Expression of the *E.coli ada* gene in yeast protects against the toxic and mutagenic effects of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine

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ABSTRACT

The *E.coli ada* gene protein coding region has been ligated into an extrachromosomally replicating yeast expression vector downstream of the yeast alcohol dehydrogenase gene promoter region to produce pADH06C. The yeast strains SX46A, 7799-4B and VV-6 are deficient in endogenous *O*⁶-alkylguanine-DNA-alkyltransferase and transformation of these strains with this shuttle vector resulted in the expression of 1730, 1260 and 374 fmoles *ada*-encoded ATase/mg protein in stationary phase yeast: transformation with the parent vector had no effect on endogenous ATase activity which remained less than 2 fm/mg. In comparison with parent vector transformed yeast, all of the pADH06C-transformed strains showed an increase in the resistance to the toxic effects of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG). In addition, 7799-4B and VV-6 were more resistant to the mutagenic effects of this agent. These results indicate that the toxic and mutagenic effects of MNNG in yeast are mediated, at least in part, by DNA lesions that can be repaired by the *E.coli ada* gene product.

INTRODUCTION

Alkylating agents are a group of environmentally relevant compounds that produce a variety of biological effects in both pro and eukaryotic organisms. These include toxicity, mutagenicity and malignant transformation and many of the responses can be correlated with the production of alkylation damage in DNA (1). Most of the oxygen and nitrogen atoms in DNA can become alkylated and a total of 13 different base and phosphate modifications have been identified (1–3). Partly because of this, the precise role of any specific DNA lesion in a particular biological response is difficult to evaluate. One approach to the problem has been provided by cloned *E.coli* genes that code for proteins that are capable of repairing specific lesions in DNA: by achieving expression of such genes, usually in repair deficient systems, the biological effect of the lesion(s) can be deduced.

Using this approach, it has been shown that expression of the *E.coli* gene *ada*, or truncated versions thereof, in mammalian cells protects them against the toxic, mutagenic and chromosome damaging effects of certain alkylating agents (reviewed in 4). This protection can be ascribed to the *O*⁶-alkylguanine-DNA-alkyltransferase (ATase) function of the *ada* gene product (4–7) that repairs *O*⁶-alkylguanine (*O*⁶-AG) and *O*⁴-alkylthymine (*O*⁴-AT) in alkylated DNA by transfer of the alkyl group to a cysteine residue within the repair protein in an autoinactivating stoichiometric reaction (8–10).

In the present report, we have explored the possibility of obtaining expression of the *ada* protein in various yeast strains and have examined how this affects their response to the toxic and mutagenic effects of the methylating agent *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.

MATERIALS AND METHODS

Yeast Strains

Saccharomyces cerevisiae strain SX46A (Dr E C Friedberg) was ura 3-52, trp 1-289, his 3-852, ade 2. Strain VV-6 was ura 3-52, arg 4-17, his 3-1 and strain 7799-4B (Dr L Prakash) was ura 3-52, his 4-17.

Culture Media

YPD medium (1% yeast extract, 2% peptone, 2% glucose) was used for nonselective growth of the yeast strains. For the selective growth of transformed yeast a synthetic medium containing 0.67% (w/v) yeast nitrogen base, 2% glucose and essential yeast nutrients without uracil was used. For growth on plates this medium containing 2% agar was used. LB medium (0.5% yeast extract, 1% tryptone, 1% NaCl) was supplemented with ampicillin (0.1mg/ml) for growth of *E.coli* HB101 harbouring plasmids: selection was carried out on plates containing 2% agar in this medium.

Plasmid Construction

YE24 is an *E.coli*-yeast shuttle plasmid (7.74 kb) that consists of 4.4 kb of pBR322, a 2.2 kb *EcoRI* fragment of the yeast 2 μ m circular DNA and a 1.1 kb *HindIII* fragment of yeast DNA that contains the URA3 gene for selection. A modified version of

this plasmid containing a 2.5 kb *SalI*-*Bam*HI fragment of yeast DNA containing the yeast alcohol dehydrogenase (ADH-1) gene promoter (pADH ref 11) was obtained from E C Freidberg. The protein coding region of the *E. coli ada* gene, together with 5 upstream and 6 downstream bases was isolated from p062C (12) by *Bam*HI digestion. The resulting 1.1 kb fragment was ligated with *Bgl*II digested pADH and transformed into competent *E. coli* strain HB101. Plasmid DNA was isolated from ampicillin resistant colonies and a construct with the *ada* fragment in the appropriate orientation was identified. The structure of this plasmid pADH06C is shown in Figure 1.

Yeast transformations were carried out essentially as described using a lithium acetate procedure (13).

Cell Survival and Mutagenesis

Logarithmically growing yeast cultures in minimal medium (5×10^6 to 1×10^7 cells/ml) were suspended in 0.05M phosphate pH 5.5. MNNG (Sigma, dissolved in 0.1M sodium acetate pH 5.0 and stored at -20°C) was added to the final concentrations indicated in the figures and incubation was continued for 20 mins. Following treatment the cells were collected by centrifugation, washed with cold 0.05M phosphate and resuspended in 0.05M phosphate. Several dilutions of cells were spread onto YPD plates for measurement of survival or onto synthetic medium plates without arginine for determination of reversion of the arg 4-17 mutation or without histidine for reversion of the his 4-17 mutation.

In the case of transformed SX46A cells, an aliquot of logarithmically growing cells were also incubated in minimal medium containing 0.3 $\mu\text{g/ml}$ MNNG for 4h at 30°C . Cells were collected by centrifugation and treated as above with higher concentrations of MNNG.

Alkyltransferase Assays

Transformed yeast cells were grown in YPD to O.D. 600 nm = 0.5 (logarithmic phase) and harvested by centrifugation. Cell

Figure 1. Structure of pADH06C. A *Bam*HI (B) fragment containing the *ada* gene protein coding region C was ligated into the *Bgl*II (Bg) site of pADH which contained the promoter region of the yeast alcohol dehydrogenase gene (ADH), a section of the yeast genome that contains an autosomal replication sequence (2 μ) and the URA3 gene for selection in appropriate medium (see text). *Sal*I (S) digestion was used to determine the orientation of C (not to scale).

pellets were resuspended in 2 ml buffer I (50 mM tris, 1 mM EDTA, 3 mM dithiothreitol pH 8.3) and sonicated for 20 seconds at 10 μ peak to peak distance. After cooling in ice the cells were resonicated for 20 seconds at 16 μ and then 0.01 volumes of a phenylmethylsulphonylfluoride solution (8.7 mg/ml in ethanol) were added to inhibit proteases. The sonicate was clarified by centrifugation at 16000 rpm and 4°C for 15 mins in a refrigerated microcentrifuge and the supernatants were removed and stored on ice. Increasing volumes of these extracts were assayed for total ATase activity as described (14). Extracts were also prepared for the separate determination of O^6 -AG and alkylphosphotriester ATase using modified substrates as previously reported (10,15). In all cases the incubation temperature was 37°C . Samples were also taken for the estimation of protein concentration using the Bio-Rad protein reagent and bovine serum albumin as a standard. ATase specific activities were calculated from the amounts of radioactivity transferred to protein under conditions of substrate excess and the specific radioactivity of the substrate (23 Ci/mmol of methyl groups) assuming a counting efficiency of 28%.

Extracts were also prepared from stationary phase yeast and a culture of *E. coli* BS73 harbouring the plasmid p062SX that contains a truncated form of the *ada* gene and expresses only O^6 -AG and O^4 -alkylthymine (O^4 -AT) ATase activity (6,9,10). These were also assayed for ATase activity.

RESULTS

Alkyltransferase Activities

Following transformation with pADH or pADH06C and selective growth, one clone of each yeast strain used was isolated and expanded, and extracts of all six clones were prepared and assayed as described. Little or no ATase activity was detected in extracts of the pADH transformed clones at stationary phase and from the amounts of protein used in the assays it can be calculated that the ATase specific activities were less than 2 fmoles/mg protein. Extracts of exponentially growing pADH-transformed SX46A or VV-6 also showed ATase specific activities of <2 fm/mg. However, in pADH-transformed 7799-4B, the activity though still very low was 6.1 and 7.3 fm/mg in two separate assays. In contrast, ATase activity in extracts of all three pADH06C transformed clones was easily measured in both exponential or stationary phase cells (Table 1). Under substrate-limiting conditions, pADH06C-transformed yeast extracts transferred twice the amount of radioactivity to protein as did extracts of *E. coli* harbouring a truncated version of *ada* that encodes only O^6 -AG ATase activity (6, Figure 2). In addition, extracts of pADH06C-transformed yeast transferred

TABLE 1. Characteristics of Transformed Yeast

Strain	Plasmid	ATase (fm/mg protein)		% Survival after MNNG (100 $\mu\text{g/ml}$)	
		Exponential	Stationary	MNNG ¹ Pretreated	Control
SX46A	pADH	2.1	<2	13.5	8.6
SX46A	pADH06C	3487	1730	61.0	43.0
7799-4B	pADH	7.3	<2	—	1.8
7799-4B	pADH06C	1373	1260	—	28.3
VV-6	pADH	<2	<2	—	15.5
VV-6	pADH06C	630	374	—	33.0

¹ see text for details

radioactivity from modified substrate DNAs that contained either only O^6 -methylguanine (O^6 -MeG/) or only methylphosphotriesters as the ATase-repairable lesions (Table 2). The close similarity of the ATase specific activities using these modified substrates provides further evidence that the ATase expressed is *ada* gene encoded.

Cell Survival

The survival of the pADH and pADH06C transformed yeast clones following exposure to increasing doses of MNNG is shown in Figure 3 and presented as % survival after exposure to given doses of MNNG in Table 1. Expression of the *E. coli* ATase provides protection against MNNG and the degree of protection was greatest in cells expressing highest ATase activity although there appears to be no direct correlation between these two parameters. In transformed SX46A clones pretreatment of the cells with MNNG (0.3 μ g/ml) 4h prior to exposure for survival measurements did not significantly change the response of either pADH or pADH06C transformed clones to MNNG (Figure 3A).

Mutagenicity

The MNNG-induced reversion frequencies ($his^- \rightarrow his^+$) of pADH or pADH06C transformed 7799-4B cells were 1280 and 211/10⁵ survivors respectively after exposure to 100 μ g/ml MNNG (Figure 4A). In the case of VV-6 cells $arg^- \rightarrow arg^+$ reversion rates were 112 and 49/10⁷ survivors for pADH and pADH06C-transformed cells respectively after the same dose of MNNG (Figure 4B).

Figure 2. Alkyltransferase activity (measured as fmoles methyl transferred to protein) in extracts of pADH (○, △, □, inset) or pADH06C (●, ▲, ■) transformed yeast strains. SX46A, ○, ●; 7799-4B, △, ▲; VV-6, □, ■. The figure also shows activity in extract of *E. coli* harbouring p062SX (▼). See text for experimental details.

TABLE 2. Alkyltransferase activity in pADH06C transformed yeast strains

Strain	Total	Alkyltransferase activity (fmoles/mg protein)	
		O^6 -alkylguanine	Alkylphosphotriester
SX46A	1203	665	675
7799-4B	1552	921	576
VV6	384	184	189

DISCUSSION

In *E. coli*, the *ada* gene encodes a 39 kDa protein that repairs O^6 -AG, O^4 -AT and the S-stereoisomer of alkylphosphotriesters in alkylated DNA by transfer of the alkyl group to cysteine residue 322 in the former 2 cases and cysteine residue 69 the latter case (16). Several groups have achieved very high levels of expression of the entire or truncated derivatives of this gene in mammalian cells (reviewed in 3) and it has been shown that the *ada* protein is able to penetrate the mammalian cell nucleus and repair (at least) O^6 -MeG in host cell DNA after treatment of the cells with [³H]-MNU (17). The toxic, mutagenic and

Figure 3. Survival of pADH (open symbols) or pADH06C (closed symbols) transformed yeast cells following treatment with increasing doses of MNNG. A, SX46A with (▼, ▼) or without (○, ●) pretreatment with MNNG (0.3 μ g/ml) 4h prior to exposure to the higher doses. See text for experimental details. The data from two individual experiments are plotted in the figure. B, 7799-4B (△, ▲) and C, VV-6 (□, ■) mean $\pm$ SE of 4 determinations.

Figure 4. Reversion of pADH (open symbols) or pADH06C (closed symbols) transformed yeast cells following exposure to increasing dose of MNNG. A, histidine reversion in 7799-4B (△, ▲). B, arginine reversion in VV-6 (□, ■), mean $\pm$ SE of 3 determinations.

chromosome damaging effects of certain methylating and chloroethylating agents in such cells are considerably reduced in comparison with control cells and from the work with truncated versions of *ada* it can be concluded that these biological effects are mediated by *O*⁶-AG and/or *O*⁴-AT (3–7).

In most cases, the entire *ada* gene including the 5' untranslated region containing bacterial promoter sequences and ribosome binding domain was engineered into vectors containing a 5' promoter sequence and 3' polyadenylation signal that are required for expression in mammalian cells. In some cases, however, most of the untranslated region of the gene was removed leaving the protein coding region together with five 5' and six 3' bases (12) and this truncated version, as a 1.1 kb *Bam*HI fragment, was used in the present experiments to avoid any complications that may have arisen from the presence of the prokaryotic control sequences in yeast cells.

Most of the mammalian cell types that have been transformed expressed extremely low levels of the endogenous ATase gene and this facilitated both the detection of the expression of the transformed *ada* gene and the interpretation of its effects on the various parameters examined. Since this would also apply to yeast, we initially measured the levels of ATase activity in a number of yeast strains and found that they were extremely low (unpublished results). This observation has been supported by the present results and those of other workers (18). Yeast was therefore a suitable organism to attempt the transfer and expression of the *E. coli* ATase gene and to examine how this might affect their response to the toxic and mutagenic effects of alkylating agents. The yeast strains we used were all *ura3-52* mutants and were therefore defective in uracil synthesis. pADH replicates extrachromosomally by virtue of the 2 μ region (Figure 1) and the URA3 gene permits selection of competent yeast by its ability to permit growth in uracil deficient medium.

ATase expression in pADH-transformed yeast was about 2 fm/mg or less, except in exponentially growing 7799-4B, which contained 7 fm/mg. This represents the first report of growth-phase dependent ATase expression in yeast, however, because of the very low activity, the yeast ATase has not yet been characterised. In pADH06C transformed 7799-4B, ATase activity was around 200 $\times$ higher and differential activity in the other strains was even greater. It is unlikely that that transformation with pADH06C (and not pADH) caused an extensive increase in endogenous ATase gene expression: the dual ATase activity (see Results and Table 2) indicates that the ATase measured was *ada*-gene encoded. At stationary phase, ATase levels in SX46A and VV-6 but not 7799-4B were reduced by approximately 50% in comparison with exponentially growing cells. This may be related to the observation that alcohol dehydrogenase expression changes with growth phase (16). The present results imply that this regulatory mechanism might be *via* alcohol dehydrogenase promoter down-regulation. Interestingly ATase specific activities were similar to those obtained on expression of *ada* in ATase deficient mammalian cells (17) but the significance of this finding is not clear.

The pADH06C-transformed yeast strains SX46A, 7799-4B and VV-6 were much more resistant to the toxic effects of MNNG than pADH transformed cells (Table 1, Figure 3). This indicates that one or more of the alkylation products that are repaired by the *ada* ATase are toxic lesions in yeast. Further work with truncated versions of the *ada* gene that code for the *O*⁶-AG ATase (that acts on both *O*⁶-AG and *O*⁴-AT) and alkylphosphotriester ATase separately (6) or the *ogt* (20) that also acts on *O*⁶-AG and *O*⁴-AT but 80 times more rapidly on the

latter than the *ada* *O*⁶-AG ATase (10) should indicate more specifically which lesion(s) are principally responsible.

The different degree of protection provided by the ATase protein may be due partly or even entirely to the different levels of ATase expressed in the pADH06C transformed cells. Thus the increase in survival, in comparison with pADH transformed strains, was greater in pADH06C transformed 7799-4B than in VV-6 and this corresponds to the higher ATase expression in the former. On the other hand, the survival in all of the pADH06C transformed strains was broadly similar and this might indicate that there is a maximum limit to the amount of protection attainable. It is not unreasonable to suggest that the residual toxicity is due to DNA damage that is not repairable by the *ada* ATase or to some other cytotoxic events. The resistance to MNNG of pADH transformed VV-6 in comparison with 7799-4B and SX46A may be related to these factors or possibly to a greater ability to tolerate or to repair by some other mechanism, the *ada* ATase repairable DNA damage.

In *E. coli*, pretreatment with low non-toxic doses of MNNG results in a large increase in the resistance to the toxic effects of a subsequent higher 'challenging' dose of MNNG, a phenomenon known as the adaptive response and controlled by the *ada* gene (16). In the present experiments, the resistance of both pADH and pADH06C-transformed SX46A cells to MNNG was unchanged or only slightly increased by an MNNG pretreatment dose that is effective in inducing the adaptive response in *E. coli* (Figure 3A, Table 1). Although this may represent a very weak adaptive response, other workers have also been unable to induce large increases in resistance to MNNG or ATase expression by pretreatment with low doses of MNNG (18,21). It therefore seems likely that the effect observed here is related to MNNG-induced changes in growth phase and that an adaptive response analogous to that in *E. coli* does not occur in yeast.

In pADH06C-transformed yeast strains there was a decrease in the MNNG-induced reversion frequency in comparison with pADH-transformed strains. Arg 4-17 is reported to revert by GC-AT transition (22) and this would be expected to be the result of *O*⁶-alkylation of DNA. Our finding that *E. coli* ATase expression reduces MNNG-induced reversion frequency supports this suggestion. As was the case for MNNG toxicity, pADH transformed VV-6 was more resistant to reverse mutation induction than 7799-4B however the difference in sensitivity was very much greater than for toxicity. The reasons for this difference have not been established but may be related to the nature of the forward mutation involved. In pADH and to a lesser extent pADH06C-transformed 7799-4B *His*⁺ reversion frequency appeared to decrease between 100 and 200 μ g/ml MNNG and this may be a reflection of the greater toxicity of MNNG in this strain in comparison with that in VV-6. Like VV-6, pADH06C-transformed 7799-4B had a much lower MNNG-induced reversion frequency than pADH transformed cells. These results are consistent with the increased capacity to repair reversion-inducing lesions conferred by expression of the *ada* ATase. However, with the present constructs we cannot dismiss the possibility that *O*⁴-AT or alkylphosphotriesters might contribute to MNNG-induced reversion.

Expression of the *denV* gene of bacteriophage T4 (11) and the *E. coli* *phr* gene (23) have been shown to complement various repair deficient mutants and to increase resistance to the toxic effects of UV light. It will be of considerable interest to extend the present work to *rad* mutant strains in order to use the ATase gene as a probe for the repair defects in such strains.

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