The EMBO Journal vol.11 no.12 pp.4439-4444, 1992

Expression of the *E.coli* 3-methyladenine DNA glycosylase I gene in mammalian cells reduces the toxic and mutagenic effects of methylating agents

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Communicated by T.Lindahl

In order to investigate the importance of 3-methyladenine in cellular sensitivity to chemical methylating agents we have constructed retroviral vectors for the integration and expression of the *Escherichia coli tag* gene in mammalian cells. The *tag* gene encodes 3-methyladenine DNA glycosylase-1 which specifically removes 3-alkyladenines from DNA. The constructs were introduced into Chinese hamster V79 cells by liposome mediated transfection or into murine haemopoeitic stem cells by cocultivation with a lipofected, virus-packaging cell line. In both cases, stable transfectants were selected for resistance to the antibiotic, G418, conferred by expression of the *neo* gene carried by the vector. Measurements of 3-methyladenine DNA glycosylase activity in cell extracts showed an up to 10-fold increase in cell lines with stably integrated *tag* gene sequences. These cell lines were significantly more resistant to the cytotoxic effects of methylmethanesulfonate and *N*-methyl-*N*-nitrosourea than their parent cell lines, indicating that 3-methyladenine repair is a limiting factor in cellular resistance to these methylating agents. Furthermore, the mutation frequency induced by methylmethanesulfonate was reduced to 50% of normal by expression of 3-methyladenine I activity in the Chinese hamster cells, indicating that m^3A is not only a cytotoxic but also a premutagenic lesion in mammalian cells. It is concluded that an alkylation repair gene function of a type only thought to be present in bacteria can yield a hyperresistant phenotype when transferred to mammalian cells.

Key words: alkylation repair/Chinese hamster V79 lung fibroblasts/gene transfection/3-methyladenine/murine haemopoietic stem cells

Introduction

Simple alkylating agents such as *N*-methyl-*N*-nitrosourea (MNU) and methylmethansulfonate (MMS) can introduce methyl groups at all of the available nitrogen and oxygen atoms in DNA bases (for reviews see O'Connor and Margison, 1979; Beranek *et al.*, 1980; Singer and Grunberger, 1983). The majority of evidence indicates that among the 11 products identified, two of the species, 3-methyladenine (m^3A) and O^6-methylguanine (m^6G), are to a greater or lesser extent responsible for the biological effects of such agents (Singer, 1976; Lawley, 1980; Margison and O'Connor, 1990).

In both prokaryotes and eukaryotes, DNA repair mechanisms exist for the elimination of these alkylation products from DNA and resistance to the biological effects of DNA alkylation frequently correlates with the increased efficiency of these processes. Repair proceeds differently for the two lesions and involves the removal of methyl groups by O^6-alkylguanine-DNA alkyltransferase acting on m^6G (Olsson and Lindahl, 1980; Mehta *et al.*, 1981) and DNA glycosylase mediated base excision for m^3A (Lindahl, 1976; Laval, 1977).

In bacteria, m^6G is considered the principal product responsible for the mutagenic effects of alkylation (Loveless, 1969; Newbold *et al.*, 1980) whereas alkylation cytotoxicity is dependent on m^3A repair ability (Karran *et al.*, 1980; Evensen and Seeberg, 1982), although it has recently been shown that cytotoxicity also to a lesser extent correlates with reduced m^6G repair capacity (Rebeck and Samson, 1991; Takano *et al.*, 1991). In mammalian cells, both the cytotoxic and mutagenic potential of alkylating agents have been attributed to the formation of O^6-alkylguanine. For example, cancer-derived cell lines lacking or expressing low levels of alkyltransferase activity, are usually much more sensitive to the cytotoxic effects of alkylating agents than normal repair proficient cell lines (Day *et al.*, 1980; Sklar and Strauss, 1980; Shiloh and Becker, 1981). Whilst such correlations may also be a consequence of differences other than alkyltransferase activity, expression of the *Escherichia coli ada* or *ogt* genes in mammalian cells increases the capacity for m^6G removal and at the same time confers resistance to alkylation toxicity and mutagenesis. This provides a clear demonstration that normal cellular sensitivity to these effects can indeed be determined by the capacity for m^6G repair (Brennand and Margison, 1986; Samson *et al.*, 1986; Margison and O'Connor, 1990).

In order to assess the contribution of m^3A to the damaging effects of alkylating agents in mammalian cells, we have adopted a similar approach to that for m^6G and used a retroviral vector to introduce the *tag* gene of *E.coli* (Clarke *et al.*, 1984) into the mammalian genome in order to supplement their capacity for m^3A excision. The *tag* gene encoded DNA glycosylase specifically removes m^3A from methylated DNA and has no reported activity on any other methylation product (Riazuddin and Lindahl, 1978; Thomas *et al.*, 1982; Bjelland and Seeberg, 1987). This is in contrast to the endogenous alkylpurine DNA glycosylase present in mammalian cells (Margison and Pegg, 1981; Singer and Brent, 1981; O'Connor and Laval, 1990; Chakravarti *et al.*, 1991) which is biochemically similar to the *alkA* gene function of *E.coli* (Evensen and Seeberg, 1982; Karran *et al.*, 1982). Such glycosylases can remove, in

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addition to m^3A, several other types of alkylated bases from DNA (McCarthy *et al.*, 1984).

In the present report we show that cell lines which permanently express the Tag DNA glycosylase have increased resistance to killing by MMS and MNU, indicating that m^3A in DNA is cytotoxic to mammalian cells.

Results

Integration of the tag gene from E.coli in the genome of mammalian cells

The coding region of the gene for 3-methyladenine DNA glycosylase I from *E.coli* was transferred to the mammalian expression vector pZipNeoSV(X)1 as outlined in Figure 1 thus creating the *tag* vector plasmid pBK204 (see Materials and methods). When this vector is integrated in the host genome, transcription will start in the 5′ terminal repeat and terminate in the 3′ terminal repeat of the retrovirus and after splicing, two mRNA species will be generated. Translation of the full-length transcript starts at the first AUG which is the initiation codon of the *tag* gene, whereas the spliced form will have lost the *tag* coding sequence and will thus express only the *neo* gene which confers G418 resistance. Previously, the same vector has been used to express the *E.coli ada*, *ogt* and *nth* genes in Chinese hamster cells (Brennand and Margison, 1986; Margison *et al.*, 1991; Harrison *et al.*, 1992) and the *ada* gene in murine haemopoietic stem cells (Jelinek *et al.*, 1988) and Indian muntjak cells (Musk *et al.*, 1989).

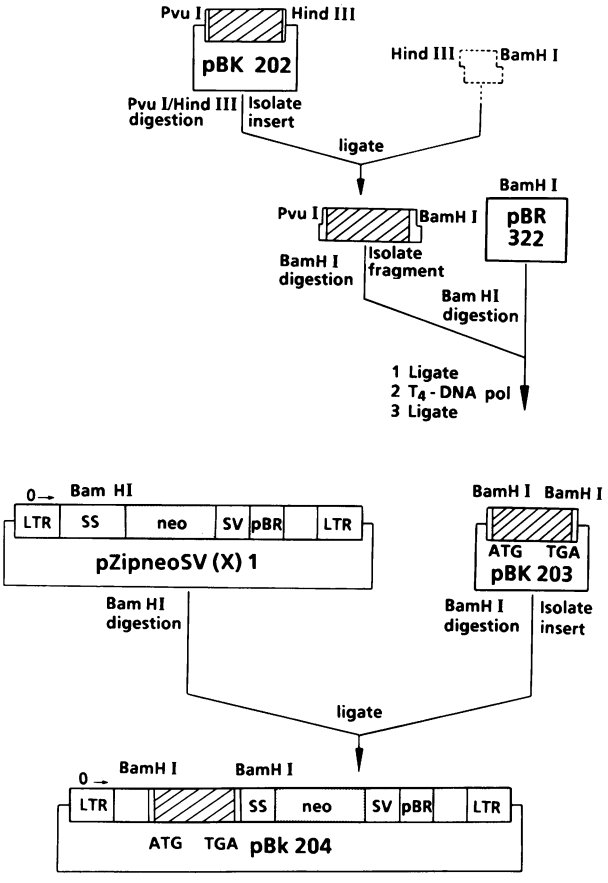

Fig. 1. Construction of the vector for *tag* expression (pBK204). The *PvuI*–*Hin*dIII fragment of pBK202 containing the *tag* gene coding sequences (30 bp from the translation initiation codon to the *PvuI* site in which there were no ATG sequences; Steinum and Seeberg, 1986) was isolated by polyacrylamide gel electrophoresis. The *Hin*dIII end was ligated to a *Hin*dIII–*Bam*HI linker, recut with *Bam*HI to eliminate multiple linkers, and ligated to one end of the *Bam*HI site of pBR322. The remaining free *PvuI*/*Bam*HI ends were made blunt by filling the 3′ end of the *Bam*HI site and degrading the protruding 3′ end of the *PvuI* site in one reaction with T4 polymerase and dNTPs. Ligation of the blunt ends thus restored a *Bam*HI restriction site. After verification of the correct construction in the pBR322 vector, the *tag* coding sequence was reisolated by *Bam*HI cleavage and gel electrophoresis, and inserted into the *Bam*HI site of pZipNeoSV(X)1. One plasmid isolate with the *Bam*HI fragment in the correct orientation was selected for transfection.

The pBK204 construct or pZipNeoSV(X)1 were introduced into the Chinese hamster lung fibroblast cell line, RJKO by lipofection and G418-resistant clones were selected. The presence of stably integrated *tag* sequences was verified after several rounds of subcultivation by PCR amplification with appropriate primers . PCR products of the correct size were generated for nine of the 12 G418 resistant clones (data not shown). The pBK204 construct or pZipNeoSV(X)1 were further introduced into the murine haemopoietic stem cell line FDCP1 by primary liposome mediated passage through a virus-packaging cell line followed by cocultivation and viral transfer to FDCP1 selecting for G418 resistance.

Expression of the Tag DNA glycosylase activity in tag gene-containing cell lines

The majority of the nine positive hamster cell clones expressed m^3A DNA glycosylase at the same level as RJKO cells transfected with pZipNeoSV(X)1, suggesting that vector sequences essential for expression had been altered or rearranged during chromosomal integration. However, two different clones expressed levels of m^3A DNA glycosylase activity at least 10 times higher than the endogenous levels normally present in the RJKO cells (Figure 2A). This activity was assessed for its ability to be inhibited by the addition of m^3A to the *in vitro* assay buffer, which is a characteristic property of the Tag enzyme (Riazuddin and Lindahl, 1978) but not the mammalian m^3A DNA glycosylase. Specific inhibition of the activity in extracts from both expressing clones (Figure 2B) demonstrated that the increased glycosylase activity was caused by expression of the *tag* gene and not by any alteration in the level of the endogenous enzyme.

Similarily, m^3A DNA glycosylase activity in a parent retrovirus infected G418-resistant FDCP1 clone was found to be 35 fmol/mg protein/h whereas it was around seven times higher than this (260 fmol/mg/h) in one of the *tag*–retrovirus infected clones. The activity in the overproducing cell line was also for FDCP1 cells found to have the characteristic property of being product inhibited by m^3A. The presence of 3 mM m^3A reduced the observed glycosylase activity to 160 fmol/mg/h in *tag*-containing virus-infected cells but had no effect on the endogenous glycosylase in cells infected with the cloning vector alone.

Effect of tag gene expression on cell sensitivity to methylating agents

The survival of the Chinese hamster clones was determined after exposure to increasing doses of the methylating agents, MMS and MNU (Figure 3). The *tag* gene expressing cell lines were considerably more resistant to the toxic effects of both MMS and MNU than were the control cells. No

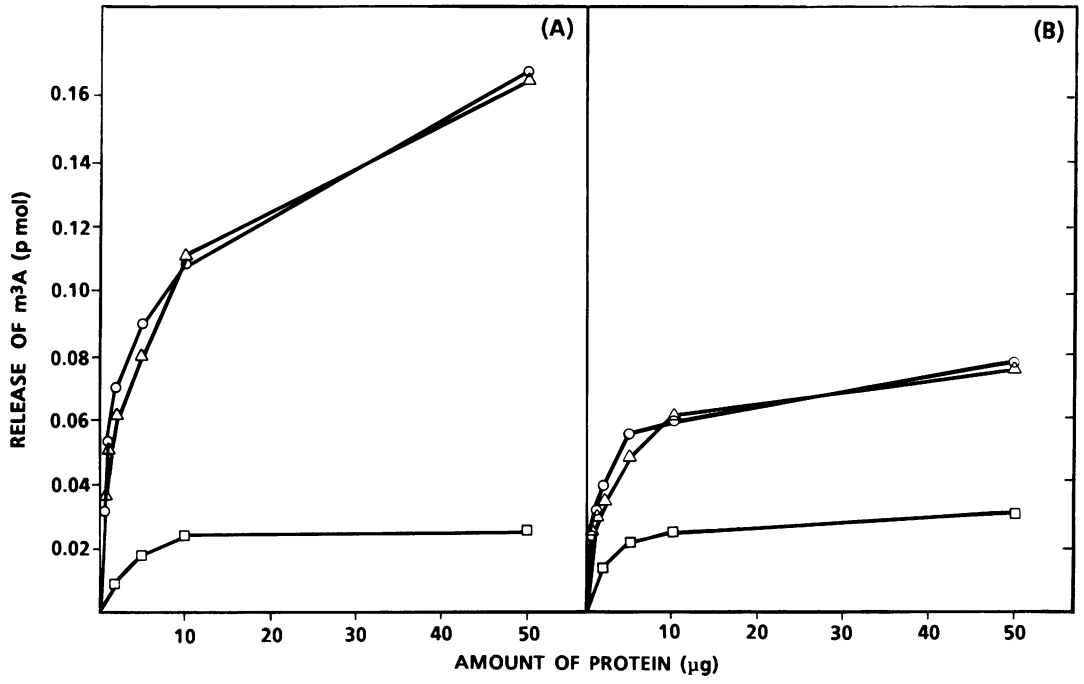

Fig. 2. m^3A DNA glycoslyase activity in extracts of RJKO cells harbouring pBK204: ○ (clone 1), △ (clone 2) or pZipNeoSV(X)1: □. Assays were performed with (**B**) and without (**A**) 3 mM m^3A in the reaction buffer.

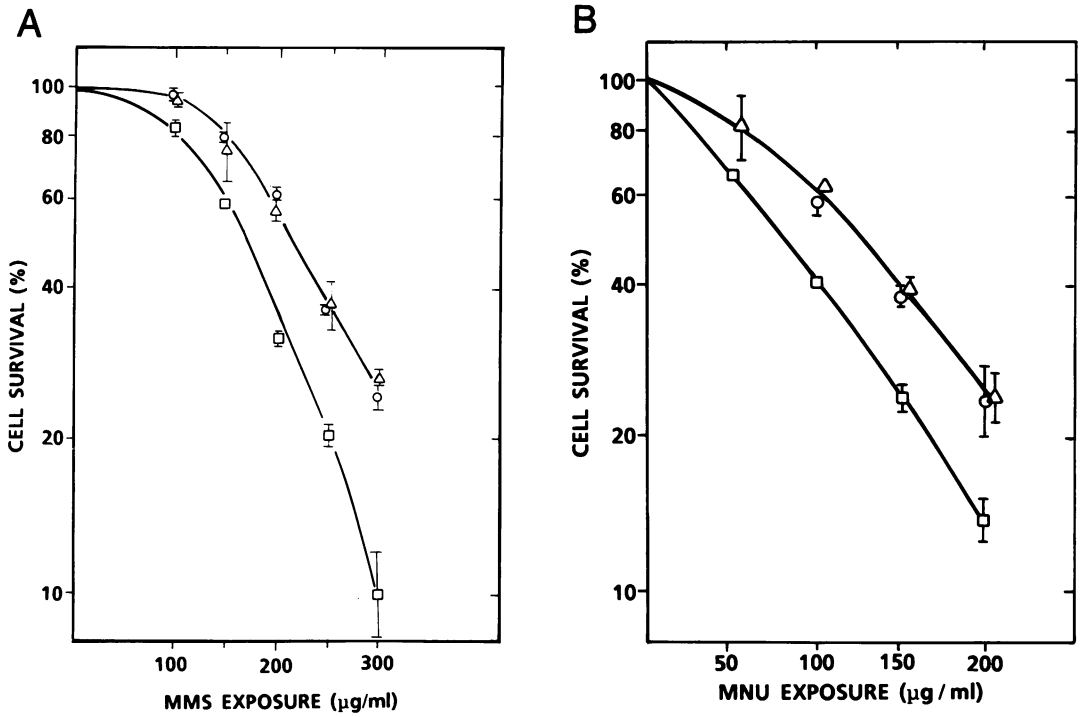

Fig. 3. Survival of RJKO cells harbouring pBK204: ○ (clone 1), △ (clone 2); or pZipNeoSV(X)1: □, after exposure to increasing doses of MMS (**A**) and MNU (**B**). The error bars indicate standard error.

significant difference was observed between the two different *tag* expressing cells. The murine cells were considerably more sensitive than the Chinese hamster cells to the toxic effects of MMS and MNU, but the extent of protection conferred against this toxicity by the Tag DNA glycosylase was greater than in Chinese hamster cells (Figure 4).

Effect of tag gene expression on MMS induced mutation frequency in Chinese hamster cells

To approach the question of whether m^3A is a premutagenic lesion we have measured mutations in the HPRT locus of *tag* expressing RJKO cells as compared with a control transfected cell line. MMS was chosen as an

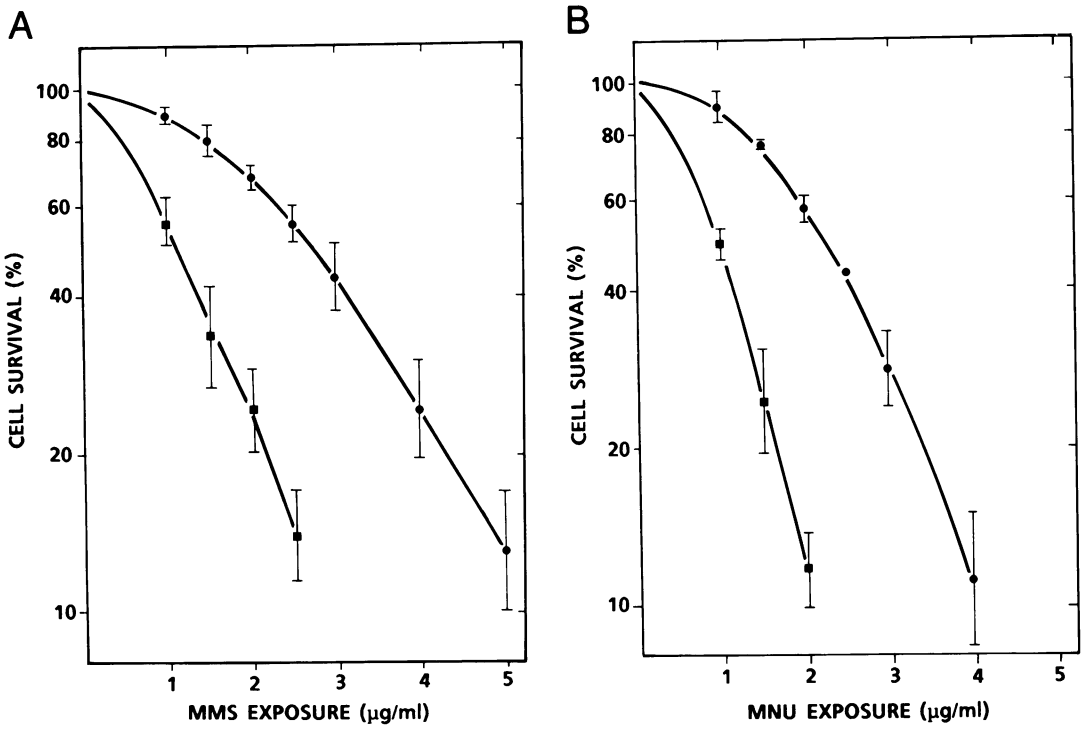

Fig. 4. Survival of FDCP1 cells infected with the *tag* retrovirus, (●) or the parent retrovirus, (■), after exposure to increasing doses of MMS (**A**) and MNU (**B**). The error bars indicate standard error.

alkylating agent for this experiment in order to minimize the contribution of mutations formed at m^6G residues. Such mutations would appear as a background at a similar frequency for both cell types and be much higher after MNU exposure because of the relatively high yield of m^6G for this methylating agent (Beranek *et al.*, 1980). The results showed that the MMS induced mutation frequency in the *tag* gene-expressing cells was only half of that observed in control cells (Table I). This is the first evidence indicating that m^3A is a premutagenic lesion in mammalian cells.

Table I. MMS induced mutations in the HPRT locus of RJKO cells harbouring pBK204 or pZipNeoSV(X)1

MMS concentration (mM)	RJKO/pBK204 (clone 1)	RJKO/pZipNeoSV(X)1
0	$<0.4\times10^{-5}$	$<0.4\times10^{-5}$
1	$1.9 \pm 0.4\times10^{-5}$	$4.3 \pm 0.8\times10^{-5}$
2	$5.2 \pm 0.9\times10^{-5}$	$11.1 \pm 0.5\times10^{-5}$

Cells were exposed to MMS for 60 min at the concentrations indicated and then scored for mutations in the HPRT locus by selection for 6-thioguanidine resistance. Numbers given represent the ratio of mutants to viable cells. See Figure 3 for a reference to the fraction of surviving cells (1 mM MMS is equivalent to 91 μg/ml).

Discussion

In mammalian cells, the mutagenic, cytotoxic and chromosome damaging effects of alkylating agents have been attributed to the formation of O^6-alkylguanine in DNA (Day *et al.*, 1980; Sklar and Strauss, 1980; Shiloh and Becker, 1981; Brennand and Margison, 1986; Samson *et al.*, 1986; Margison and O'Connor, 1990). However, since no mammalian systems have been available with different endogenous capacities for 3-alkyladenine repair it has been difficult to assess the potential biological significance of 3-alkyladenine in DNA in mammalian cells (Margison and O'Connor, 1973).

We have addressed this problem by providing Chinese hamster or murine haemopoietic cells with an increased capacity for m^3A repair by means of transfection and expression of the *E.coli tag* gene. The results show that high level expression of the *tag* gene function gives a substantial increase in cell survival following exposure to methylating agents. Since the *tag* encoded glycosylase is specific for 3-alkyladenines, this clearly demonstrates that m^3A is a potential cytotoxic lesion in mammalian cells and hence that m^3A repair can be a limiting factor in cellular resistance to alkylating agents. If it is assumed that, for the cell types that they represent, RJKO and FDCP1 cells are not repair deficient with respect to m^3A, then *tag* expression might be considered to have provided a hyperresistant phenotype. In related studies, complementation of mutant or deficient cell lines has clearly been demonstrated *via* expression of *ada* (Brennand and Margison, 1986; Samson *et al.*, 1986) *ogt* (Margison *et al.*, 1991) and *denV* (Valerie *et al.*, 1985), but an increase in resistance to above that of the normal phenotype has not previously been reported.

In *E.coli* it had been assumed that only m^3A was responsible for lethality. This was supported by data showing that *ada* mutants, defective in the adaptive repsonse and in alkyltransferase activity, were efficiently complemented for survival by plasmids carrying glycosylase genes (Clarke *et al.*, 1984; Kaasen *et al.*, 1986). However, the discovery of a second *E.coli* alkyltransferase, *ogt* (Margison *et al.*, 1985; Potter *et al.*, 1987), and the subsequent demonstration that *ada/ogt* double mutants could be complemented by expression of endogenous alkyltransferases (Rebeck and

Samson, 1991; Takano *et al.*, 1991), indicates that m^6G is also toxic to *E.coli* but probably to a lesser extent than m^3A because glycosylase mutants are much more sensitive than alkyltransferase mutants. Taken with the present data it can now be concluded that both m^6G and m^3A are potential cytotoxic lesions in both mammalian cells and *E.coli*.

Although the *tag* transfected cells express much higher levels of m^3A glycosylase activity and are more resistant to the methylating agents than control cells, the protection conferred is not proportional to the degree of glycosylase activity. A similar situation occurs in cells expressing high levels of the *ada* encoded alkyltransferase (e.g. Brennand and Margison, 1986). In both cases, this is likely to be a consequence of other lesions in DNA now becoming more predominant factors for toxicity.

Another important question frequently raised concerning the effects of alkylating agents is whether m^3A is a premutagenic lesion. This was approached by analysing the formation of MMS induced mutations in the Chinese hamster cells. It is shown that *tag* gene expression significantly reduces the level of induced mutations, indicating that m^3A is indeed a premutagenic lesion. From the numbers obtained it can be estimated that more than half of the MMS induced mutations in normal cells would occur directly at m^3A residues. Alternatively, the mutations in normal cells could be formed indirectly, perhaps by the use of an endogenous error-prone repair pathway which is bypassed when the error-free *tag* gene mediated pathway is introduced. However, endogenous repair also appears to involve a 3-methyladenine DNA glycosylase (Margison and Pegg, 1981; Singer and Brent, 1981; O'Connor and Laval, 1990; Chakravarti *et al.*, 1991) and the latter possibility therefore seems unlikely. It should now be possible to analyse the type of mutation that results from the introduction of m^3A into mammalian cells.

Since the mammalian repair enzyme has a broader substrate specificity than the Tag enzyme, it seems possible that increased m^3A repair effected by introduction of *tag*, could promote more efficient repair of other lesions by the endogenous glycosylase. Such lesions would be quantitatively minor but could still be biologically important. If such repair was limiting for survival or reduced mutations it might be argued that this could explain much or part of the rescuing effect of *tag* gene expression. Although such an interpretation cannot immediately be excluded we consider this unlikely as the major substrate for the endogenous enzyme in quantity in any case will be m^7G. More rapid repair of m^3A will only represent a relatively small reduction (at most 25%, see Beranek *et al.*, 1980) in the total number of alkylated residues acted upon by the endogenous enzyme.

In conclusion, it is shown that mammalian expression of the bacterial *tag* gene, which encodes a well characterized DNA repair enzyme with known specificity, can provide insight into the biological effects of a specific lesion induced among others in cells exposed to alkylation. Further work will allow studies of the effects on sister-chromatid exchanges and chromosome abberations as well as on the cancerogenic potential using transgenic mice harbouring the *tag* gene function.

Materials and methods

Plasmids and DNA manipulations

Plasmids pBK202 (Kaasen *et al.*, 1986) and pZipNeoSV(X)1 (Cepko *et al.*, 1984) have been described. Plasmids pBK203 and pBK204 (this work, see Figure 1) contain the tailored *tag* gene-containing *Bam*HI fragment in pBR322 and pZipNeoSV(X)1, respectively. All DNA manipulations were performed using standard methodology (Maniatis *et al.*, 1982). The *Bam*HI–*Hin*dIII adaptor (5′-GATCCTCGAGCGGCCGA-3′/3′-GAGCT-CGCCGGCTTCGA-5′) and primers for PCR were synthesized on an ABI model 381A automatic DNA synthesizer using the phosphotriester method and β-cyanoethylphosphoramidites as monomers.

Construction of a vector for tag gene expression in mammalian cells

The entire coding region of the *tag* gene (561 bp) is contained within a 742 bp *Pvu*I–*Hin*dIII fragment present in the *tag* plasmid subclone pBK202 (Steinum and Seeberg, 1986). This fragment was isolated by agarose gel electrophoresis and modified using an adaptor and standard recombinant DNA techniques to generate *Bam*HI termini as outlined in Figure 1. The correct *Bam*HI fragment construction was selected from subclones in pBR322. From this construct the 763 bp *Bam*HI fragment was reisolated and ligated into the unique *Bam*HI site of pZipNeoSV(X)1 (Cepko *et al.*, 1984): the version with the insert in the correct orientation was called pBK204.

Mammalian cell culture

The Chinese hamster fibroblast cell line RJKO (Gillin *et al.*, 1972) was maintained in minimal essential Eagle's medium (MEM), 10% fetal calf serum at 37°C in a humidified atmosphere of 5% CO_2/95% air. Cells resistant to the antibiotic G418 (GIBCO/BRL) were selected in the same medium containing 1.5 mg/ml of the drug, and grown in medium containing 0.5 mg/ml of the drug, unless otherwise indicated.

The FDCP1 cell line was maintained in Iscove's modification of Dulbecco's medium (IMDM) supplemented with 20% donor horse serum, 4 mM glutamine, 2% x63Ag8-653-conditioned medium (a source of interleukin 3; Karasuyama and Melchers, 1988), 50 mg/ml streptomycin sulfate and 5×10^6 units/ml penicillin. For clonogenic assay this was supplemented with 0.33% agar-noble (Difco) and cells were plated in 1 ml aliquots at 5×10^2/ml which typically gave rise to 200–300 colonies in the absence of the drug.

Cell transfection

Exponentially growing RJKO cells were seeded at 6×10^5 cells/80 cm^2 culture flask 1 day before transfection. Cationic liposome mediated transfection was performed as described (Felgner *et al.*, 1987) and modified according to the manufacturer's instructions (GIBCO/BRL). Three days after transfection cells were subcultured to 4×10^5 cells/80 cm^2 culture flask, and colonies resistant to 1500 μg/ml G418 were subcultured until stable G418 resistance was obtained and conserved for further analysis of *tag* gene sequences and expression of m^3A DNA glycosylase DNA activity in extracts. With this selection an average transfection frequency of 5×10^{-3} was obtained.

Production of retroviruses and infection of haemopoietic cells

Lipofection was also used to introduce the above vectors into the packaging line, psi-2 (Mann *et al.*, 1983). FDCP1 cells (5×10^6) were infected by cocultivation with a confluent irradiated (30 Gy, 3.6 Gy/min, ^{137}Cs source) monolayer of retroviral producers in a T25 flask containing 10 ml of IMDM/horse serum/interleukin 3 medium with 2 mM polybrene. After 24 h, the FDCP1 cells were removed and selected by plating in soft agar at a seeding density of 5×10^4 cells/ml in the presence of 1 mg/ml G418. Seven days later, colonies of resistant cells were transferred into liquid medium for expansion.

PCR analysis of cell lysates

Monolayer cells were washed twice in phosphate buffered saline, and trypsinized (0.05% trypsin for 3 min at 37°C). Phosphate buffered saline was added (8 ml) and the cells were collected by centrifugation for 5 min at 1000 *g*. Cells were resuspended in PCR buffer (50 mM KCl; 10 mM Tris–HCl, pH 8.3; 2.5 mM $MgCl_2$; 0.1 mg/ml gelatin) with non-ionic detergents (0.45% Nonidet P40; 0.45% Tween 20) and Proteinase K (60 μg/ml) and incubated at 55°C for 1 h. Proteinase K was inactivated by heating the sample at 95°C for 5 min. PCR was carried out for 30 cycles (1 min at 55°C annealing, 1 min at 72°C extension, 1 min at 95°C denaturation) in a total volume of 25 μl, using cell lysate corresponding to 7.5×10^4 cells (0.5 μg genomic DNA) and 1 U of Taq polymerase. The primers used were 5′-ACTCTAGAGGGAAAGTATGGAACGTTG-3′ (start codon underlined) and 3′-ACGAATTCGTTGCGCTTTCCGAATCAT-5′ (complement to stop codon underlined).

Assays of m3A DNA glycosylase activity in cell extracts

Chinese hamster cells were harvested by trypsination and murine cells by centrifugation, rinsed in phosphate buffered saline (PBS), and resuspended at $1-5\times10^7$ cells/ml in 70 mM MOPS pH 7.5, 1 mM EDTA and 1 mM DTT. Cells were disrupted by sonication on ice for two periods of 10 s. Cellular debris was removed by centrifugation at 14 000 *g* for 8 min at 4°C, and the supernatants were used as extracts for assays of m^3A DNA glycosylase activity by the method of Riazuddin and Lindahl (1978) as slightly modified (Bjelland and Seeberg, 1987) or by the method of Margison and Pegg (1981). Proteins were measured according to Bradford (1976).

Survival measurements

Chinese hamster cells were harvested from log phase, plated out at 150 cells per well in 100 μl MEM, 10% FCS in 96 well plates and incubated at 37°C for 3 h to allow cells to settle before exposure. MMS or MNU was added at the concentrations indicated in 200 μl medium. After 1 h exposure the medium was changed and the plates were incubated for 5 days, until control cells were almost confluent. Surviving cells were stained with MTT [3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] for 3 h at 50 mg/ml in PBS (Mosmann, 1983). The staining solution was removed by aspiration and the cell bound dye dissolved in 200 μl DMSO. Absorbance at 570 and 630 nm was measured using a multiwell plate reader.

Murine cells were plated in soft agar (see above) in the presence of the indicated amounts of MNU or MMS. After 7 days, colonies comprising >50 cells were counted.

Mutation assay

Mutation frequency at the HPRT locus was measured as described with minor modifications (O'Neill and Hsie, 1977). Briefly, cells were plated at 2×10^5 cells/80 cm^2 culture flask 3 h prior to exposure to allow the cells to settle. Cells were then exposed to MMS in MEM medium without fetal calf serum at the concentrations indicated for 1 h, rinsed in phosphate buffered saline, and replenished with fresh medium. After 7 days of incubation to allow expression of the mutant phenotype the cells were subcultured in 9 cm dishes at 4×10^5 for selection of mutants and at 4×10^2 for the counting of viable cells. Selective growth of hprt mutants was obtained by addition of 6-thioguanidine at 10 μg/ml. Cells were stained after 6 days for counting viable cells and after 8 days for the mutants.

Acknowledgements

We are grateful to Elsebeth Hoff for excellent technical assistance. A.Klungland is a fellow of the Norwegian Cancer Society and acknowledges the receipt of a travel fellowship from the European Molecular Biology Organization and thanks Phil Potter, Joe Rafferty and Ian Frehling (Paterson Institute) for helpful discussions. E.Seeberg acknowledges the receipt of grants from the Norwegian Cancer Society (89050 and 90048) and from the Norwegian Research Council for Science and the Humanities (RMF 316.91). Work in the Paterson Institute was supported by the Cancer Research Campaign, UK.

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Received on January 9, 1992; revised on August 5, 1992

Note added in proof

After submission of this manuscript a paper was published describing the expression of the *tag* gene function in NIH-3T3 murine fibroblasts [Taverna *et al.* (1992) *Biochem. Biophys. Res. Commun.*, **185**, 41–46]. However, no data were included on the biological effects of *tag* gene expression.