Induction of *S.cerevisiae* MAG 3-methyladenine DNA glycosylase transcript levels in response to DNA damage

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ABSTRACT

We previously showed that the expression of the *Saccharomyces cerevisiae* MAG 3-methyladenine (3MeA) DNA glycosylase gene, like that of the *E. coli* *alkA* 3MeA DNA glycosylase gene, is induced by alkylating agents. Here we show that the MAG induction mechanism differs from that of *alkA*, at least in part, because MAG mRNA levels are not only induced by alkylating agents but also by UV light and the UV-mimetic agent 4-nitroquinoline-1-oxide. Unlike some other yeast DNA-damage-inducible genes, MAG expression is not induced by heat shock. The *S. cerevisiae* MGT1 O⁶-methylguanine DNA methyltransferase is not involved in regulating MAG gene expression since MAG is efficiently induced in a methyltransferase deficient strain; similarly, MAG glycosylase deficient strains and four other methylmethane sulfonate sensitive strains were normal for alkylation-induced MAG gene expression. However, *de novo* protein synthesis is required to elevate MAG mRNA levels because MAG induction was abolished in the presence of cycloheximide. MAG mRNA levels were equally well induced in cycling and G1-arrested cells, suggesting that MAG induction is not simply due to a redistribution of cells into a part of the cell cycle which happens to express MAG at high levels, and that the inhibition of DNA synthesis does not act as the inducing signal.

INTRODUCTION

The genome of every organism is exposed to numerous natural and synthetic agents that damage DNA and the expression of certain sets of prokaryotic and eukaryotic genes can be induced in response to that DNA damage (1–4). Some of these DNA-damage-inducible gene products are involved in DNA repair and so protect cells against mutation and cell death. To date, three DNA-damage-inducible responses have been characterized in *E. coli*, namely the SOS response (4), the adaptive response to alkylation damage (2, 5) and the adaptive response to oxidative damage (3). The inducing signal for the SOS response seems

to be single-stranded regions of DNA which activate RecA to facilitate the proteolytic cleavage of the transcriptional repressor of the SOS genes, namely LexA; at least 17 genes are induced and some of the products are involved in DNA repair, mutagenesis, recombination and filamentation. The adaptive response to alkylating agents is induced by sublethal levels of DNA alkylation damage; at least four genes (*ada*, *alkA*, *alkB* and *aidB*) are induced, and their products provide protection against the killing and mutagenic effect of further alkylation damage (2,5). Ada is a DNA methyltransferase (MTase) which transfers methyl groups from O⁶-methylguanine (O⁶MeG) and O⁴-methylthymine (O⁴MeT) to Cys-321 of the Ada polypeptide, and from methylphosphotriester (MePT) lesions to Cys-69; O⁶MeG and O⁴MeT repair protects cell from mutations caused by base mispairing during replication and MePT repair converts the Ada protein into a transcriptional activator for the *ada-alkB* operon and the *alkA* and *aidB* genes (2). Thus, MePTs serve as the inducing signal and Ada serves as the sensor and effector for the induction of this adaptive response (6). The functions of AlkB and AidB are not known, but AlkA is a 3-methyladenine (3MeA) DNA glycosylase that repairs 3MeA, 3-methylguanine (3MeG), O²-methylthymine (O²MeT) and O²-methylcytosine (O²MeC) lesions and specifically protects cells from alkylation-induced killing (7,8). The adaptive response to oxidative damage involves the induction of a considerable number of genes regulated by the *oxyR*, *soxR* and *katF* genes, depending on the oxidative stress signal and cell growth conditions (3). The OxyR protein itself senses the hydrogen peroxide and oxidized OxyR becomes a transcriptional activator of nine genes including *katG* and *ahpC* (9). Whether SoxR and KatF also act as sensors of oxidative damage and/or transcriptional activators is not yet known. The SOS response, the adaptive response to alkylating agents and the adaptive responses to oxidative damage are induced by different signals and controlled by different regulators. Whether distinct sets of DNA-damage inducible genes exist in eukaryotes is not yet clear.

In *S. cerevisiae*, DNA-damage-inducible genes have been identified in several ways. Ten genes were directly identified as DNA-damage-inducible using *lacZ* operon fusions and differential nucleic acid hybridization (10–12) and the expression of a

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number of known DNA repair genes (*RAD2*, *RAD7*, *RAD18*, *RAD23*, *RAD54* and *PHR1*) was shown to be induced by UV (13–18). Further, several genes involved in nucleotide and DNA metabolism and protein modification (*CDC9*, *POLI*, *RNR2*, *RNR3* and *UBI4*) were also found to be induced by UV (19–23). Despite the identification of so many DNA damage-inducible genes, little is known about the mechanism of their induction or the biological effects of their inducibility.

We recently identified and isolated from *S. cerevisiae* a 3MeA DNA glycosylase gene (*MAG*) (24). The *MAG* glycosylase shares significant amino acid homology with the *E. coli* AlkA glycosylase, and they each protect cells against alkylation-induced cell death but not mutation (7, 8, 25). The level of *MAG* mRNA, like that of *alkA*, is dramatically increased when yeast cells are exposed to alkylating agents (25). Here we further characterize the process of inducing *MAG* expression and show that it is quite different from *alkA* gene induction in *E. coli*.

MATERIALS AND METHODS

Yeast strains

S. cerevisiae haploid strains were: DBY747 (a, *his*- Δ -1, *leu*2-3, *leu*2-112, *trp*1-289, *ura*3-52) and its alkylation sensitive derivatives WX1991 (*mgt1::LEU*) and JC9005 (*mag-2::URA3*) (generated in this lab); 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*) (26) (gifts from Louise Prakash, University of Rochester, Rochester, NY); RC634 (a *sst1-3*, *rme1*, *ade2*, *his6*, *met1*, *ura1*, *can1*, *cyh2*) was obtained from Ronald Davis (Stanford University, Stanford, CA).

Northern blot hybridization

Northern blot hybridization was performed as described previously (25). Briefly, 25 μ g of yeast total RNA was separated by electrophoresis in a formaldehyde/1% agarose gel, transferred to a nylon membrane and probed with 32 P-labelled DNA. RNA blots were washed at high stringency (30mM sodium chloride/3 mM sodium citrate at 58°C for 2h).

DNA damage and heat shock treatments

Chemical DNA damaging agents were added directly to log phase cells (1×10^7 /ml) growing in YPD medium (yeast extract 1%/peptone 2%/dextrose 2%) at 30°C and left in for the duration of the assay. For UV, log phase cells were washed once in water, resuspended in 5 ml of water and exposed to a UV fluency of 1.6 J/m²sec while shaking the cells in a petri dish to minimize the effects of shielding. After irradiation, cells were grown at 30°C in the dark to avoid photoreactivation. For the heat shock, cells were grown at 23°C in YPD, then resuspended in 37°C YPD and grown at 37°C for various times. Control samples were treated similarly to experimental samples except for DNA damage or heat shock treatment.

Cell cycle arrest

Strain RC634 was used to determine whether the cell cycle stage affects *MAG* induction. RC634 is sensitive to α factor because the *sst1* mutation eliminates an α -factor protease. α -factor (300ng/ml), which arrests cells in G1 (27), was added to log phase cells and after 3 hrs 99% of the cells exhibited 'schmooing' morphology, the characteristic elongated shape which indicates

Figure 1. Dose response of *MAG* mRNA accumulation after alkylation damage. Total RNA (15 μ g per lane) was isolated from wildtype yeast strain DBY747 that had been exposed to the indicated concentrations of MMS or MNNG for 30 min. The RNA was analyzed by Northern hybridization with *MAG* and *ACTIN* probes.

G1 arrest. RNAs were isolated from treated and control cells and subjected to Northern blot analysis as described previously (25).

Protein synthesis inhibition

To determine whether the induction of *MAG* by MMS was dependent on protein synthesis, cycloheximide (Sigma) was added to log phase cells to a final concentration of 100 μ g/ml and incubated at 30°C for 15 min. MMS was then added at 0, 0.025%, 0.05% and 0.1% to control and cycloheximide treated cells, and incubated at 30°C for 30 min. RNA was prepared from these cells and analyzed by Northern hybridization.

DNA glycosylase assay

MAG DNA glycosylase activity was measured as described previously (24). Briefly, cell extracts were incubated with 3 H-DMS-treated calf thymus DNA (1.2 Ci/mmol) for various periods. The reaction was stopped by ethanol precipitation and the alkylated bases released into the supernatant were separated by descending paper chromatography in 2-propanol/ammonium hydroxide/water, 7:1:2 (vol/vol). The dried chromatogram was sliced into 1-cm strips, eluted into 1 ml of water and assayed for tritium in a Beckman scintillation counter.

RESULTS

MAG mRNA levels increase in response to a variety of DNA damaging agents

The expression of the *MAG* gene in response to DNA damaging agents was studied quantitatively by Northern blot analysis. We previously reported that *MAG* transcripts accumulate with time in response to methyl methanesulfonate (MMS) and methyl-N-nitro-N'-nitrosoguanidine (MNNG) treatment (25). Here we show that the *MAG* mRNA level increases linearly with increasing MMS and MNNG doses (Figure 1); even the highest of these doses was relatively non-toxic, leaving more than 60% of the cells viable (data not shown). When cells were exposed to more toxic doses (0.3%, 0.5% and 1% MMS) the *MAG* mRNA levels decreased (data not shown).

Table 1. Induction of MAG by various DNA damaging agents

Agent	Dose	Time(min)	Fold increase
None	—		1 ^a
MMS	0.1%	(30)	11 ^a
MNNG	5 μ g/ml	(30)	6 ^b
4NQO	2 μ g/ml	(30)	5 ^c
UV	25J/m ²	(30)	3 ^c
Heat shock	23°C—37°C	(30, 60 and 120)	1 ^c

^a, ^b and ^c are the average of 7, 6 and 2 experiments respectively.

Figure 2. DNA glycosylase activity in *S. cerevisiae* cell-free extracts. 3MeA (left panel) and 7MeG (right panel) released during incubation with 74 μ g calf thymus DNA (911.6 d.p.m./ μ g) alkylated by ³H-DMS (1.2 Ci/mmol) at 25°C by 500 μ g of extracted proteins from DBY747 control (○) and pretreated with 0.05% MMS for 1 hr (●).

In *E. coli*, the expression of the *alkA* DNA glycosylase gene is induced by the alkylating agents MNNG and MMS and is not induced by UV (28–30). However, in *S. cerevisiae*, all the alkylation damage-inducible genes tested so far (namely, *DIN*, *DDR*, *RAD2*, *RAD54*, *PHR1*, *CDC9*, *RNR2*, *RNR3/DIN1*, *UBI4*) can also be induced by DNA damaging agents such as UV and 4-nitroquinoline-1-oxide (4NQO) (12, 13, 18, 19, 21–23, 25, 31). We therefore examined the specificity of the response of the *MAG* gene to various DNA damaging agents. Table 1 shows that, like other *S. cerevisiae* DNA-damage-inducible genes, the *MAG* gene is not exclusively induced by one type of agent, in this case alkylating agents, but it is also induced by UV and 4NQO. McClanahan and McEntee (32) reported that a number of *S. cerevisiae* damage-inducible genes are also induced by a brief heat shock at 37°C, suggesting that these genes respond to a general stress signal. However, no induction of the *MAG* transcript was observed in heat shocked cells shifted from 23°C to 37°C (Table 1), suggesting that the induction of *MAG* mRNA may be part of a DNA-damage stress response rather than a general stress response.

MAG DNA glycosylase activity is moderately induced in response to DNA damage

We determined whether the alkylation induction of *MAG* mRNA levels leads to an increase in *MAG* DNA glycosylase activity. Figure 2 (left panel) shows that exposure to 0.05% MMS for 1 hour, which induces *MAG* mRNA levels about 10 fold, increased the *MAG* 3MeA DNA glycosylase activity by about two fold; notably, under our assay conditions no increase in the

Figure 3. Induction of *MAG* transcripts in *mgt1::LEU2* mutants that deficient in methyltransferase activity. *mgt1::LEU2* mutants were exposed to the indicated concentrations of MMS for 30 min. RNA (25 μ g per lane) was isolated and analyzed by Northern hybridization with *MAG* and *ACTIN* probes.

rate of 7MeG release was observed (Figure 2 right panel) as one would expect from our previous determination that, under our assay conditions, the *MAG* glycosylase does not recognize this alkylated base (24). This 2 fold increase in *MAG* glycosylase activity is comparable to the 2–3 fold induction of 3MeA DNA glycosylase activity seen in mammalian cells exposed to various different DNA damaging agents (33). This moderate level of *MAG* enzyme induction (relative to the higher level of mRNA induction) is reminiscent of the response of *alkA* in *E. coli* where a 300 fold increase in transcription only produces about a 20 fold increase in glycosylase activity (28–30).

MAG transcript levels are not regulated by the yeast MGT1 methyltransferase

In *E. coli* the methylated Ada DNA MTase activates the transcription of the *alkA* gene (2). We recently identified, cloned and characterized a *S. cerevisiae* O6MeG/O4MeT DNA repair MTase and its gene *MGT1* (34–36). To test whether the induction of *MAG* mRNA levels in *S. cerevisiae* is regulated by the methylated *MGT1* methyltransferase, the alkylation inducibility of the *MAG* transcript was examined in yeast *mgt1* mutants deficient in DNA MTase activity (36). In the absence of the *MGT1* DNA MTase, the *MAG* mRNA level was efficiently induced by MMS (figure 3) and MNNG (36). We infer that the *MGT1* DNA MTase activity is not involved in the regulation of *MAG* gene expression.

Induction of MAG transcripts in various MMS sensitive mutants

More than twenty MMS sensitive yeast mutants were isolated by Prakash and Prakash (26). Among them, mutants from 5 complementation groups, *mms1*, *mms2*, *mms4*, *mms5* and *mms22*, are sensitive to MMS alone while the other mutants are also sensitive to radiation damage. We previously found the *mms5* mutant is deficient in *MAG* glycosylase activity and that *MAG*

Figure 4. Induction of *MAG* transcripts in MMS sensitive yeast mutants. The indicated yeast strains were exposed to MNNG (5 μ g/ml) for 30 min. RNA (25 μ g per lane) was isolated and analyzed by Northern hybridization with *MAG* and *ACTIN* probes.

Figure 5. Induction of *MAG* transcripts in *mms5* and *mag-2::URA3* mutants. wild type cells (○), *mms5* (●) and *mag-2::URA3* (▲) were exposed to 0% (lanes 1,5,9), 0.025% (lanes 2, 6, 10), 0.05% (lanes 3, 7, 11) and 0.10% (lanes 4, 8, 12) of MMS for 30 min. RNA was isolated and analyzed by Northern hybridization.

and *MMS5* are probably allelic (25). It seemed possible that one of the other four mutants may be specifically sensitive to MMS because it can not regulate the *MAG* gene in response to DNA damaging agents. We therefore examined whether *MAG* gene expression could be induced in these *mms* mutants, including *mms5* which is deficient in *MAG* glycosylase activity. All five *mms* mutants showed an increase in *MAG* transcript levels comparable to that of wildtype (figure 4), suggesting that none of these gene products are likely to be required for *MAG* gene induction.

Although *mms5* and *mag-2::URA3* mutants are deficient in *MAG* glycosylase activity, the level of *MAG* mRNA was still induced in response to MMS in a dose dependent manner with induction kinetics similar to that of wildtype (figure 5). These results indicate that the *MAG* 3MeA DNA glycosylase activity *per se* (i.e., the release of 3MeA and the production of abasic sites) plays no direct role in *MAG* gene induction.

Figure 6. Induction of *MAG* transcripts by MMS and MNNG in cycling and cells arrested in G1 by α -factor. Yeast strain RC634 (containing the *ssr1* mutation which eliminates a protease that normally degrades α -factor) was exposed to α -factor at a concentration of 300ng/ml for 3h whereupon 99% cells exhibited 'shmooing' morphology (G1 arrest). RNA from untreated RC634 cells, 0.05% MMS and 5 μ g/ml MNNG treated cells in the absence (left panel) and presence (right panel) of α -factor was analyzed by Northern hybridization with *MAG* and *ACTIN* probes.

Figure 7. The effect of cycloheximide on alkylation induction of the *MAG* transcript. RNA from wildtype yeast DBY747 with and without pretreatment of cycloheximide (100 μ g/ml) for 15 min and then exposed to 0% (lanes 1, 5), 0.025% (lanes 2, 6), 0.05% (lanes 3, 7) and 0.10% (lanes 4, 8) of MMS for 30 min in the presence (●) or absence (○) of cycloheximide analyzed by Northern hybridization with *MAG* and *ACTIN* probes.

***MAG* induction is primarily due to DNA damage rather than cell cycle arrest**

DNA damaging agents such as MMS can transiently arrest cells at the late S/early G2 boundary (37). Thus, one explanation for the accumulation of *MAG* mRNA in DNA damaged cells could be that the *MAG* gene is cell cycle regulated and its expression increases in S/G2 phase. To test whether the *MAG* gene is directly

induced by DNA damage, RNA was prepared from alkylation treated yeast cells arrested and maintained in G1 phase by α -factor (27). To confirm that the arrest of cells in G1 phase by α -factor was complete, the RNA blot was probed with the histone *H2A* gene, which is almost exclusively expressed in S phase (38); the *H2A* transcript was barely detectable in the G1 arrested cells and abundant in the cycling cells (data not shown). Figure 6 shows that the *MAG* mRNA levels increased in response to MMS and MNNG in G1-arrested cells, and that the increase in *MAG* transcript levels was very similar to that in cycling cells. Thus, induction of *MAG* expression does not appear to be a cell-cycle phenomenon but rather a direct response to DNA damage.

Induction of *MAG* transcripts depends on *de novo* protein synthesis

As a first step towards understanding the mechanism of *MAG* induction, we tested whether this induction requires *de novo* protein synthesis. Figure 7 shows that the inhibition of protein synthesis by cycloheximide had two effects; the basal level of the *MAG* transcript decreased and the induction of the *MAG* gene in response to MMS was totally abolished. We infer that cycloheximide either inhibits the synthesis of transcription factors responsible for the constitutive and induced transcription of the *MAG* gene, enzymes that modify these transcription factors, or proteins that stabilizes the *MAG* transcript.

DISCUSSION

Although more than a dozen DNA-damage-inducible genes have been identified in *S. cerevisiae*, little is known about their mechanism of induction. In contrast to various sets of *E. coli* damage-inducible genes which respond to specific kinds of DNA damaging agents, each of the *S. cerevisiae* DNA-damage-inducible genes studied so far appear to be induced by a wide variety of DNA damaging agents (10–14, 16–23). To further understand the regulation of DNA-damage-inducible eukaryotic genes, we have characterized the inducibility of a recently discovered *S. cerevisiae* DNA repair gene, namely the *MAG* 3MeA DNA glycosylase gene whose product specifically repairs DNA alkylation damage and protects cells from alkylation-induced cell death.

In *E. coli*, the expression of the *alkA* 3MeA DNA glycosylase gene is induced by alkylating agents but not by UV irradiation or other agents, because methylated DNA phosphates provide the only known induction signal (2, 6). Since the *S. cerevisiae* *MAG* glycosylase shares structural and functional similarity with the *E. coli* *AlkA* glycosylase, we expected to find that the induction of the *MAG* gene was also alkylation specific. However, to our surprise, the *MAG* gene was not only induced by alkylating agents but also by UV irradiation and 4NQO (table 1). Thus, the induction signal for *MAG* gene expression can be produced by three very different kinds of DNA damaging agent. It is currently unclear whether there is more than one mechanism for *MAG* induction, but at least one must involve a common signal produced as a consequence of the DNA damage produced by MMS, MNNG, UV and 4NQO. However, the most obvious common signal, namely the inhibition of DNA replication, appears not to be involved because *MAG* induction can occur in non-replicating, G1-arrested cells.

Increased cellular levels of a particular mRNA can be achieved either by increasing its rate of synthesis or by increasing its stability. Here we examined increases in *MAG* glycosylase

mRNA steady-state levels, and therefore have not yet differentiated between these two induction mechanisms. Induced transcription is usually regulated by the binding of a trans-acting activator protein to an upstream element (UAS) and its interaction with transcription factors and RNA polymerase II to increase the frequency of transcription initiation (39); genes induced by the same stimulus usually contain similar UAS sequences. It is not yet clear whether DNA damage-inducible genes belong to one or several regulons because the upstream regions of most DNA damage-inducible genes have not yet been characterized. However, the sequence CG(A/T)GG(A/T)NG(C/A)A has been identified upstream of three DNA damage-inducible genes, *PHR1*, *RAD2* and *RNR2*, and is known to be required for damage inducibility of *RAD2* and *RNR2* (18, 31, 40, 41). This sequence is not present in the 227 base pairs so far sequenced upstream of the *MAG* gene (42) and the rest of the *MAG* promoter region is currently being analyzed. We have examined a number of alkylation sensitive mutants in search of the genetic requirements for *MAG* induction. Our results suggest that the MGT1 MTase, the *MAG* glycosylase and 4 *MMS* gene products are not involved in *MAG* regulation (Fig3–5). However, it is clear that induction requires *de novo* protein synthesis and a more direct genetic approach should identify the genes involved in the regulation of *MAG* gene expression.

It is currently unclear whether the induction of the *MAG* transcript and glycosylase levels by DNA damage plays any *in vivo* role in the response of *S. cerevisiae* cells to alkylating agents. Ultimately, the isolation of mutants that can not induce *MAG* expression in response to DNA damage may reveal the biological significance of such regulation. Our results (data not shown) and those of others (43, 44) clearly show that the treatment of *S. cerevisiae* with doses of alkylating agents which efficiently induce *MAG* expression does not enable these cells to resist the killing effects of these agents. It seems that *S. cerevisiae* simply lacks an adaptive response to alkylating agents, and we speculate that this may be because these cells are deficient in the induction of some other enzyme(s) that participate in the base excision repair pathway. Thus, even if the *MAG* glycosylase is induced, if AP endonuclease, DNA polymerase or DNA ligase remain rate limiting the overall rate of 3MeA repair would not change and cells would not become resistant to the killing effects of alkylating agents. It would be premature to conclude that all yeast strains lack an adaptive response based on our experiments. Indeed, of the eighteen bacterial strains studied so far, adaptation to alkylating agents was demonstrated in only eleven (45, 46). At least one fungus strain, namely *Aspergillus nidulans*, has been shown to adapt in response to alkylating agents (47) and we are currently screening a number of yeast strains for their ability to acquire alkylation resistance upon exposure to sublethal levels of alkylating agents.

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