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Saccharomyces cerevisiae 3-methyladenine DNA glycosylase has homology to the AlkA glycosylase of *E.coli* and is induced in response to DNA alkylation damage

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We previously cloned a DNA fragment from *Saccharomyces cerevisiae* that suppressed the alkylation sensitivity of *Escherichia coli* glycosylase deficient mutants and we showed that it apparently contained a gene for 3-methyladenine DNA glycosylase (*MAG*). Here we establish the identity of the *MAG* gene by sequence analysis and describe its *in vivo* function and expression in yeast cells. The MAG DNA glycosylase specifically protects yeast cells against the killing effects of alkylating agents. It does not protect cells against mutation; indeed, it appears to generate mutations which presumably result from those apurinic sites produced by the glycosylase that escape further repair. The *MAG* gene, which we mapped to chromosome V, is not allelic with any of the *RAD* genes and appears to be allelic to the unmapped *MMS-5* gene. From its sequence the MAG glycosylase is predicted to contain 296 amino acids and have a molecular weight of 34 293 daltons. A 137 amino acid stretch of the MAG glycosylase displays 27.0% identity and 63.5% similarity with the *E.coli* AlkA glycosylase. Transcription of the *MAG* gene, like that of the *E.coli alkA* gene, is greatly increased when yeast cells are exposed to relatively non-toxic levels of alkylating agents.

Key words: alkylation/glycosylase/homology/inducible/yeast

Introduction

Escherichia coli and mammalian cells contain various DNA methyltransferases and DNA glycosylases that protect against DNA alkylation (Gallagher and Brent, 1983; Male *et al.*, 1985, 1987; Lindahl *et al.*, 1988). Two *E.coli* 3-methyladenine (3MeA) DNA glycosylases encoded by the *tag* and *alkA* genes have been characterized (Karran *et al.*, 1980, 1982; Evensen and Seeburg, 1982; Nakabeppu *et al.*, 1984a,b; Sakumi *et al.*, 1986). The Tag DNA glycosylase is constitutively expressed and repairs 3MeA lesions; the AlkA DNA glycosylase is inducible by DNA alkylation and repairs 3MeA, 3-methylguanine (3MeG), O^2-methylthymine (O^2MeT) and O^2-methylcytosine (O^2-MeC) lesions. *E.coli* mutants deficient in the Tag and AlkA DNA glycosylases are extremely sensitive to killing by alkylating agents (Clarke *et al.*, 1984). 3MeA DNA glycosylases have also been identified and purified from various mammalian sources (Gallagher and Brent, 1983; Male *et al.*, 1985, 1987), but it is not clear whether, like the *E.coli* enzymes, their synthesis is induced by DNA alkylation (Laval, 1985). Since mutants defective in these mammalian genes have not yet been isolated, it is not known how important 3MeA DNA glycosylase is in protecting mammalian cells against alkylating agents.

A large number of DNA alkylation sensitive mutants have been identified in *Saccharomyces cerevisiae* (Friedberg, 1988) and more than a dozen alkylation inducible genes have been found (Johnston and Nasmyth, 1978; McClanahan and McEntee, 1984; Ruby *et al.*, 1983; Peterson *et al.*, 1985; Ruby and Szostack, 1985; Madura and Prakash, 1986; Robinson *et al.*, 1986; Cole *et al.*, 1987; Hurd *et al.*, 1987; Treger *et al.*, 1988; Elledge and Davis, 1987, 1989), but so far the products of only three of these inducible genes have been identified, namely, DNA ligase (Johnston and Nasmyth, 1978; Peterson *et al.*, 1985), ribonucleotide reductase (Hurd *et al.*, 1987; Elledge and Davis, 1987, 1989) and ubiquitin (Treger *et al.*, 1988).

Despite the identification of so many genes involved in the response of yeast cells to alkylating agents, it is only recently that *S.cerevisiae* has been shown to repair DNA alkylation damage using the same sorts of repair enzymes as *E.coli* and mammalian cells. Recent studies showed that *S.cerevisiae* contains both DNA glycosylase and DNA methyltransferase activities (Nisson and Lawrence, 1986; Sassanfar and Samson, 1990) and we have isolated a clone for a 3MeA DNA glycosylase gene (*MAG*) (Chen *et al.*, 1989) and for an O^6MeG DNA methyltransferase gene (L.Samson and B.Derfler, unpublished results). By generating an alkylation sensitive yeast mutant that is deficient in 3MeA DNA glycosylase activity, we showed that the MAG glycosylase increases survival after DNA alkylation damage (Chen *et al.*, 1989). Here, we describe the nucleotide sequence, chromosomal location and biological function of the *MAG* gene, and its response to DNA damaging alkylating agents. Our results show that the predicted MAG protein is significantly homologous to the *E.coli* AlkA DNA glycosylase and, like *alkA*, the *MAG* gene is induced upon exposure to relatively non-lethal levels of alkylating agents and specifically protects yeast cells against alkylation induced cell death.

Results

Sequencing and localization of the MAG gene on a yeast genomic DNA fragment

We have reported the isolation of a 2.1 kb yeast genomic DNA fragment that suppresses the alkylation sensitivity of an *alkA tag E.coli* strain (Chen *et al.*, 1989). This has now been subcloned in both orientations in the M13mp18 phage vector, and its nucleotide sequence determined by the dideoxy chain termination method. One strand was sequenced using a nested set of unidirectional deletions spanning the entire 2.1 kb DNA fragment, and the other strand was sequenced using synthetic oligonucleotides as primers. We found two non-overlapping large open reading frames (ORFs). ORF1 is 1011 nucleotides and appears to extend beyond the end of the 2.1 kb *Eco*RI fragment. ORF2 is 888

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nucleotides and would encode a 296 amino acid polypeptide. In order to determine which ORF provides methylmethanesulphonate (MMS) resistance, two shortened fragments which had lost either part of ORF1 or part of ORF2 were prepared by digestion with *Kpn*I or *Xba*I (Figure 1). These fragments were subcloned into both pUC18 and pUC19. The plasmids that carried intact ORF2 conferred MMS resistance to *E.coli alkA tag* cells, and the plasmids that carried ORF1 did not (Figure 1). Since ORF2 is expressed in *E.coli* in both orientations with respect to the plasmid's *lacZ* promoter, the yeast sequence upstream of ORF2 can apparently be recognized by the *E.coli* RNA polymerase.

Before the complete sequence was available, we had generated a yeast deletion mutant that was very sensitive to MMS and contained much less 3MeA DNA glycosylase activity (Chen *et al.*, 1989). However, sequencing revealed that this was an ORF1 and ORF2 double mutant. To confirm that it is ORF2, not ORF1, that confers MMS resistance in yeast cells and directs the synthesis of 3MeA DNA glycosylase, an ORF2::*URA*3 disruption was constructed *in vitro* and used to replace the wild-type gene (Figure 2A). The ORF2::*URA*3 gene disruption was confirmed by Southern blot analysis (Figure 2C). The haploid ORF2::*URA*3 strain proved to be just as sensitive to MMS as the ORF1/ORF2 double mutant (Figure 2B) and contained much less 3MeA DNA glycosylase activity than wild-type cells (Table I).

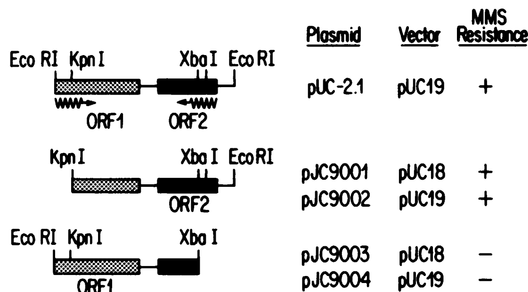

Fig. 1. Identification of the glycosylase ORF on the cloned yeast genomic DNA fragment. The depicted DNA fragments from the 2.1 kb yeast genomic DNA containing the *MAG* gene were subcloned into pUC18 or pUC19 and tested for their ability to restore MMS resistance to *E.coli alkA tag* mutants. Solid and stippled bars represent open reading frames. Arrows indicate direction of transcription.

The amino acid sequence for ORF2 shares homology with the E.coli AlkA glycosylase

The nucleotide sequence and the predicted amino acid sequence of ORF2 are shown in Figure 3. The open reading frame begins with the ATG codon at position +1 and extends to a TAA codon at position +888. ORF2 would encode a protein of 296 amino acids, with a predicted molecular weight of 34 293 daltons. When the predicted amino acid sequence was checked against the National Biomedical Research Foundation (NBRF) protein database (Lipman and Pearson, 1985), it was found to have significant homology to the *E.coli* AlkA DNA glycosylase (Figure 4) but not to the *E.coli* Tag glycosylase which is known to be unrelated to AlkA (Steinum and Seeburg, 1986). A one-to-one comparison revealed 27.0% identity and 63.5% similarity in a stretch of 137 amino acids in the middle of the reading frame. Because of this homology and the experiments described above, we now believe that ORF2 is established as the gene for the yeast MAG 3MeA DNA glycosylase.

Identification of transcription and translation signals in the cloned MAG gene

The 138 nucleotide sequence upstream of the *MAG* initiation codon is 64.5% AT and contains two poly(dA-dT) stretches. We found no perfect matches for the proposed yeast TATA promoter element, which can be up to 700 nucleotides upstream of the translation initiation codon (Guarente, 1987; Cigan and Donahue, 1987), and so our clone may not contain the normal promoter. However, the poly(dA-dT) stretch found between −51 and −35 upstream of the ATG initiation codon could be the promoter recognized by the *E.coli* RNA polymerase. The nine nucleotides, AGAGAGGGA, just 5′ of the initiation codon bear close resemblance to the Shine−Dalgarno ribosomal binding site in *E.coli* (Shine and Dalgarno, 1974) and probably serve to ensure efficient translation of the *MAG* transcript in *E.coli*. The AATAAA consensus sequence believed to represent the signal for poly(A)$^+$ addition in higher eukaryotes (Fitzgerald and Shenk, 1981) was observed in the 3′ non-coding region (Figure 3). The *MAG* mRNA uses 56 of 61 codons, indicating an absence of bias toward the major yeast isoacceptor tRNA species that characterizes some highly expressed yeast genes (Bennetzen and Hall, 1982). This suggests that the *MAG* gene is not highly expressed in yeast cells, which is consistent with our

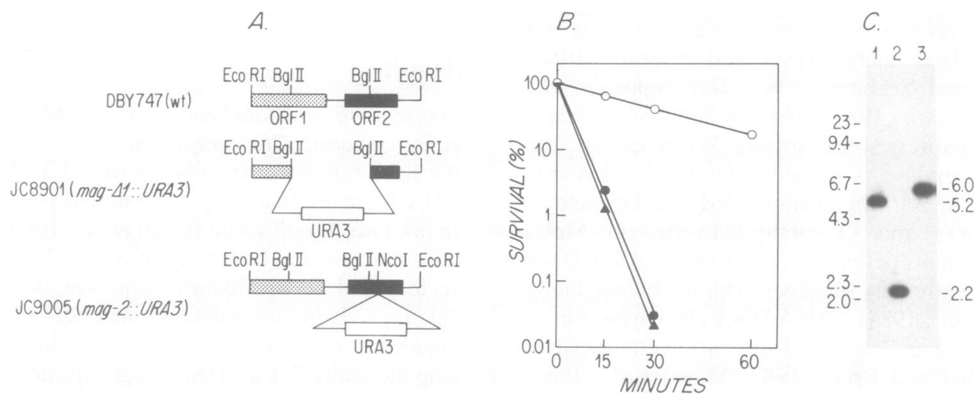

Fig. 2. ORF1 and ORF2 disruptions. **(A)** The molecular structure of ORF1 and ORF2 in DBY747, JC8901 and JC9005 *S.cerevisiae* strains. **(B)** MMS (0.28%) induced cell killing of DBY747 (○), JC8901 (●) and JC9005 (▲). **(C)** Southern blot analysis of 3 μg DNA from JC8901 (lane 1), DBY747 (lane 2) and JC9005 (lane 3) hybridized with 2.1 kb *Eco*RI fragment carrying the *MAG* gene.

observation of a low abundance of the *MAG* transcript (relative to the actin transcript) in wild-type yeast cells (see below).

MAG mRNA levels increase in response to DNA alkylation damage

Our initial Northern blot analysis of RNA isolated from *S.cerevisiae* DBY747 had shown that the 2.1 kb *Eco*RI fragment hybridized to a single transcript of 1.8 kb (Chen *et al.*, 1989). However, using a ^{32}P-labelled probe with a 40-fold higher specific activity (1×10^9 c.p.m./μg DNA), we have now found that the 2.1 kb *Eco*RI fragment actually hybridizes to two transcripts of 1.8 kb and 1.2 kb (Figure 5A, lane 1), which is consistent with the existence of two open reading frames in the fragment. In order to determine which mRNA was transcribed from the *MAG* gene, two 0.7 kb DNA fragments, *Eco*RI−*Nde*I and *Nco*I−*Eco*RI, were isolated from each end of the 2.1 kb *Eco*RI DNA fragment, and used to probe the RNA blot. The *Eco*RI−*Nde*I probe, which is part of ORF1, hybridized to the 1.8 kb transcript (Figure 5A lane 2), and the *Nco*I−*Eco*RI probe containing part of the *MAG* gene hybridized to the 1.2 kb transcript (Figure 5A lane 3). Thus, the *MAG* gene directs the synthesis of the 1.2 kb mRNA.

Table I. Comparison of the *mag* mutant to five MMS sensitive *S.cerevisiae* strains

	Response to MMS[a]	3-MeA released (fmol)[b]	Complementation of $\alpha mag\Delta$-*1::URA3*[c]
wild-type	R	58.0	Yes
mag-2::URA3	S	4.3	ND
mag-Δ1::URA3	S	7.1	ND
mms-5	S	7.3	No
mms-4	S	145.3	Yes
mms-1	S	63.9	ND
mms-2	S	57.4	ND
mms-22	S	80.1	ND

[a]R, resistant; S, sensitive.
[b]1 mg of extract proteins were incubated with 74 μg alkylated calf thymus DNA (455.8 c.p.m./μg) at 25°C for 1 h as previously described (Chen *et al.*, 1989).
[c]The complementation assay was conducted by testing the sensitivity of diploid strains to MMS on an MMS gradient plate. ND, not done.

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-138 GAATTCTTACATAAAGTTTAAGTTATCTATGAATCAATGAGAATTGGCCACTGCCCTCTGATATGACGATGGAAGTGGT
                                                                      +1
 -59 ACTTTTCCTTTTTAATTTTTTACTGAGAATCAAGAGAAGCTAGAGAGGGATTGGTCTCA ATG AAA CTA AAA AGG
                                                                 Met Lys Leu Lys Arg

  16 GAG TAT GAT GAG TTA ATA AAA GCA GAC GCT GTT AAG GAA ATA GCA AAA GAA TTA GGG TCT
     Glu Tyr Asp Glu Leu Ile Lys Ala Asp Ala Val Lys Glu Ile Ala Lys Glu Leu Gly Ser

  76 CGA CCT CTA GAG GTT GCT CTT CCT GAG AAA TAT ATT GCT AGA CAT GAA GAA AAG TTC AAT
     Arg Pro Leu Glu Val Ala Leu Pro Glu Lys Tyr Ile Ala Arg His Glu Glu Lys Phe Asn

 136 ATG GCT TGC GAA CAC ATT TTA GAG AAA GAT CCA TCA CTT TTT CCC ATA CTT AAG AAT AAT
     Met Ala Cys Glu His Ile Leu Glu Lys Asp Pro Ser Leu Phe Pro Ile Leu Lys Asn Asn

 196 GAA TTT ACG TTG TAC TTG AAG GAG ACT CAA GTC CCT AAT ACA CTC GAA GAT TAT TTT ATT
     Glu Phe Thr Leu Tyr Leu Lys Glu Thr Gln Val Pro Asn Thr Leu Glu Asp Tyr Phe Ile

 256 AGG CTT GCA AGC ACA ATT TTG TCT CAA CAG ATC AGT GGC CAA GCA GCT GAA AGC ATC AAG
     Arg Leu Ala Ser Thr Ile Leu Ser Gln Gln Ile Ser Gly Gln Ala Ala Glu Ser Ile Lys

 316 GCA AGG GTT GTC AGT CTT TAT GGC GGT GCA TTT CCT GAT TAC AAA ATC CTT TTC GAA GAC
     Ala Arg Val Val Ser Leu Tyr Gly Gly Ala Phe Pro Asp Tyr Lys Ile Leu Phe Glu Asp

 376 TTC AAA GAC CCA GCA AAA TGT GCA GAA ATC GCA AAA TGT GGA TTG AGT AAA AGG AAA ATG
     Phe Lys Asp Pro Ala Lys Cys Ala Glu Ile Ala Lys Cys Gly Leu Ser Lys Arg Lys Met

 436 ATA TAT CTA GAG TCT CTT GCT GTC TAT TTT ACT GAA AAA TAT AAG GAT ATC GAA AAG CTC
     Ile Tyr Leu Glu Ser Leu Ala Val Tyr Phe Thr Glu Lys Tyr Lys Asp Ile Glu Lys Leu

 496 TTC GGT CAA AAA GAT AAT GAT GAG GAA GTG ATT GAA AGT TTA GTT ACG AAT GTA AAA GGT
     Phe Gly Gln Lys Asp Asn Asp Glu Glu Val Ile Glu Ser Leu Val Thr Asn Val Lys Gly

 556 ATA GGC CCA TGG AGT GCC AAA ATG TTC TTG ATC TCC GGA TTG AAA AGA ATG GAT GTA TTT
     Ile Gly Pro Trp Ser Ala Lys Met Phe Leu Ile Ser Gly Leu Lys Arg Met Asp Val Phe

 616 GCT CCT GAA GAT CTA GGT ATT GCT AGG GGT TTT TCA AAA TAC CTT TCA GAT AAG CCA GAA
     Ala Pro Glu Asp Leu Gly Ile Ala Arg Gly Phe Ser Lys Tyr Leu Ser Asp Lys Pro Glu

 676 TTG GAA AAA GAA TTA ATG CGT GAA AGA AAA GTA GTT AAA AAG AGT AAG ATT AAG CAT AAG
     Leu Glu Lys Glu Leu Met Arg Glu Arg Lys Val Val Lys Lys Ser Lys Ile Lys His Lys

 736 AAA TAC AAC TGG AAA ATA TAT GAC GAC GAC ATA ATG GAA AAA TGC TCT GAA ACA TTT TCT
     Lys Tyr Asn Trp Lys Ile Tyr Asp Asp Asp Ile Met Glu Lys Cys Ser Glu Thr Phe Ser

 796 CCG TAT AGG TCT GTG TTT ATG TTC ATA CTT TGG AGG CTC GCG AGC ACA AAT ACA GAT GCC
     Pro Tyr Arg Ser Val Phe Met Phe Ile Leu Trp Arg Leu Ala Ser Thr Asn Thr Asp Ala

 856 ATG ATG AAG GCA GAA GAA AAT TTC GTG AAA TCC TAACTTAAAGATATCATGTATTACTGACATTTATA
     Met Met Lys Ala Glu Glu Asn Phe Val Lys Ser End

 924 TAAGAAAAAAAAAAATAAAAATAAATACACCCTATTGTTGTGTGTGATGCCTACTAAAGAAATTCATATTTACAGCTTC

1003 TCGTAGGGATACCAACATATAATAAGAAAAAGCCTCACACATACAATCCAACCATTGGAGCGACAAAGCGGAAAAGCGA
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Fig. 3. Nucleotide sequence of ORF2. The numbering is in relation to the first base of the ATG translation initiation codon, which is indicated at position +1. The amino acid sequence of the predicted protein is indicated below the nucleotide sequence. Sequences which resemble TATA promoter elements, the *E.coli* Shine−Dalgarno ribosomal binding site and the eukaryotic polyadenylation signal are underlined.

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               60        70        80        90        100       110
ORF2    LEKDPSLFPILKNNEFTLYLKETQVPNTLEDYFIRLASTILSQQISGQAAESIKARVVSL
                                    * * : :::**:* :* : *::::***: *
AlkA    FDLQCNPQIVNGALGRLGAARPGLRLPGCVDAFEQGVRAILGQLVSVAMAAKLTARVAQL
               90        100       110       120       130       140

               120       130       140       150       160       170
ORF2    YGGAFPDYKILFEDFKDPAKCAEIAKCGLSKRKMIYLESLA-VYFTEKYKDIEKLFGQKD
        **: : *:    : * :*:: *: :   :*:   *     : * :::::    : :  :: :
AlKA    YGERLDDFPE-YICFPTPQRLAAADPQALKALGMPLKRAEALIHLANAALEGTLPMTIPG
               150       160       170       180       190       200

               180       190       200       210       220       230
ORF2    NDEEVIESLVTNVKGIGPWSAKMFLISGLKRMDVFAPEDLGIARGFSKYLSDKPELEKEL
        : *:::::* *   ***:*:*: * ::* : :*** *:*  * : *:
AlkA    DVEQAMKTLQT-FPGIGRWTANYFALRGWQAKDVFLPDDYLIKQRFPGMTPAQIRRYAER
               210       220       230       240       250

               240       250       260       270       280       290
ORF2    MRERKVVKKSKIKHKKYNWKIYDDDIMEKCSETFSPYRSVFMFILWRLASTNTDAMMKAE

AlkA    WKPWRSYALLHIWYTEGWQPDEA
     260       270       280
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Fig. 4. Comparison of the MAG and AlkA amino acid sequences. Exact matches are indicated by asterisks, similar amino acids are indicated by colons and gaps are indicated by a dash.

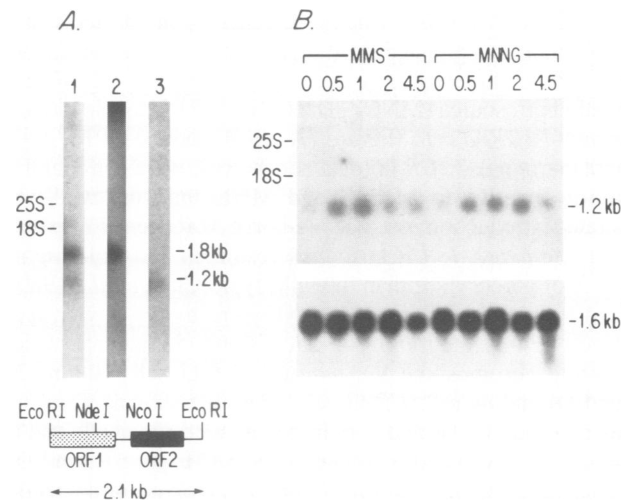

Fig. 5. (**A**) Northern blot analysis of ORF1 and ORF2 (*MAG*) expression in *S.cerevisiae*. In each lane in A and B, 15 μg of total yeast RNA was separated in a formaldehyde/1% agarose gel and transferred to a nylon membrane. Panel **A**. Lane 1 was probed with the 2.1 kb fragment containing both ORF1 and ORF2; lanes 2 and 3 were probed with the *Eco*RI−*Nde*I fragment (for ORF1) and *Nco*I−*Eco*RI fragment (for ORF2) respectively. Panel **B**. RNA was extracted from MMS (0.05%) or MNNG (5 μg/ml) treated cells exposed for 0, 0.5, 1, 2, 4.5 h as indicated at the top of each lane. The blot was hybridized with the 0.7 kb *Nco*I−*Eco*RI fragment (ORF1) (top of panel B), then dehybridized and re-probed with an actin gene fragment to quantitate the amount of RNA present in each lane (bottom of panel B). The positions of the 25S and 18S rRNA are indicated.

When *E.coli* is exposed to low concentrations of alkylating agents, the activity of the AlkA glycosylase is induced ~20-fold (Evensen and Seeberg, 1982; Karran *et al.*, 1982). Figure 5A and B showed that the 1.2 kb *MAG* transcript was barely detectable in untreated yeast cells, but increased ~15-fold within 1–2 h after exposure to relatively non-toxic levels of MMS or *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) (Figure 5B). Laser densitometry showed that alkylation treatment did not significantly alter the level of actin mRNA, and the levels of the MAG transcript were normalized to the actin transcript levels (Figure 5B).

The MAG gene is located on chromosome V

A yeast genomic DNA fragment containing part of the *MAG* gene was radiolabelled and hybridized to a pulse field yeast chromosome gel. The *MAG* probe hybridized uniquely to chromosome V (Figure 6, lane 1). The location of the *MAG* gene on chromosome V was confirmed by hybridization of the *URA3* gene (known to be on chromosome V) to the same chromosome (data not shown). Because of the close proximity of chromosomes V and VIII on the chromosome gel, the *ARG4* gene (known to be on chromosome VIII) was used to distinguish between chromosomes V and VIII. As expected, the *ARG4* probe hybridized to a band lower than that of chromosome V, excluding the possibility that the *MAG* gene might be on chromosome VIII (Figure 6, lanes 2 and 3). Five *rad* mutants, *rad-3, -4, -23, -24* and *-51*, that cause sensitivity to UV or ionizing radiation, have also been mapped to chromosome V. To determine whether the *MAG* gene was any of these *RAD* genes, we further characterized the phenotype of the *mag* mutants.

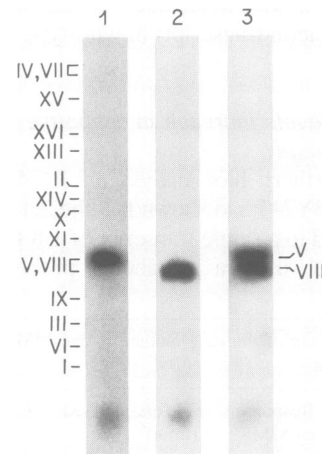

Fig. 6. Chromosomal location of the *MAG* gene. Yeast chromosomes separated by pulse field electrophoresis (Clontech) were hybridized with a ^{32}P-labelled 0.9 kb *Bgl*II fragment containing part of the *MAG* gene (lane 1), a 3.2 kb *Pst*I fragment containing the *ARG4* gene (lane 2) and the mixture of both probes (lane 3). The positions of yeast chromosomes are indicated.

The MAG gene specifically protects yeast from DNA alkylation damage

In order to determine whether the *MAG* gene protects cells from monofunctional alkylating agents other than MMS, we determined the sensitivity of the *mag* mutants to MNNG and methylnitrosourea (MNU). The ORF1/ORF2 double mutant, *mag*Δ*-1*::*URA3* cells (Figure 7A and B) and the ORF1 single mutant, *mag*Δ*-2*::*URA3* (data not shown), were considerably more sensitive to MNNG and MNU than the wild-type strain. Introduction of a plasmid-borne *MAG* gene (YEp13A) into the *mag*Δ*-1*::*URA3* mutant results in a 10-fold higher level of glycosylase than the wild-type (Chen *et al.*, 1989), and restored MNNG and MNU sensitivity to wild-type levels. Unlike the *rad-3, -4, -23, -24* and *-51* mutants (which map to chromosome V), *mag*Δ*-1*::*URA3* cells (figure 7C and D) and *mag-2*::*URA3* cells (data not shown) were just as resistant to UV and γ-radiation as wild-type cells and as *mag*Δ*-1*::*URA3* cells carrying YEp13A (Figure 7C and D). This suggests that the *MAG* gene is involved in a specific pathway that protects yeast cells from alkylation damage but not from radiation damage.

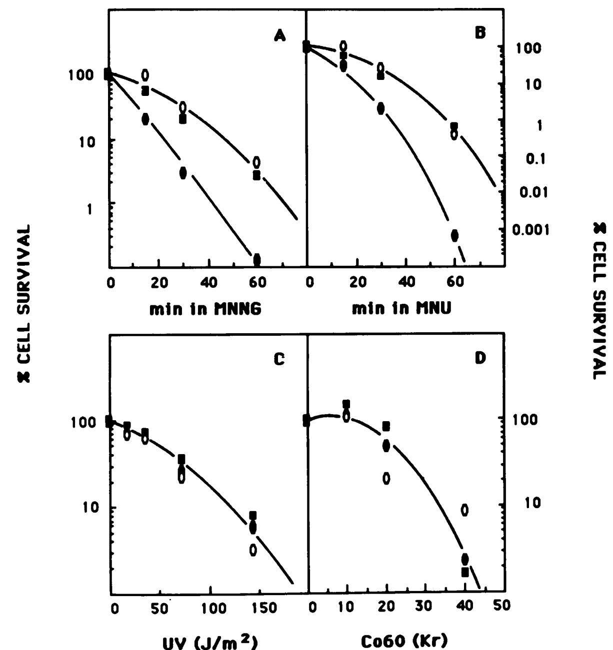

Fig. 7. Cell survival upon treatment of DNA damaging agents. The colony forming ability of *S.cerevisiae* strains DBY747 (○), JC8901 (●), JC8901/YEp13A (■) was measured after treatment with MNNG at 30 μg/ml (panel **A**) and MNU at 2 mg/ml (panel **B**) for the indicated times, and after various exposures to UV (panel **C**) or γ-radiation (panel **D**).

Comparison of mag mutants with other MMS sensitive yeast mutants

The phenotype of *mag* mutants suggests that they are distinct from the five known *rad* mutants on chromosome V. But six other mutants have been isolated that, like *mag* mutants, are sensitive to MMS but not sensitive to radiation (Prakash and Prakash, 1977; Nisson and Lawrence, 1986). One of these, *ngs1*, is known to contain 3MeA DNA glycosylase activity (Nisson and Lawrence, 1986). We have now measured the glycosylase activity in the other five. Mutants *mms-1, -2* and *-22* showed wild-type levels of enzyme activity, and *mms-4* showed ~2.5-fold higher glycosylase activity than wild-type cells. However, *mms-5* showed 8-fold less activity (Table I). The *mag*$^-$ phenotype is recessive because *mag*/wild-type and *mag*/*mms-4* diploid strains were resistant to MMS (Table I), but a diploid strain constructed from the *mag* and *mms-5* haploid mutants was still sensitive to MMS (Table I). The decreased 3MeA DNA glycosylase activity in the *mms-5* strain, and the failure of the *mag* mutant to complement *mms-5*, suggests that the *mms-5* and *mag*

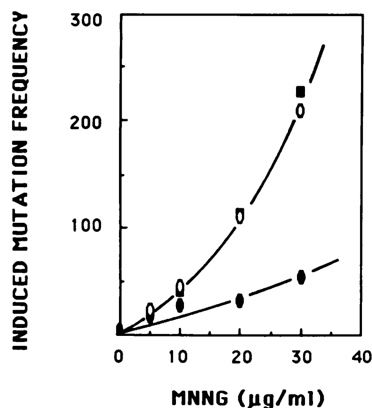

Fig. 8. MNNG induced mutation. The frequency of canavanine resistant mutants (canavanine resistant colonies per 10^6 surviving cells) in *S.cerevisiae* strains DBY747 (○), JC8901 (●), and JC8901/YEp13A (■) was measured after treatment with MNNG at the indicated doses.

mutant strains bear defects in the same gene. The relatively high glycosylase activity in *mms-4* cells is not currently understood.

Expression of the MAG gene and sensitivity to alkylation induced mutation

In *E.coli*, the mutagenic lesions induced by alkylating agents are repaired by methyltransferase (Lindahl *et al.*, 1988) and via other pathways such as the Uvr nucleotide excision pathway (Van Houten and Sancar, 1987; Samson *et al.*, 1988), but not apparently by the various glycosylases, which protect against killing rather than against mutagenesis (Lindahl *et al.*, 1988). To test whether the *MAG* gene protects yeast cells from alkylation induced mutation, we measured the mutation frequency in *mag*Δ*-1*::*URA3* mutants after MNNG (Figure 8) and MMS (data not shown) treatment. Figure 8 shows that a low MNNG dose (5 μg/ml) is equally mutagenic for *mag*Δ*-1*::*URA3* and wild-type cells. Surprisingly, at higher MNNG doses (10, 20 and 30 μg/ml), the *mag*Δ*-1*::*URA3* strain (Figure 8) and the *mag-2*::*URA3* strain actually showed a lower mutation frequency than wild-type cells. MMS mutagenesis exactly paralleled MNNG mutagenesis: 0.025% MMS was equally mutagenic for *mag-2*::*URA3* and wild-type cells, but higher MMS doses (0.05% and 0.1%) were much more mutagenic for wild-type than for *mag-2*::*URA3* cells (data not shown).

Discussion

E.coli prevents alkylation induced killing and mutation via several different DNA repair pathways. A similar picture is emerging for *S.cerevisiae*. At very low levels of DNA alkylation damage in *E.coli*, the constitutively expressed Tag glycosylase and Ogt methyltransferase are responsible for alkylation repair (Lindahl *et al.*, 1988; Rebeck *et al.*, 1988, 1989). When DNA alkylation levels increase, the AlkA glycosylase and Ada methyltransferase are induced to augment the process of repair (Karran *et al.*, 1982; Evensen and Seeberg, 1982; Nakabeppu and Sekiguchi, 1986; Teo *et al.*, 1986; Lindahl *et al.*, 1988), and at high DNA alkylation levels the nucleotide excision repair pathway begins to play a major role (Samson *et al.*, 1988). At still higher levels, the SOS regulon is induced and this may be why SOS defective *recA* and *lexA* mutants are sensitive to alkylating agents (Boiteux *et al.*, 1984; Walker, 1984). Thus, at least four sets of pathways can be recruited to protect *E.coli* against alkylation; two of these pathways are specific for the repair of DNA alkylation damage, and the other two, namely nucleotide excision repair and the SOS response, can repair radiation (and other) DNA damage in addition to alkylation damage.

Mutations in a large number of *S.cerevisiae* genes raise sensitivity to alkylation, and these mutants can be divided into two broad classes: those that are specifically sensitive to alkylation and those that are also sensitive to alkylation and radiation (Friedberg, 1988; Prakash and Prakash, 1977; Nisson and Lawrence, 1986; Cooper and Waters, 1987). The radiation/alkylation sensitive mutants fall into three epistasis groups (*RAD3*, *RAD6* and *RAD52*) which loosely parallel the radiation/alkylation sensitive *uvrABC*, *lexA* and *recA E.coli* mutants. *S.cerevisiae* mutants specifically sensitive to alkylation were initially presumed to be analogous to *E.coli* mutants defective in glycosylase, methyltransferase and other alkylation repair functions, but until recently these enzymes and forms of repair had not been detected in yeast. It is now clear that *S.cerevisiae* contains a 3MeA DNA glycosylase gene (the *MAG* gene) which parallels the *E.coli* AlkA glycosylase gene in being alkylation inducible and in protecting cells specifically against killing by alkylating agents, though it remains to be determined whether *S.cerevisiae* contains a constitutively expressed 3MeA DNA glycosylase analogous to the Tag glycosylase. *S.cerevisiae* also contains an O6MeG DNA methyltransferase (Sassanfar and Samson, 1990), and now that its gene has been cloned (L.Samson and B.Derfler, unpublished results) it should be a simple matter to determine whether it specifically protects against alkylation and whether it is alkylation inducible like the Ada methyltransferase or expressed constitutively like the Ogt methyltransferase.

E.coli exposed to sublethal levels of MNNG and MMS for one or two generation times adapts to become extremely resistant to alkylation induced killing and mutation, due in part to the induction of the *ada* and *alkA* genes (Samson and Cairns, 1977; Jeggo, 1979; Lindahl *et al.*, 1988). This response is detectable only by a narrow range of alkylation doses (Jeggo *et al.*, 1977; Schendel *et al.*, 1978). More than a dozen alkylation inducible genes have been found in *S.cerevisiae* and these include genes for DNA ligase, ribonucleotide reductase and ubiquitin genes (Johnston and Nasmyth, 1978; Peterson *et al.*, 1985; Elledge and Davis, 1987, 1989; Hurd *et al.*, 1987; Treger *et al.*, 1988). There have, however, been two reports that *S.cerevisiae* lacks an adaptive response to alkylating agents (Maga and McEntee, 1985; Polakowska *et al.*, 1986). We show here that one alkylation specific DNA repair gene, the *MAG* gene, is very efficiently induced by relatively non-toxic doses of MNNG and MMS, and this suggests that an adaptive response may be detectable in *S.cerevisiae* under certain conditions.

It remains to be determined whether the alkylation induced increase in *MAG* transcript levels leads to an increased resistance to alkylation induced killing. We have previously shown that increasing the level of MAG glycosylase above the normal wild-type level (by expression of the glycosylase from a plasmid) does not provide any extra alkylation resistance over and above that found in wild-type cells (Chen

et al., 1989; Figure 7), suggesting that some other enzyme in the base excision repair pathway is usually rate limiting. To have an adaptive increase in resistance to killing by alkylating agents, the cell might have to increase the levels of several enzymes, for example; apurinic/apyrimidinic (AP) endonuclease (Popoff *et al.*, 1990), DNA polymerase I (Johnston *et al.*, 1987) and DNA ligase (Peterson *et al.*, 1985). In contrast, to have an adaptive increase in resistance to mutagenesis, it might only be necessary to increase the synthesis of one enzyme, because an inducible methyltransferase would be expected to operate without assistance from other enzymes.

We made the unexpected observation that MAG glycosylase deficient yeast cells are less sensitive to alkylation mutagenesis than wild-type cells. The explanation may be as follows. The effect of 3MeA DNA glycosylase is to produce AP sites in DNA. Their repair requires the action of AP endonucleases, and then DNA polymerase and DNA ligase (Friedberg, 1985; Wallace, 1988). If left unrepaired, they are mutagenic in both prokaryotic and eukaryotic cells (Loeb, 1985). Thus, the balance of DNA glycosylase and AP endonuclease activities will clearly influence mutation induction. Presumably fewer AP sites are produced in alkylated *mag*$^-$ cells since they do not remove 3MeA efficiently from their alkylated genome. When wild-type *S.cerevisiae* cells are exposed to high levels of alkylation the repair of 3MeA rescues the cells from death but apparently at the cost of a certain amount of mutation induction from unrepaired AP sites in DNA.

It has become plain that widely divergent organisms employ similar enzymes to rid their genomes of damage. Physical and functional homologies have been found among DNA repair enzymes from organisms such as bacteriophage, bacteria, yeast, fish, insects and humans (Valerie *et al.*, 1985; Doolittle *et al.*, 1986; van Duin *et al.*, 1986, 1989; Samson *et al.*, 1986; Nakatsuru *et al.*, 1987; Friedberg, 1988; Downes, 1988; Chen and Bernstein, 1988; Percival *et al.*, 1989; Bernstein and Bernstein, 1989; Kelley *et al.*, 1989; Banga *et al.*, 1989). Like bacteria, yeast cells have the ability to recruit a number of different pathways to prevent the toxic effects of DNA alkylation damage. The more these pathways are characterized the more they turn out to be similar to those employed by bacteria.

Materials and methods

Strains and vectors

The *E.coli* strain HB101 was used for subcloning and propagation of plasmids and the strain JM101 was used for the color selection of pUC derivatives and the propagation of M13mp18 phage. *S.cerevisiae* haploid strains were: DBY747 (a, *his-Δ-1, leu2-3, leu2-112, trp1-289, ura3-52*) and its alkylation sensitive derivatives JC8901 (*mag-Δ1::URA3*) and JC9005 (*mag-2::URA3*) (generated in this lab); DBY745 (α, *ade1-100, ura3-52, leu2-3, leu2-112*) and its alkylation sensitive derivatives JC9001 (*mag-Δ1::URA3*); B635 (a, *cyc1-115, his1, lys2, trp2*) and its MMS sensitive derivatives MD-1 (*mms1-1*), MD-2 (*mms2-1*), MD-10 (*mms4-1*), MD-24 (*mms5-1*) and MD-85 (*mms22-1*) (gifts from Louise Prakash, University of Rochester, Rochester, NY). The *S.cerevisiae* diploid strains used for complementation tests were, JC9002 (*MAG/mag-Δ1::URA3*), JC9003 (*mms-4/mag-Δ1::URA3*) and JC9004 (*mms-5/mag-Δ1::URA3*); these strains were obtained from crosses of B635 × JC9001, MD-10 × JC9001 and MD-24 × JC9001, respectively. A plasmid containing the *ARG4* gene was a gift from Jack Szostack (Harvard Medical School, Boston, MA). YEp13A, a YEp13 plasmid carrying the *MAG* gene, was isolated in this lab from a yeast genomic library purchased from American Type Culture Collection (Chen *et al.*, 1989).

DNA sequencing

The 2.1 kb *Eco*RI yeast genomic DNA fragment containing the *MAG* gene was subcloned into M13mp18. A nested set of unidirectional deletions spanning the insert was generated by exonuclease III and S1 nuclease using the Promega 'Erase-a-Base System', based on the procedure developed by Henikoff (Henikoff, 1984). Synthetic oligonucleotides were used as primers to complete the sequencing of both strands of DNA. Sequencing was by the dideoxy chain termination method of Sanger (Sanger *et al.*, 1977), using deoxyadenosine 5'-[α-^{35}S]thiotriphosphate (NEN) and sequenase enzyme (United States Biochemical Corp).

Nucleic acid hybridizations

Southern blot and Northern blot analyses were performed as described previously (Chen *et al.*, 1989). Briefly, 2.5 μg yeast genomic DNA was digested with *Eco*RI, separated by electrophoresis in 1% agarose gel, transferred to a nylon membrane, and probed with the ^{32}P-labelled 2.1 kb *Eco*RI fragment containing the *MAG* gene. 15 μg of yeast RNA was separated by electrophoresis in a formaldehyde/1% agarose gel, transferred to a nylon membrane, and probed with ^{32}P-labelled DNA. Both DNA and RNA blots were washed at high stringency (30 mM sodium chloride/3 mM sodium citrate at 58°C for 2 h).

Direct chromosomal gel hybridization

A yeast chromosomal gel with most of the chromosomes separated by pulsed field electrophoresis (except for XII and XVII) was purchased from Clontech, and was probed with a ^{32}P-labelled 0.9 kb *Bgl*II fragment (containing part of the *MAG* gene) at 60°C for 20 h. The gel was washed at low stringency (180 mM NaCl, 10 mM NaH_2PO_4 pH 7.4, 1 mM EDTA, 0.1% SDS at 60°C for 2 h, and then at high stringency (18 mM NaCl, 1 mM NaH_2PO_4 pH 7.4, 0.1 mM EDTA, 0.1% SDS at 65°C for 1 h).

Targeted MAG gene disruption

A 3.8 kb *Bam*HI–*Bgl*II fragment containing the yeast *URA3* gene was isolated from plasmid pNKY51 (Alani *et al.*, 1987) and inserted into the *Nco*I site of pUC-2.1 to disrupt the *MAG* gene (see Figure 2). *Eco*RI DNA fragments containing the disrupted *MAG* gene were used to transform yeast DBY747; DNA isolated from *URA*$^+$ transformants was analysed on Southern blots to confirm the disruption of the *MAG* gene.

Yeast cell killing

Cell killing was measured as described (Chen *et al.*, 1989). Yeast cells were grown in rich medium (YPD 1% yeast extract, 2% peptone, 2% dextrose) to 2×10^7 cells per ml. For measurement of alkylation sensitivity, MNNG and MNU were added to cell cultures to a final concentration of 30 μg/ml and 2 mg/ml respectively. For UV or γ-radiation induced killing, log phase cells were washed and resuspended in water, and irradiated in a Petri dish either by UV or by ^{60}Co γ-ray at the indicated doses. Aliquots were removed from the culture at the indicated times, diluted and spread on YPD plates to estimate cell survival.

Mutagenesis experiments

Logarithmically growing cultures (2×10^7/ml) in YPD were treated with 0, 5, 10, 20, 30 μg/ml MNNG for 15 min. 10 ml cells were washed once with water, concentrated 20-fold by centrifugation, diluted and plated on both YPD plates and synthetic media plates lacking arginine and containing 2% canavanine. Mutation frequency was calculated as the number of canavanine resistant colonies per 10^6 surviving cells.

DNA glycosylase activity

MAG DNA glycosylase activity was measured as described previously (Chen *et al.*, 1989).

Complementation test

Diploid strains used for the complementation tests were generated as described (Sherman *et al.*, 1986) and tested for MMS sensitivity on MMS gradient plates. Gradient plates were prepared by pouring 30 ml of YPD agar containing 0.01% MMS into a square Petri dish at an angle of about 6° from the horizontal; after the agar wedge solidified 30 ml YPD agar was added to the horizontal dish. Overnight cultures were printed across the gradient and resistance was scored by the percent of confluent growth along the gradient.

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