The *Saccharomyces cerevisiae* MGT1 DNA repair methyltransferase gene: its promoter and entire coding sequence, regulation and *in vivo* biological functions

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ABSTRACT

We previously cloned a yeast DNA fragment that, when fused with the bacterial *lacZ* promoter, produced O⁶-methylguanine DNA repair methyltransferase (MGT1) activity and alkylation resistance in *Escherichia coli* (Xiao *et al.*, *EMBO J.* 10,2179). Here we describe the isolation of the entire *MGT1* gene and its promoter by sequence directed chromosome integration and walking. The *MGT1* promoter was fused to a *lacZ* reporter gene to study how *MGT1* expression is controlled. *MGT1* is not induced by alkylating agents, nor is it induced by other DNA damaging agents such as UV light. However, deletion analysis defined an upstream repression sequence, whose removal dramatically increased basal level gene expression. The polypeptide deduced from the complete *MGT1* sequence contained 18 more N-terminal amino acids than that previously determined; the role of these 18 amino acids, which harbored a potential nuclear localization signal, was explored. The *MGT1* gene was also cloned under the *GAL1* promoter, so that MTase levels could be manipulated, and we examined MGT1 function in a MTase deficient yeast strain (*mgt1*). The extent of resistance to both alkylation-induced mutation and cell killing directly correlated with MTase levels. Finally we show that *mgt1 S.cerevisiae* has a higher rate of spontaneous mutation than wild type cells, indicating that there is an endogenous source of DNA alkylation damage in these eukaryotic cells and that one of the *in vivo* roles of *MGT1* is to limit spontaneous mutations.

INTRODUCTION

Alkylating agents represent a large group of toxic chemicals present in the environment. Simple alkylating agents such as methyl methanesulfonate (MMS) and N-Methyl-N'-nitro-N-nitrosoguanidine (MNNG) are known to be toxic, mutagenic and carcinogenic (1). Virtually all organisms tested so far employ

two kinds of pathway for the specific repair of DNA alkylation damage. DNA glycosylases remove the replication-blocking 3-methyladenine (3MeA) lesion from alkylated DNA, and thus protect cells from killing. DNA repair methyltransferases (MTases) transfer methyl groups from O⁶-methylguanine (O⁶MeG) and O⁴-methylthymine (O⁴MeT) to form S-methylcysteine in the MTase itself, and since the S-methylcysteine is not subsequently demethylated the MTase is inactivated by the act of repair (2). During DNA synthesis, O⁶MeG and O⁴MeT can mispair with thymine and guanine (3,4), respectively, and thus their repair prevents alkylation-induced transition mutations.

Escherichia coli expresses two MTases. The *ogt* MTase gene is constitutively expressed and protects cell against low levels of DNA alkylation damage (5,6). The *Ada* MTase gene is induced several thousand fold when cells experience low levels of DNA alkylation (2,7). Both MTases have one active cysteine that repairs O⁶MeG or O⁴MeT, and *Ada* has a second active cysteine that repairs methylphosphotriesters (MePTs). The transfer of a methyl group from MePT DNA lesions to Cys-69 of *Ada* provides a signal for the induction of *Ada* expression (8); the methylated *Ada* MTase becomes a positive transcriptional regulator and elevates transcription of its own gene and three other genes, namely *alkA*, *alkB* and *aidB*. *alkA* encodes a 3MeA DNA glycosylase, *alkB* (located on the same operon as *ada*) provides MMS resistance, and the function of *aidB* is currently unknown (2).

Mammals appear to express a single MTase which repairs O⁶MeG (9) and which may have a very low affinity for O⁴MeT (10,11). MTase levels in mammalian cells are tissue and cell type specific, e.g., human liver contains much higher MTase level than brain (12). Interestingly, about 60% of virus transformed cell lines and 20% of other human tumour cells are deficient in MTase activity (termed Mer⁻ or Mex⁻), and are very sensitive to alkylation induced mutation and killing (13). However these Mer⁻ cell lines still contain an intact *MGMT* gene encoding the mammalian MTase (14–16), and the absence of MTase activity in Mer⁻ cells appears to be achieved by switching off *MGMT* transcription (17). This transcriptional regulation is thought to

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involve alterations in methylation of the *MGMT* gene (18). Furthermore, *MGMT* down-regulation is often accompanied by the down-regulation of a number of other gene products (19) and the coordinate regulation of several genes in *Mer⁻* cells makes it difficult to deduce the exact biological function of mammalian MTase.

We recently described the molecular cloning of a *Saccharomyces cerevisiae* O⁶MeG DNA repair MTase gene (*MGT1*) by its ability to suppress the alkylation sensitivity of MTase deficient *ada⁻ ogt⁻ E.coli* (20). The yeast genomic library was made by inserting yeast DNA fragments in the polylinker of *E.coli* expression vector pUC19. The *MGT1* clone was later found to be fused to the *lacZ* coding region of pUC19 at 5' terminus and its expression was thus controlled by the *lacZ* promoter. A *mgt1* null mutant was created by homologous recombination between the yeast genome and an *in vitro* disrupted *MGT1* allele, to produce a eukaryotic strain lacking MTase activity in a well defined genetic background. Like MTase deficient strains of *E.coli* and mammalian cells, the yeast *mgt1* mutant was very sensitive to alkylation-induced killing and mutation (20). Here we describe the isolation and sequencing of the full length *S.cerevisiae* *MGT1* gene and its promoter (GenBank accession number M94227). The cloned sequences were used to generate *lacZ* fusions and constructs for regulated *MGT1* expression, and with these we determined the following: (i) the presence of an upstream repression sequence in the *MGT1* promoter; (ii) the role of a putative nuclear localization sequence at the N-terminus of the predicted full length *MGT1* polypeptide; (iii) the ability of different levels of the *MGT1* MTase to prevent alkylation-induced cell killing and mutation; (iv) the *MGT1* MTase limits spontaneous mutation in *S.cerevisiae*.

MATERIALS AND METHODS

Strains and plasmids

E.coli strain GWR111, a MTase deficient mutant (*ada⁻ ogt⁻*) of AB1157, was constructed by Rebeck and Samson (6) and used as recipient to test some yeast MTase gene clones. *E.coli* DH5 α was purchased from Bethesda Research Laboratory (Bethesda, MD, USA) and used for the preparation of plasmid DNAs.

S.cerevisiae strain DBY747 (*mata, ura3-52, his3- Δ 1, leu2-3,112, trp1-289*) was obtained from E.Eisenstadt (Office of Naval Research, Arlington, VA, USA); *S.cerevisiae* FY86 (*mata, his3 200, ura3-52, leu2- Δ 1, GAL⁺*) and FY40 (*mata, ura3-52, trp1- Δ 63, leu2- Δ 1, GAL⁺*) were gifts from F.Winston (Harvard Medical School, Boston, USA) and used for galactose induction of the *GAL* promoter. WX9102, an *mgt1* deletion mutant derivative of FY40, was created by gene disruption using a Δ *mgt1::LEU2* cassette from plasmid p Δ *mgt1::LEU2* (Fig.1). p Δ *mgt1::LEU2* was constructed by replacing a 0.75-kb *Bgl*III fragment containing the carboxyl terminal *MGT1* coding region (including the potential methyl receptor Cys-169) and some sequences downstream of *MGT1* with a DNA fragment containing the *LEU2* gene. The Δ *mgt1::LEU2* cassette was obtained by *Hind*III digestion of p Δ *mgt1::LEU2* and used to replace genomic *MGT1* by homologous recombination. WX1991 was a *mgt1* derivative of DBY747 constructed in the same way as WX9102 (20). The yeast cells were grown in complete YPD or synthetic SD medium supplemented with necessary nutrients (21).

Plasmids pTZ18R was purchased from Pharmacia LKB (Piscataway, NJ, USA). The yeast expression vector pYES2.0 (2 μ m-*STB*, *URA3*, *P_{GAL1}*, *T_{CYC1}*) was purchased from Invitrogen (San Diego, CA, USA).

Yeast transformation

A lithium acetate method (22) was used for yeast transformation. For targeted DNA integration, the plasmid DNA was digested with the appropriate restriction enzymes to generate recombinogenic ends, purified and used for transformation. Positive colonies were streaked once on selective plates and total yeast DNA was extracted (23) for Southern hybridization to confirm the integrant structure.

Yeast cell extracts and MTase assay

Yeast cell extracts were prepared in a MTase buffer (50 mM Hepes, pH7.8, 10 mM DTT, 1 mM EDTA and 5% glycerol) as described previously (24). For the MTase gel assay, yeast cell extracts were incubated with 6.3 μ g O⁶-[³H]-MeG DNA substrate (260 cpm/ μ g DNA) at 25°C for 1 hr and proteins were separated in a 12% polyacrylamide-SDS gel. The substrate was made by labelling *Micrococcus luteus* DNA with [³H]methyl nitrosourea (10 Ci/mmol; Amersham International) and then enriched for O-alkyl lesions as described (25). The gel was sliced into 2 mm slices (containing different molecular size proteins) and each slice was incubated in 10 ml of ScintiLene (Fisher, Pittsburg, PA, USA) plus 5% (v/v) Protosol (New England Nuclear, Boston, MA, USA) at 55°C overnight and the radioactivity was measured by scintillation counting.

For the rapid MTase assay, various amounts of crude cell extract were incubated with 4.2 μ g O⁶-[³H]-MeG DNA substrate at 25°C for 1 hr; samples were adjusted to 1–4 mg of total protein by adding bovine serum albumin (BSA) to the reaction mixture. After hydrolyzing DNA at 70°C for 1 hr in 1 M perchloric acid (PCA), total protein was precipitated by centrifugation, washed twice with 1 M of PCA and resuspended in 0.4 ml of 10 mM NaOH for scintillation counting. MTase activity was measured at several protein levels to establish the linear range.

Cell killing and mutagenesis

For both cell killing and mutagenesis studies, yeast cells were cultured in SD selective media at 30°C overnight and then used to inoculate YPD at 1:25 dilution. Incubation was continued for 4–6 hrs to reach 2 $\times$ 10⁷ cells/ml. For galactose induction, the SD overnight culture was harvested by centrifugation, washed with sterile distilled water and resuspended in the same volume of synthetic medium plus 2% potassium acetate and 2% glycerol as carbon source. After 2 hr, the culture was used to inoculate YP galactose (YPG) medium and incubation continued to reach 2 $\times$ 10⁷ cells/ml.

For the killing assay, cells were treated with 30 μ g/ml MNNG for various times, washed, diluted, and plated onto YPD or YPG; colonies were counted after 2 days at 30°C. For the mutagenesis assay, cells were incubated for 15 min in various doses of MNNG, washed, resuspended in sterile distilled water, diluted, and plated onto YPD or YPG for total surviving cells and onto SD or SG plus 40 μ g/ml canavanine to score for canavanine resistant mutants (26).

DNA sequencing

Plasmid DNA was purified by CsCl-EtBr gradient centrifugation and used as template for double-stranded DNA sequencing. Sequencing was by dideoxy termination method (27) using a T7 polymerase DNA sequencing kit (Pharmacia LKB). Standard and synthesized oligonucleotides were used as primers.

Fig. 1. Strategy for the cloning of the entire *MGT1* gene. (A) A diagram of plasmid pΔ*mgt1*::*LEU2*, which was constructed by cloning a 2.7-kb *Bgl*III fragment from YEp13 containing *LEU2* into, and replacing the 0.75-kb *Bgl*III fragment of, pUC11-3 (20). This plasmid served two purposes: to generate an *mgt1* null mutation (by *Hind*III digestion and using the Δ*mgt1*::*LEU2* cassette to replace the endogenous *MGT1*) and to clone *MGT1* upstream sequence (see Fig. 1B). Single line (including *ori* and *Amp*^R), pUC19 backbone; open box, sequence from *LEU2* locus of YEp13; solid box, *MGT1* sequence; and dotted box, yeast DNA sequence at *MGT1* locus. (B) Strategy of integrative transplacement and chromosome walking at *MGT1* locus. Plasmid pΔ*mgt1*::*LEU2* was integrated into *MGT1* locus in Chromosome IV by *Stu*I (S) directed homologous recombination. The intergrat structure was arranged in the genome so that digestion of the total genomic DNA with *Xba*I (X) will generate a fragment containing the pUC19 backbone (single line) and the 5.3-kb yeast genomic DNA (same size as in pUC11-3) plus upstream of *MGT1* region until the first *Xba*I site. This fragment was cloned by DNA ligation and *E. coli* transformation. (∇) indicates the truncated *MGT1* in pΔ*mgt1*::*LEU2*.

Oligonucleotide synthesis and polymerase chain reaction (PCR)

Oligonucleotides used for DNA sequencing and PCR were synthesized in a Biosearch 8700 DNA Synthesizer. To clone *MGT1* alleles containing the upstream and downstream ATGs (*MGT1L*), or only the downstream ATG (*MGT1S*), three oligonucleotides were synthesized according to 5' and 3' sequences of *MGT1* plus a restriction site to facilitate the cloning. They are: P1, 5'-CCGGATCCGGTGGAAACAAGGAAGA-3' (5' *MGT1L*); P2, 5'-CCGGATCACCGGTCGCATTTTGTATC-3' (5' *MGT1S*); and P3, 5'-CCGTCGACGCCAGCCATAT-TGTAAC-3' (3' *MGT1*). Standard PCR protocols (28) were followed for DNA amplification.

Plasmid construction

Plasmids pGAL1-*MGT1L* and pGAL1-*MGT1S* were made by cloning *MGT1L* and *MGT1S* PCR products (digested by *Bam*HI and *Sal*I) into the *Bam*HI-*Xho*I sites of pYES2.0. For the *MGT1-lacZ* fusion and upstream deletion analyses, the 1.2-kb *Spe*I-*Bgl*III fragment containing 2/3 of the *MGT1* coding region plus 718-bp upstream region (Fig. 2) was cloned into the *Xba*I-*Bam*HI sites of YEp356 (29) so that *lacZ* is fused in-frame with *MGT1* (pWX1142). The 0.9-kb *Pst*I-*Hind*III (−718 to +167) fragment of *MGT1* from pWX1142 was cloned into YEp357 (29) in-frame with *lacZ* to form pWX1152. pWX1151 and pWX1156 were obtained by partial and complete *Eco*RI digestion of pWX1151 (Fig. 3).

β-galactosidase assay

Yeast cells containing various *MGT1-lacZ* fusion genes were grown overnight in SD selective medium and 0.5 ml cells were used to inoculate 2.5 ml of SD. After 6 hr incubation, 1 ml of culture was used to determine cell density at OD_{600nm}, and 2 ml was used for β-galactosidase assay. Cells were harvested, resuspended in 1 ml of Z buffer (30), then permeabilized by the addition of 50 μl of 1% SDS and 50 μl of chloroform and

vortexing at top speed for 10 seconds. 0.2 ml of 4% ortho-nitrophenyl-β-galactoside (ONPG) was added to the permeabilized cells which were incubated at 28°C for 20 min and the reaction was stopped by adding 0.5 ml of 1 M Na₂CO₃. Cell debris was removed by centrifugation before measuring the OD at 420 nm. β-galactosidase activity was expressed as Miller units (30).

Fluctuation test

Fluctuation test (31) was used to measure the spontaneous mutation rate in *S. cerevisiae*. Overnight cultured yeast cells were used to inoculate SD medium (21) limited in tryptophan (1.5 μM) to approximately 4,000 cells per ml. Other required amino acids and nucleoside were supplemented in excess. The culture was divided at 1 ml per well using 24-well tissue culture plates (Costar) to a total of 240 wells for each treatment. The plates were sealed and incubated at 30°C for 11 days before counting wells that did not contain colonies. At this stage, approximately half of the wells contain colonies in the *mgt1* mutant strain and final cell density in unreverted wells was about 10⁷ cells. The spontaneous mutation rate was calculated as described (31).

RESULTS

Cloning the entire *S. cerevisiae* *MGT1* coding sequence and its promoter

We previously described the molecular cloning of the yeast *MGT1* gene by functional complementation of *ada*[−] *ogt*[−] MTase deficient *E. coli*. The cloned *MGT1* coding sequence was in-frame with the *lacZ* translation start codon in the pUC19 plasmid and *MGT1* expression was driven by the *lacZ* promoter (20). We noted an in-frame ATG near the 5' end of the yeast DNA fragment containing *MGT1* and suggested that this could be the translation start codon used in *S. cerevisiae* because the predicted molecular size of *MGT1* MTase (21.5 kDa) agreed with that previously reported (24). In order to determine whether this ATG

Fig. 2. (A) Restriction map of the 11.3-kb yeast chromosome IV genome at *MGT1* locus. The entire DNA fragment was cloned in plasmid pMGT1-Xb (see Fig.1 and results for the strategy of cloning). Restriction sites: B, *Bgl*III; E, *Eco*RI; H, *Hind*III; HII, *Hinc*II; P, *Pst*I; S, *Sal*I; Sp, *Spe*I and X, *Xba*I. Bracketed restriction sites indicate unsolved location. The *MGT1* coding region is indicated by an arrow. **(B)** Complete DNA sequence of *MGT1*. The *MGT1* upstream sequence contains several potential TATA boxes (underlined) and a consensus sequence, GTGGAGGC (sandwiched). The additional 18 amino acids at N-terminus and their coding sequence are in boldface as is the presumed active site cysteine residue. Location of three primers (P1, P2 and P3) used for *MGT1* and *MGT1S* PCR cloning and certain restriction sites are underlined and labelled.

was indeed the authentic start codon, we cloned the entire *S.cerevisiae* *MGT1* coding sequence plus its promoter. To do this, we applied the strategy of integrative transplacement (32) and chromosome walking. The plasmid pΔmgt1::LEU2 (Fig.1A) contains a *LEU2* gene replacing the 3' end of the *MGT1* gene that was originally cloned in plasmid pUC11-3 (20); the *LEU2* insert in pΔmgt1::LEU2 is flanked by yeast genomic DNA from around the *MGT1* locus. As illustrated in Fig.1B, pΔmgt1::LEU2 was integrated into the DBY747 *S.cerevisiae* genome at the *MGT1* locus by *Stu*I site-directed integration (33) and the integrants were selected as leucine prototrophs. Total genomic DNA from the integrant strain was digested by *Xba*I, the fragments were self-ligated and used to transform *E.coli*. Plasmids from ampicillin resistant colonies were expected to contain the pUC19 backbone (from pΔmgt1::LEU2) and the host

Fig. 3. Upstream deletion analysis of the *MGT1* promoter using the *MGT1-lacZ* fusion. Construction of the deletion plasmids is described in Materials and Methods. The open bar represents the *MGT1* promoter fragments and the hatched bar the *lacZ* gene. The two *Eco*RI sites (E) are located at -199 and -480 upstream of first *MGT1* translation start site (Fig.2). β -galactosidase activity in log phase FY86 cells containing each plasmid was measured in Miller units (30).

MGT1 coding sequence plus the 5' region up to the first upstream *Xba*I site, which turned out to be located about 6 kb from the 5' end of *MGT1*. A restriction map of the entire 11.3-kb yeast genomic DNA encompassing the *MGT1* locus is shown in Fig.2A. The *Xba*I clone was named pMGT1-Xb and used for subsequent studies.

DNA sequence of the *MGT1* gene and its upstream region

The intact *MGT1* gene and its upstream sequence was determined from pMGT1-Xb and is shown in Fig.2B. Unexpectedly, sequencing of the pMGT1-Xb insert revealed an in-frame ATG site upstream of the putative ATG start codon previously identified (20). The DNA sequence of the *MGT1* ORF originally cloned in pUC11-3 was identical to the equivalent region in pMGT1-Xb (Fig.2B), indicating that the MNNG selection used to isolate the *MGT1* gene did not produce any mutations in this sequence. If *MGT1* is translated from the upstream ATG, 18 amino acids and about 2 kDa would be added to the 188 amino acid, 21.5 kDa *MGT1* protein previously described (Fig.2B, bold face); this difference would not be detected in our MTase gel assay. We have renumbered the *MGT1* nucleotides with the upstream ATG at +1, 2 and 3, since there is an in-frame TAG stop codon immediately 5' to this upstream ATG (Fig.2B). It is currently unclear which ATG is the true translational start site and so the two possible *MGT1* gene products have been called *MGT1S* and *MGT1L* for the short and long polypeptides, respectively. Interestingly, the putative 18 additional amino acid residues at the N-terminus contain a highly basic domain with the sequence His-Lys-Lys-Lys found to serve as a nuclear localization signal (NLS) in several eukaryotic proteins (34).

Previous studies of MTase activity (24) and Northern hybridization (20) indicate that *MGT1* is expressed at a very low level in *S.cerevisiae*. It was therefore not surprising to find that the codon usage of *MGT1* is extremely low (0.128 for *MGT1L* and 0.127 for *MGT1S*) and that the *MGT1* promoter does not contain a long stretch of d(A-T) known to support high levels of constitutive expression (35). However, we were surprised to identify the octanucleotide sequence 5'-GTGGAGGC-3' at -209, because the same sequence is present in the promoter of the *RAD2* gene (36,37) whose product is involved in the excision repair of DNA damage produced by ionizing radiation. Similar sequences are also present upstream of at least four other yeast DNA repair genes, namely *RAD4* (38,39), *RAD10* (40), *PHR1* (41), *MAG* (Xiao and Samson, in preparation) and the ribonucleotide reductase small subunit gene, *RNR2* (42,43). These

Fig. 4. Yeast MTase activity assay. (A) Gel electrophoretic assay of MTase activity of crude cell extracts from FY40 (wild type) and the WX9102 (*mgt1*) transformants growing in galactose medium. 0.4 mg of total protein was used for WX9102/pGAL1-MGT1L (●) and WX9102/pGAL1-MGT1S (○) transformants and 2 mg of protein was used for FY40 (▲) and WX9102/pYES2.0 transformant (□). Slice 0 is the origin of electrophoresis. Molecular size was determined by Rainbow protein molecular weight markers (Amersham); the MTase peak corresponds to 24–25 kDa. (B) Rapid MTase assay of wild type (FY40), *mgt1* mutant (WX9102/pYES2.0), WX9102/pGAL1-MGT1S and WX9102/pGAL1-MGT1L in glucose (YPD, open bars) and galactose (YPG, hatched bars) media. Several protein concentrations were assayed for each sample to determine the linear range of MTase activity. MTase activity (cpm transferred per mg protein) in galactose medium was an average from three experiments: wild type, 47.8 ± 15.8 ; *MGT1S*, 1976 ± 413 ; *MGT1L*, 1610 ± 297 . MTase activity (cpm transferred per mg protein) in glucose medium was an average from two experiments: wild type, 118.1 ± 1.1 ; *MGT1S*, 27.8 ± 6.0 ; *MGT1L*, 49.3 ± 6.6 . The MTase activities of *MGT1* transformants in the gel assay might reach the saturation point and were less quantitative.

seven octanucleotide regions share the consensus sequence $G^A/TGG^A/TNG^A/C$. The transcription of *MAG* (44), *RAD2* (45) *PHR1* (46) and *RNR2* (43,47) has been shown to be induced in response to DNA damage. We have previously shown that *MGT1* was not induced by alkylating agents (20,24). However, the consensus sequence shared between *MGT1* and *MAG*, which codes for a yeast 3MeA DNA glycosylase specific for the repair of DNA alkylation damage, and the observation by Chen and Samson (48) that *MAG* is induced by UV and 4-nitroquinoline-1-oxide in addition to alkylating agents led us to examine whether or not *MGT1* can be induced by other DNA damaging agents. We found that the *MGT1-lacZ* fusion containing 719-bp *MGT1* upstream sequence (e.g. pWX1152, Fig.3) was not induced by either MMS or UV light (data not shown).

The *MGT1* promoter contains an upstream repression sequence (URS)

Two upstream deletions were made from the *MGT1-lacZ* construct pWX1152 to form pWX1156 (–480) and pWX1151 (–199) (see Materials and Methods, Fig.3). The wild type *S.cerevisiae* strain FY86 was transformed with the three constructs and β -galactosidase activity was determined. As can be seen from Fig.3, the deletion up to –480 had little effect on *MGT1-lacZ* expression, but the deletion to –199 dramatically increased *MGT1-lacZ* expression. This result indicates the presence of a URS in the –480 to –199 region. Note that the $G^A/TGG^A/TNG^A/C$ consensus sequence resides in this region, i.e. –209 5'-GTGGAGGC-3' and may be the candidate URS. A more detailed analysis of the *MGT1* promoter is in progress.

Fig. 5. The effect of *MGT1S* and *MGT1L* expression on the alkylation sensitivity of *S.cerevisiae mgt1* mutants. (A) MNNG induced mutation (15 min treatment) in YPG. (B) MNNG-induced (30 µg/ml) killing in YPG. (C) MNNG-induced mutation (15 min treatment) in YPD. (D) MNNG-induced (30 µg/ml) killing in YPD. (□) WX9102/pYES2.0; (●) WX9102/pGAL1-MGT1L and (○) WX9102/pGAL1-MGT1S.

Expression of *MGT1L* and *MGT1S* in *E.coli* and yeast MTase deficient mutants

To study the biological functions of *MGT1*, we amplified *MGT1L* and *MGT1S* by PCR and cloned each of them into bacterial and yeast expression vectors. *MGT1L* and *MGT1S* were expressed equally well in *E.coli* and protected *ada⁻ ogt⁻* cells from killing by MNNG to similar levels (data not shown). Thus the *MGT1L* and *MGT1S* genes produce MTases that appear to be equally stable, at least in *E.coli* cells. To determine whether the additional 18 amino acids in *MGT1L* are essential for *in vivo* function in *S.cerevisiae*, *MGT1L* and *MGT1S* were cloned into the yeast 2µm expression vector pYES2.0 under the control of the *GAL1* promoter and the plasmids were introduced into the *GAL⁺ mgt1* *S.cerevisiae* strain, WX9102. Since eukaryotic translation normally starts at the first in-frame AUG of mRNA (49), and translation initiation in *S.cerevisiae* has no strong preference to the AUG context (50), we assume that the *MGT1L* and *MGT1S* clones will produce the expected MTases. Both the MTase gel assay (Fig.4A) and the rapid MTase assay (Fig.4B) showed that, in the presence of galactose, *MGT1L* and *MGT1S* were overexpressed from the *GAL1* promoter (about 40 fold the wild type MTase level estimated from quantitative rapid assay), whereas the *mgt1* mutant strain with pYES2.0 vector alone had no detectable MTase activity.

If the putative NLS (present only in *MGT1L*) were required for *MGT1* entry into the nucleus, one would expect *MGT1L*,

Fig. 6. (A) Mutagenesis assay of WX9102/pGAL1-MGT1L transformant. Cells were treated with MNNG at indicated doses in YPD (○) and YPGal (●) media for 15 min. (B) MNNG-induced (30 μg/ml) cell killing. (Δ) DBY747; (▲) DBY747(*mag-Δ1::URA3*) and (□) DBY747(*Δmgt1::LEU2*).

but not MGT1S, to protect *mgt1* mutant cells from alkylation-induced killing and mutation. However, upon induction by galactose, both *MGT1L* and *MGT1S* conferred dramatic resistance to MNNG induced mutation (Fig.5A) and killing (Fig.5B), suggesting that MGT1S without the N-terminal 18 amino acids can enter the nucleus and repair alkylated DNA.

Since proteins smaller than 40 kDa may travel across the nuclear membrane without a NLS (51), it was possible that the tremendous overexpression of *MGT1S* allows diffusion of enough MGT1S into nucleus to provide full protection against DNA alkylation damage. We therefore limited *P_{GAL1}-MGT1* expression by growing both *MGT1L* and *MGT1S* in glucose medium. Under these conditions, the *MGT1L* clone produced slightly more MTase activity than *MGT1S* clone, but both clones produced MTase levels lower than that of wild type cells, but higher than that of *mgt1* cells (Fig.4B). In glucose medium, *MGT1L* and *MGT1S* provided equal resistance to MNNG induced mutation (Fig.5C); surprisingly, the extent of resistance appeared to be almost as great as that observed in galactose medium (Fig.5A), where MTase levels are up to 100 fold higher (Fig.4B). However, if the MNNG challenge dose is increased, it is clear that galactose-induction of MTase activity provides extensive resistance to MNNG mutation (shown for *MGT1L*, Fig.6A). For killing, the resistance conferred by the *MGT1L* and *MGT1S* clones in glucose medium was significant (Fig.5D), but much less extensive than that seen in galactose (Fig.5B). Further, the *MGT1L* clone repeatedly provided slightly higher killing resistance than the *MGT1S* clone (Fig.5D), consistent with the production of slightly higher MTase levels. We infer that the 5' end of the *MGT1L* message or the amino terminus of MGT1L protein may influence MTase levels. The slightly higher MTase levels produced from the *MGT1L* clone (Fig.4B) may be due to a more stable mRNA or a more stable polypeptide; alternatively the NLS may support slightly more efficient entry into the nucleus and result in a slower MTase turnover.

Finally we compared the *mgt1* strain to another MNNG-sensitive yeast strain, namely the *S.cerevisiae mag* mutant which is deficient in 3MeA DNA repair glycosylase (44). Although both *mag* and *mgt1* mutants are very sensitive to MNNG-induced killing, compared to wild type strains, the MTase deficient mutant

Table 1. Spontaneous mutation rates

Strains	Expt 1 ^b	Mutations (10 ⁻⁸)/cell/generation ^a		
		Expt 2	Expt 3	Average
DBY747 (<i>MGT1</i>)	1.69	1.5	1.41	1.53 ± 0.14
WX1991 (<i>mgt1</i>)	5.03	5.55	4.48	5.02 ± 0.5

^a Spontaneous mutation rates were determined by fluctuation test as described in (31).

^b For each experiment mutation in 240 separate cultures was monitored for each strain (see Materials and Methods).

is much more sensitive than the glycosylase deficient strain (Fig.6B).

MTase deficient *S.cerevisiae* have an increased spontaneous mutation rate

DNA repair MTase deficient *E.coli* strains have an elevated spontaneous mutation rate, presumably because MTase repairs endogenous DNA alkylation damage (6). To determine whether the *MGT1* MTase provides such a function in yeast cells, we measured spontaneous mutation rates in wild type and *mgt1* cells. DBY747 contains an Amber mutation allele *trp1*-289 that can be reverted to Trp⁺ either by point mutations in the *trp1* gene or by other Amber suppressor mutations. Three independent fluctuation tests (Table 1) consistently showed that the MTase deficient strain (WX1991) has about a three-fold higher spontaneous mutation rate than the isogenic parental strain (DBY747). We infer that one of the biological functions of the *MGT1* gene is to limit spontaneous mutation.

DISCUSSION

Four eukaryotic O⁶MeG DNA MTases genes have been cloned to date, namely the human (14–16), rat (52) and mouse (53) *MGMT* genes, and the *S.cerevisiae MGT1* gene (20). In principle, each of these cloned genes could be disrupted *in vitro* by selectable genetic markers, and used to generate MTase null mutants via homologous recombination in the appropriate cells. In practice, this is much easier to do in haploid yeast cells than in diploid mammalian cells, and we recently generated a null MTase (*mgt1*) strain of *S.cerevisiae* (20). Here we describe the use of this null MTase mutant and the newly cloned full length *MGT1* gene to further define the *in vivo* roles of the O⁶MeG DNA repair MTase in eukaryotes.

The fact that all organisms have been found to express MTases and glycosylases for the repair of O-alkyl and N-alkyl DNA damage suggests that the pressure to maintain an efficient means of DNA alkylation repair is high and that alkylating agents are commonly encountered. Indeed, we recently described *in vivo* evidence that *E.coli* contains natural metabolites that continually alkylate DNA. *E.coli* strains devoid of DNA repair MTase activity have an elevated rate of spontaneous mutation (6), and, characteristic of alkylation-induced mutation, the vast majority of the excess mutations appear to be G:C to A:T transitions which are presumably induced by unrepaired O⁶-alkylguanine (W.Mackay and L.Samson, in preparation). Similarly, we now find that *S.cerevisiae* MTase null mutants display an elevated rate of spontaneous mutation, indicating that eukaryotic cells also contain natural metabolites that alkylate DNA, and that DNA repair MTases limit spontaneous mutation rates in eukaryotic cells. We are currently investigating whether the extra

spontaneous mutations in MTase deficient yeast cells are mainly G:C to A:T transitions, as they are in MTase deficient *E. coli*. We do not yet know which metabolites are responsible for 'spontaneous alkylation' in *E. coli* and *S. cerevisiae*, but several possibilities have been suggested, namely *S*-adenosylmethionine, alkyl radicals and nitrosamines. *S*-adenosylmethionine is an efficient methyl donor for the enzymatic methylation of DNA, RNA and protein, and has been shown to alkylate DNA *in vitro*, under physiological conditions (54–56). Lipid peroxidation reactions generate alkyl radicals capable of alkylating DNA (57) and the endogenous nitrosation of amines generates reactive nitrosamine species also capable of alkylating DNA (58,59). It is perhaps because of endogenous cellular alkylating agents such as these that specific DNA alkylation repair functions have been found in all organisms examined to date.

In addition to limiting spontaneous and MNNG-induced mutation, the *S. cerevisiae* MGT1 MTase provides tremendous resistance to MNNG-induced cell killing. In fact, *mgt1* MTase deficient yeast mutants are much more sensitive to killing by MNNG than *mag* 3MeA DNA glycosylase deficient mutants. At first sight this result was very surprising because MNNG produces roughly equal quantities of O⁶MeG and 3MeA DNA lesions (60), and because, at least in *E. coli*, 3MeA is considered to be a potent killing lesion and O⁶MeG a weak killing lesion (2,6). However, while *mgt1* mutants express no detectable MTase activity, *mag* mutants express about 10% the wild type level of 3MeA DNA glycosylase activity (44). It is therefore possible that *S. cerevisiae* completely deficient in 3MeA repair would be more sensitive than *mgt1* cells to alkylation-induced killing. 3MeA is believed to cause cell death by inhibiting DNA replication (2) but the mechanism by which O⁶MeG causes cell death is not yet clear. Previously (6), we proposed that cell death might result from some fraction of the O⁶MeG-induced mutations being lethal, or from O⁶MeG lesions in certain regions of the genome inhibiting DNA replication; alternatively, the repair of O⁶MeG by MTase may somehow signal the induction of other pathways that prevent alkylation-induced cell death. The mechanism by which unrepaired O⁶MeG cause cell death in *S. cerevisiae* remains to be determined, but it is quite clear that the resistance of yeast cells to both alkylation-induced killing and mutation, correlates with the cellular level of O⁶MeG DNA MTase.

MGT1-mediated protection of *S. cerevisiae* against killing and mutation is probably achieved directly by O-alkyl DNA repair, and for this the MGT1 protein must enter the nucleus. However, as discussed, it remains formally possible that MGT1, like the *E. coli* Ada protein, regulates the expression of other genes whose products provide some of the protection, and this too might require MGT1 entry into the nucleus. Proteins under about 40 kDa apparently do not require NLSs to traverse the nuclear membrane (51). It was therefore surprising to find a typical NLS in the predicted N-terminal region of the 23.5 kDa MGT1 protein. However, MGT1S without this potential NLS turned out to be functional *in vivo*, even when the number of MGT1 molecules per cell was extremely low.

We have established that a *cis*-acting URS element lies within a defined region of the *MGT1* promoter. The most unexpected observation in this study was that this same region of the promoter contains an octanucleotide sequence that appears is common to several other yeast DNA repair and DNA metabolism genes, namely *MAG*, *RAD2*, *RAD4*, *RAD10*, *PHR1*, and *RNR2* (36–43). Upstream deletion analyses of *RNR2* (61) and *MAG* (WX and LS, manuscript in preparation) also defined small URS

regions, both of which contain the octanucleotide consensus sequence. These observations strongly suggest that a common URS element contributes to controlling the expression of a regulon involved in DNA repair and metabolism in *S. cerevisiae*.

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