lesions previously shown to be repaired by 3MeA DNA glycosylases, underscoring the unexpectedly wide variety of damaged bases which serve as substrates for this enzyme.

Inherited defects in DNA repair pathways have been associated with cancer predisposition, e.g. defective repair of mismatched bases is associated with hereditary non-polyposis colorectal cancer (Fishel *et al.*, 1993; Leach *et al.*, 1993; Papadopoulos *et al.*, 1994), and defective nucleotide excision repair is associated with skin cancer (Cleaver, 1968). We want to understand the role of mammalian 3MeA DNA glycosylases in protecting against endogenous and exogenous DNA-damaging agents, since this class of enzyme may be important in protecting against cancer or other diseases. We are thus generating a mouse specifically lacking Aag, which, if viable, will allow us to assess the role of this enzyme in a mammal. *Aag* null cells have a significantly increased level of alkylation-induced chromosome damage compared with wild-type controls (Figure 6), and this result clearly emphasizes the potential importance of Aag in protecting against disease.

In summary, Aag appears to be the major 3MeA DNA glycosylase in mouse ES cells and protects against cytotoxicity and chromosome damage caused by a wide variety of agents. Aag plays a role in the cellular response to certain cancer chemotherapeutics and it may play a role in the prevention of cancer.

Materials and methods

Cells, plasmids, genomic library and enzymes

The pBluescript vector and a 129SV mouse genomic library in the λ-FIX II vector were obtained from Stratagene. AB1 ES cells were from A.Bradley, Baylor University. All genetic markers for ES cells were under a PGK promotor and carried a PGK polyadenylation signal. The pPGK-*neo* (described by Tybulewicz *et al.*, 1991) and pPGK-*hyg* (te Riele *et al.*, 1990) vectors were transfected into LIF-expressing mouse STO cells (SNL76/7, McMahon and Bradley, 1990; and SNL76/7-hyg^R, GenPharm) and were also used for construction of the targeting vectors. The HSV-*tk* expression cassette is described by Tybulewicz *et al.* (1991). Restriction enzymes, T4 DNA ligase and Klenow were from New England Biolabs. The Wizard system (Qiagen) was used for plasmid isolation, and fragments of DNA were purified using Qiaex (Qiagen).

Sequencing reactions were done using Sequenase (United States Biochemical). *Pfu* polymerase was purchased from Stratagene. All radioactive probes were prepared by random priming using NEBlot (New England Biolabs).

Cloning a fragment of the mouse Aag gene and identification of exons and introns

A mouse genomic library with average insert sizes of 9–22 kb was screened using the full 1.1 kb *Aag* cDNA as a probe (Engelward *et al.*, 1993). Two positive clones were obtained from 1.2×10^6 plaques screened, and an 8.5 kb *Not*I fragment from a 15 kb genomic clone was subcloned into pBluescript. The location of the exon and intron borders was determined by a combination of nucleotide sequencing, restriction enzyme site analysis and PCR. The open reading frames were compared with the genomic structure of the human AAG (Vickers *et al.*, 1993) and the mouse exons were numbered according to their human homologs. The cloned genomic fragment contained exons 1, 2 and 3 (Figure 1). Whether other exons are contained in this fragment was not determined.

Construction of targeting vectors

Restriction analysis revealed the location of two *Sal*I sites in the subcloned 8.5 kb *Not*I–*Aag* genomic fragment (Figure 1A). *Xho*I and *Not*I digestion released a 1.7 kb fragment containing one of the *Sal*I sites. The neomycin marker was released by digestion with *Xho*I and *Sal*I from pPGK-*neo* and ligated directly into the remaining *Sal*I site. A pPGK-*tk* vector was opened at *Xho*I and ligated to *Xho*I–*Not*I adaptors (Stratagene) to allow the HSV-*tk* marker to be released with *Not*I alone and ligated into the unique *Not*I site at the 5′ end of the *Aag* genomic fragment. The final *Aag–neo* targeting construct contains the pBluescript vector, HSV-*tk*, a 5.5 kb *Aag* fragment, *neo* and a 1.3 kb *Aag* fragment (Figure 1A). *Aag–neo* was linearized with *Kpn*I for transfection into AB1 cells.

A similar targeting vector was made with the hygromycin gene in place of *neo*. A PGK-*hyg* expression cassette was released from pPGK-*hyg* by digestion with *Bgl*II. The overhangs were filled by Klenow and *hyg* was blunt-end ligated into the filled *Sal*I site of exon 2. The *tk* gene was inserted into the unique *Not*I site to create *Aag–hyg*. Prior to transfection, *Aag–hyg* was linearized with *Xba*I, which cut twice to release pBS.

ES cell culture

The AB1 ES cells were cultured on SNL76/7 mitotically inactive feeder cells as described (McMahon and Bradley, 1990). To maintain an undifferentiated state, ES cells were maintained in the presence of LIF (Gibco-BRL or purified without the HPLC step according to Mereau *et al.*, 1993).

Subconfluent ES cells were trypsinized for 20 min in 0.25% trypsin at 37°C, resuspended in complete media and counted. Pelleted cells were resuspended in cold Mg^{2+}/Ca^{2+}-free phosphate-buffered saline (PBS; Gibco BRL) at 1.1×10^7 cells/ml. Cells (0.9 ml) were combined with 20 μg of DNA and electroporated in a 0.4 cm cuvette (Bio-Rad) at 250 V and 500 μF (Bio-Rad Gene Pulser). Cells were then plated at $1–2\times10^6$ cells/100 mm dish of selection-resistant irradiated feeder cells. After 24 h, drug-resistant cells were selected with either G418 sulfate (400 μg/ml) or G418 with FIAU [0.5 μM; 1-(2′-deoxy, 2′-fluoro-β-D-arabinofuranosyl)-5 iodouracil; Oclassen Pharmaceuticals, Inc.]. After 7–9 days, surviving colonies were trypsinized and divided into one 96 well and one 24 well feeder-coated dish. Once confluent, cells were frozen in the 96 well plates as described by Ramirez-Solis *et al.* (1993) and DNA was extracted from cells in the 24 well plates. One *Aag* heterozygote (clone 96) was expanded and targeted a second time with *Aag–hyg*. Transfection was repeated, as described above, except with 180 units/ml hygromycin in place of G418.

To control for any possible phenotypic effect of insertion of *neo* and *hyg* into the *Aag* null clones, one wild-type (clone 33), two heterozygotes (clones 34 and 101) and two *Aag* null clones (clones 29 and 38) were expanded from the pool of doubly transfected hyg^R/$FIAU^R$ for further analysis. The *Aag* wild-type and heterozygous clones were resistant to both G418 and hygromycin B due to either random insertion of both *Aag–neo* and *Aag–hyg* (wild-type) or one targeting event and one random insertion event (heterozygotes). Untransfected AB1 ES cells were also characterized to control for possible effects of random insertion of the targeting vectors into *Aag* wild-type clone 33.

For many experiments, ES cells were grown in the absence of feeders. Readily adherent SNL76/7 feeder cells were removed from trypsinized mixed cultures of ES cells and feeders by three 30 min incubations on gelatinized dishes.

Southern and Northern analysis

DNA was isolated from ES cells by a rapid *in situ* extraction protocol (Laird *et al.*, 1991). Genomic DNA was digested with the indicated restriction enzymes and separated on a 0.8% agarose gel. Total RNA was isolated from $1–2\times10^7$ ES cells grown without feeders by a single-step protocol, and standard protocols were used for transfer onto Nytran (Schleicher and Schuell) and probing (Ausubel *et al.*, 1995).

DNA glycosylase activity

Log phase ES cells grown without feeders were washed on ice three times with cold PBS, scraped from dishes and pelleted. Cells were resuspended in cold glycosylase buffer (20 mM Tris pH 7.8, 100 mM KCl, 2 mM EDTA, 5 mM 2-mercaptoethanol) supplemented with protease inhibitors [100 μM benzamidine HCl; 1 μg/ml: leupeptin, pepstatin A and aprotinin (Sigma)] to a final volume of 0.2–0.4 ml. Cell pellets were flash-frozen in liquid N_2 and stored at –80°C. Thawed pellets supplemented with 100 μM phenylmethylsulfonyl fluoride (PMSF) were sonicated in short bursts for 7.5 s at low intensity. After centrifugation at 14 000 *g* for 20 min at 4°C, supernatants (crude cell extracts) were stored at –80°C. Extract protein was incubated for 1 h at 37°C with 44 μg of 3.6 Ci/mmol [^{3}H]dimethyl sulfate-treated calf thymus DNA (1228 c.p.m./μg), prepared according to Samson and Linn (1987). The reactions were supplemented with bovine serum albumin (BSA) to equalize protein concentration. DNA was ethanol-precipitated and the supernatants were lyophilized and analyzed by descending paper chromatography as described previously (Chen *et al.*, 1989). Counts released from DNA by active repair were determined by subtracting counts released in the presence of BSA alone. Similar results were observed in two independent analyses.

Growth rates

Log phase ES cells grown without feeders were plated at ~50 000–80 000 cells per gelatin-coated 24 well. Cells were counted at 23, 34.5, 41.5 and 61 h. Medium was changed after 34 h. For each time point, nine wells were trypsinized and counted three times each using a Coulter Counter. Using Excel (Microsoft), regression analysis was performed using all 27 data points for each estimate of cell number. Doubling time was calculated with 95% confidence intervals.

Survival curves

ES cells were treated with the following agents: MMS (Aldrich), BCNU (Bristol Laboratories), mitomycin C (Sigma) or $MeOSO_2Et$-Lex (a generous gift of B.Gold). ES cells were trypsinized, counted and diluted by factors of 10 down to 100 cells/ml. Various cell dilutions were aliquoted onto feeder-coated plates (6 well or 24 well). After 12–24 h, cells were incubated for 1–2 h in drug-containing serum-free media. For UV irradiation, media were replaced with PBS prior to exposure. After 5–9 days, dried colonies were Giemsa stained (Sigma) and counted. All survival curves were repeated 3–6 times. Figure 5 shows representative curves; experiments shown in Figure 5A–D were done simultaneously.

Sister chromatid exchanges

For MMS- and BCNU-induced SCE measurements, 1×10^6 log phase ES cells (without feeders) were seeded into gelatin-coated 25 cm^2 flasks. The following day, media were removed and replaced with serum-free media supplemented with LIF, 10 μM bromodeoxyuridine (BU), and MMS or BCNU. After 1–2 h, media were replaced with McCoy's medium supplemented with LIF and 10 μM BU. Cells were cultured for two rounds of DNA replication (25–30 h). Two hours after the addition of 0.2 μM colcemid, mitotic cells were collected by shaking, treated in hypotonic solution (0.2% KCl, 0.2% sodium citrate, 10% fetal bovine serum) at 37°C for 15 min, and fixed in Carnoy's solution. To produce 'harlequin' chromosomes, we used a modified fluorescence plus Giemsa technique (Perry and Wolff, 1974). Slides were stained in Hoechst 33258 (5.0 μg/ml) for 20 min, mounted in 0.067 M Sorensen's buffer (Na_2HPO_4/KH_2PO_4 pH 6.8) with a coverslip, exposed to a General Electric 15 W blacklight (F15T8, BLB) at 55°C for 20 min, heated at 65°C in 20× SSC for 20 min, rinsed, and stained in a 5% Giemsa solution. At least 20 cells in metaphase were counted per data point. The mean of three MMS-induced SCE experiments with standard deviations is shown in Figure 6A. Figure 6B shows two separate BCNU-induced SCE experiments.

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