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Excision repair of nitrogen mustard–DNA adducts in *Saccharomyces cerevisiae*

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ABSTRACT

The bifunctional alkylating anticancer drug nitrogen mustard forms a variety of DNA lesions, including monoadducts and intrastrand and interstrand crosslinks. Although it is known that nucleotide excision repair (NER) is important in processing these adducts, the role of the other principal excision repair pathway, base excision repair (BER) is less well defined. Using isogenic *Saccharomyces cerevisiae* strains disrupted for a variety of NER and BER genes we have examined the relative importance of the two pathways in the repair of nitrogen mustard adducts. As expected, NER defective cells (*rad4* and *rad14* strains) are extremely sensitive to the drug. One of the BER mutants, a 3-methyladenine glycosylase defective (*mag1*) strain also shows significant hypersensitivity. Using a *rad4*/*mag1* double mutant it is shown that the two excision repair pathways are epistatic to each other for nitrogen mustard sensitivity. Furthermore, both *rad14* and *mag1* disruptants show elevated levels of nitrogen mustard-induced forward mutation. Measurements of repair rates of nitrogen mustard *N*-alkylpurine adducts in the highly transcribed *RPB2* gene demonstrate defects in the processing of mono-adducts in *rad4*, *rad14* and *mag1* strains. However, there are differences in the kinetics of adduct removal in the NER mutants compared to the *mag1* strain. In the *mag1* strain significant repair occurs within 1 h with evidence of enhanced repair on the transcribed strand. Adducts however accumulate at later times in this strain. In contrast, in the NER mutants repair is only evident at times greater than 1 h. In a *mag1*/*rad4* double mutant damage accumulates with no evidence of repair. Comparison of the rates of repair in this gene with those in a different genomic region indicate that the contributions of NER and BER to the repair of nitrogen mustard adducts may not be the same genome wide.

INTRODUCTION

Nitrogen mustard (HN2) is a well-established anticancer drug which has been extensively studied as a DNA damaging agent (reviewed in 1). HN2 is a bifunctional alkylating agent which forms a variety of adducts including monoadducts and crosslinks. Monoadducts form principally at the N7 position of guanines and to a lesser extent at the N3 position of adenine. Crosslinks can be intrastrand, interstrand (most favourably between guanine N7 positions in the sequence 5'-GNC-3'/3'-CNG-5'; 2) and between DNA and protein. The vast majority (~90%) of adducts are monoadducts, with inter- and intrastrand crosslinks comprising only a small fra4ction of total lesions. Despite their rarity, there is good evidence that interstrand crosslinks (ICLs) are the critical cytotoxic adduct produced by nitrogen mustards (3).

The yeast *Saccharomyces cerevisiae* is well established as an excellent model organism for the study of DNA repair processes, since considerable genetic and biochemical conservation of these processes between yeast and mammalian cells has been demonstrated in recent years (4). A body of work exists examining the potential DNA repair processes acting on HN2 adducts in yeast, principally obtained by examining the sensitivity of various DNA repair mutants to the drug (5–9). However, since these studies were completed developments in yeast genetics now mean that it is possible to disrupt strains for the DNA repair genes under examination in a defined, isogenic genetic background and to analyse the role of more recently characterised pathways in the repair of HN2 adducts.

The two principal excision repair pathways in all cells from *Escherichia coli* to humans are nucleotide excision repair (NER) and base excision repair (BER) (for in-depth reviews of NER and BER see 10). In NER a multi-component repair complex assembles at the lesion and incises an approximately 24–32mer oligonucleotide containing the lesion. The gap created is filled in by a DNA polymerase and ligation completes the repair. The products of the *RAD1*, *RAD2*, *RAD3*, *RAD4*, *RAD10*, *RAD14* and *RAD25* genes are absolutely required for the recognition and incision steps of NER in *S.cerevisiae*. In contrast, during BER the damaged base is removed by a glycosylase which recognises the lesion and the resulting apurinic deoxyribose is excised by a separate apurinic endonuclease or by an apurinic lyase function inherent to the glycosylase. The resulting single nucleotide gap is usually filled in as a single nucleotide repair patch or more rarely is displaced in the 5'→3' direction to

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create a 'flap' structure which is subsequently cleaved by a 'flap' endonuclease DNase IV/FEN1 (Rad27 in *S.cerevisiae*) (11). This gap is filled in resulting in a nucleotide repair patch. NER occurs more rapidly in expressed genes, perhaps as a consequence of several components of the transcription factor TFIIH and the NER apparatus sharing subunits (resulting in preferential repair of the transcribed strand) and also the association of the NER apparatus with Cockayne Syndrome group A and B gene products (Rad26 and Rad28 in *S.cerevisiae*). It is also possible that accessibility to damage is enhanced in active genomic locations due to the chromatin structure and this may enhance repair rates. Genomic heterogeneity of BER has been demonstrated in both yeast and human cells. The basis for this is not well understood, but it has recently been demonstrated that the product of the human XPG gene plays a role in the transcription-coupled repair of oxidative lesions which is distinct from its role in NER (12).

It is of particular interest to examine the role of BER processes in the repair of HN2 adducts. Although it well established that NER plays a key role in the repair of HN2 adducts, as evidenced by the extreme sensitivity of NER mutants (5,9), the role of BER has not been examined in yeast. Purified *E.coli* AlkA (3-methyladenine glycosylase II) is able to excise guanine and adenine bases damaged by HN2, as well as uracil mustard, phenylalanine mustard and chlorambucil (13). In addition, rat and human 3-methyladenine glycosylases excise bases damaged by nitrogen mustards *in vitro*, but less efficiently than the AlkA protein (13). However, mouse cells disrupted for this gene do not demonstrate hypersensitivity to HN2 (14). It should be possible to obtain evidence which may help further resolve this issue using defined yeast mutants.

By screening a set of isogenic *S.cerevisiae* strains disrupted for essential components of the NER and BER pathways for sensitivity to HN2 it was possible to identify processes likely to be important in the repair of HN2 adducts. A role for both NER and BER (specifically the 3-methyladenine glycosylase) in the repair of HN2 adducts in this organism was established. However, an NER defect had a more dramatic effect on cytotoxicity, as this pathway is probably required for the repair of the critical ICLs, as well as the more abundant but less toxic monoadducts. Additionally, there appears to be considerable genomic heterogeneity in the removal of HN2 adducts with marked variation in the contributions of BER and NER.

MATERIALS AND METHODS

Chemicals and enzymes

Analytical grade Mechlorethamine (nitrogen mustard or HN2) was purchased from Sigma Chemical Co. (Poole, UK). All enzymes used were purchased from Promega UK.

Yeast strains and cell culture

The yeast strains used in this study are listed in Table 1. Cells were grown at 28°C in YEPD, or in synthetic complete medium (SC) supplemented with the appropriate amino acids and bases at recommended levels (20).

Survival analysis

For exponential cultures liquid YEPD medium was inoculated with a single colony picked from a freshly streaked (YEPD) stock plate and grown overnight at 28°C with vigorous shaking. Cells were counted microscopically and only cultures with between 2×10^7 and 4×10^7 cells/ml were used. For stationary cultures cells were grown for 48 h until the density was between 1×10^8 and 2×10^8 cells/ml. Cells were resuspended in phosphate-buffered saline (PBS) at a density of 2×10^7 cells/ml and 2 ml aliquots treated with the desired concentration of HN2 (dissolved in cold sterile water) for 60 min at 28°C with vigorous shaking. Cells were harvested, washed twice with 2 ml PBS and then diluted and plated on in triplicate onto YEPD plates at a density giving rise to 200 colonies/plate in untreated controls. Plates were incubated for 3 days at 28°C and then scored. Any experiments giving rise to >250 colonies/plate in untreated controls were rejected.

Canavanine resistance forward mutation assay

Cells were treated with HN2 as described for survival analysis. Aliquots containing 2×10^6 cells were then plated in duplicate or triplicate onto SC –Arg plates supplemented with 50 μg/ml canavanine (Sigma, Poole, UK). Plates were incubated for 4–5 days at 28°C and resistant colonies arising were scored.

Measurement of *N*-alkylpurine formation by Southern blotting

Cells were grown overnight in YEPD to exponential phase, harvested, washed, resuspended in PBS and treated with the desired dose of HN2 (dissolved in water) at a density of 2×10^7 cells/ml for 2 h at 28°C with vigorous shaking. All cells were harvested

Table 1. *Saccharomyces cerevisiae* strains used in this study

Strain	Genotype	Source (Ref)
DBY747	MAT**a**, *his3-Δ1 leu2-3, 112 trp1-289 ura3-52*	L. Samson (15)
WXY9105	DBY747 with *apnΔ::HIS3*	L. Samson (15)
WXY1991	DBY747 with *mgtΔ::LEU2*	L. Samson (15)
JC8901	DBY747 with *mag1Δ::URA3*	W. Xiao (16)
WXY9394	DBY747 with *rad4Δ::hisG-URA3-hisG*	W. Xiao (17)
WXY9395	DBY747 with *mag1Δ::hisG rad4Δ::hisG-URA3-hisG*	W. Xiao (17)
LP14Δ	DBY747 with *rad14Δ::URA3*	R. Waters (18)
W303a	MAT**a** *ade2-1 trp1-1 kan1-100 leu2-3 his3-11, 15 ura3-3*	S. Boiteux (19)
CD132	W303a with *ogg1Δ::TRP1*	S. Boiteux (19)

and washed twice in PBS. Samples which were not permitted to repair were immediately placed on ice. Repair samples were resuspended in pre-warmed YEPD and incubated with vigorous shaking at 28°C for the desired period.

DNA was extracted from samples using the Nucleon Yeast DNA miniprep kit (Nucleon, UK). Restriction digests were performed according to the manufacturer's instructions and the DNA subsequently precipitated and resuspended in sterile water. DNA was quantified by fluorimetry according to Kowalski (21). Samples (1 μg in 20 μl) were heated at 70°C for 30 min to depurinate *N*-alkylated bases. Apurinic sites were converted to single-strand breaks by the addition of 2 μl of freshly prepared 1.1 M NaOH for 30 min at 37°C. Samples were electrophoresed on 0.8% agarose gels, Southern blotted and hybridised as described (22). Probes for the *RPB2* gene were generated from a 0.64 kb PCR product of the gene made using the following primers: forward, 5'-GTGAGTTGTAATTCATGCAAT-3'; reverse, 5'-CATGGGTAACACCATCAGATT-3'. Thirty rounds of linear extension by *Taq* DNA polymerase were carried out on this product using the following primers to generate probes incorporating [α-^{32}P]dCTP: transcribed strand (TS), 5'-TTCATGCAATAAGGAACTGAC-3'; non-transcribed strand (NTS), 5'-ACCATCAGATTCATTTACCAT-3'. The probe for the 4.3 kb chromosome XIV fragment was generated from a 0.58 kb PCR product of the *MFA2* gene generated using the following primers: forward, 5'-CATTGACATCACTAGAGACA-3'; reverse, 5'-TTGATGCACGTGAAAAACCATTAT-3'. Thirty rounds of linear extension by *Taq* DNA polymerase were carried out on this product using the following primer to generate probes incorporating [α-^{32}P]dCTP: 5'-AGCGAGCTATCATCTTCATACAA-3'.

To calculate the fraction of undamaged DNA present in individual autoradiograph bands were scanned, in the linear response range of the film, using a Bio-Rad Model GS-670 imaging densitometer and band volumes determined.

RESULTS

To determine a potential role for NER and BER enzymes in the repair of HN2 adducts, a set of exponentially growing isogenic *S.cerevisiae* strains completely deficient in these activities were screened for sensitivity to the drug using a colony forming survival assay. Two completely NER defective disruptants, *rad4* and *rad14* strains, show an identical very high level of sensitivity to HN2 compared to the parental strain, DBY747 (Fig. 1A). This is consistent with previous observations based upon the sensitivity of NER mutant strains to crosslinking drugs. This sensitivity is independent of cell cycle stage since both exponentially growing (mainly in S phase) and stationary phase cells (principally G_1 phase cells) demonstrate the same level of hypersensitivity (data not shown).

To examine the role of BER and a specific alkyltransferase activity strains disrupted for 3-methyladenine glycosylase (*mag1*), 8-oxoguanine/fapy glycosylase (*ogg1*), apurinic endonuclease 1 (*apn1*) and a strain lacking O^6-alkylguanine transferase (*mgt1*) were tested for HN2 sensitivity relative to the isogenic parent DBY747, or W303a in the case of the *ogg1* strain (Fig. 1B and C). Only *mag1* showed significant hypersensitivity over the parental strain, demonstrating an ~10-fold increased sensitivity, the LD_{50} of DBY747 being 110 μM and that of *mag1* 10 μM. As with NER, the same level of sensitivity

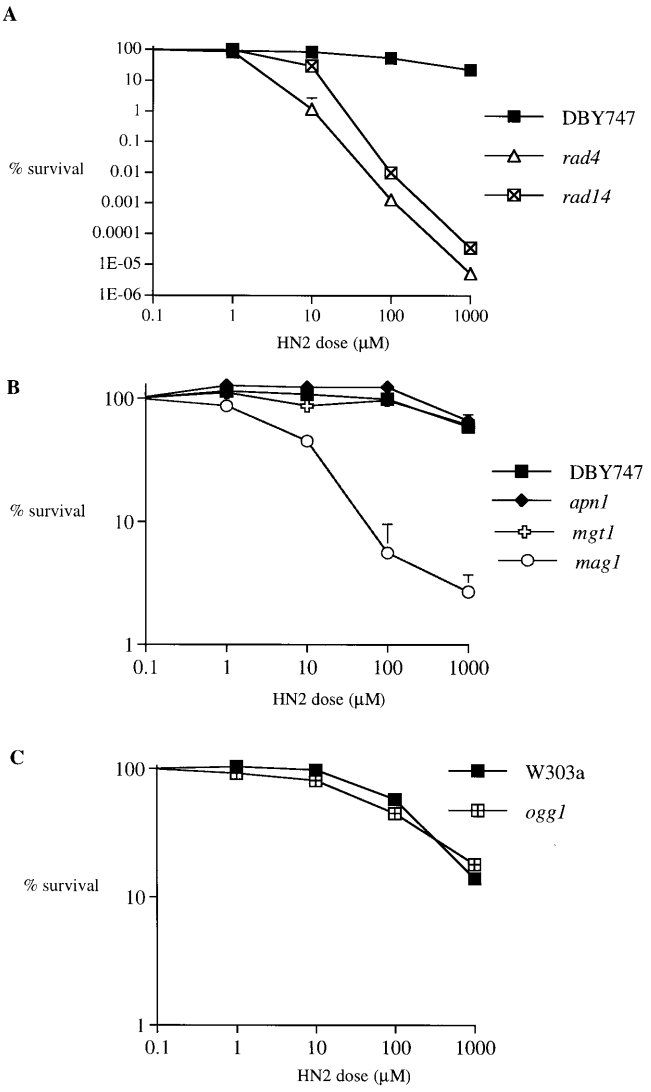

Figure 1. HN2 sensitivity of NER, BER and methyltransferase mutants. (**A**) Parental (DBY747), *rad4* and *rad14* cells were treated with the stated doses of HN2 for 1 h at 28°C. Appropriate dilutions were spread onto YEPD plates and incubated for 3 days at 28°C. Results are the average of at least three independent experiments; vertical error bars show the standard error of the mean. (**B**) The HN2 sensitivity of *apn1*, *mgt1* and *mag1* mutants compared to DBY747. (**C**) Sensitivity of an *ogg1* mutant compared to its isogenic parent W303A.

to HN2 was observed in exponentially growing and stationary phase cells (data not shown).

An unfortunate consequence of HN2 in the clinic is its relatively high level of carcinogenicity, sometimes leading to secondary malignancies in treated individuals (1). This is not surprising since it is well established that HN2 is a powerful mutagen in a variety of test systems from bacteria through to mammalian cells (1). Since both NER and BER can influence the repair response to HN2 it is relevant to determine what effect the excision repair status of the cell has on the mutagenicity of HN2. Using a forward mutation assay which relies upon

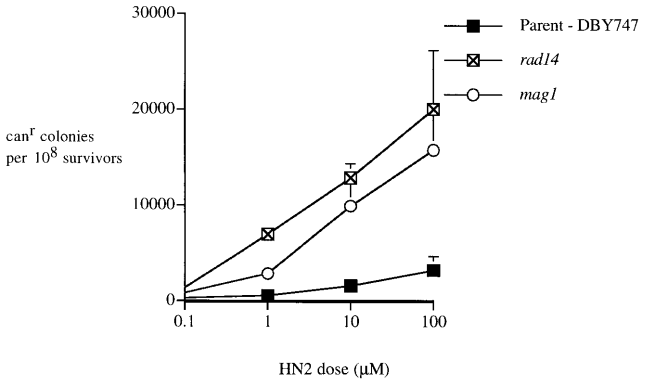

Figure 2. HN2-induced forward mutation frequency in the *ARG1* gene. Parental (DBY747), *rad14* and *mag1* cells were treated with the stated doses of HN2 for 1 h at 28°C. Aliquots containing 2×10^6 cells were plated onto SC –Arg plates supplemented with canavanine and scored following 4–5 days growth at 28°C. Values were calculated by scoring the fraction of canavanine-resistant colonies arising per 10^8 survivors where survival was simultaneously measured as in Figure 1. Results are the average of three independent experiments and error bars show the standard error of the mean.

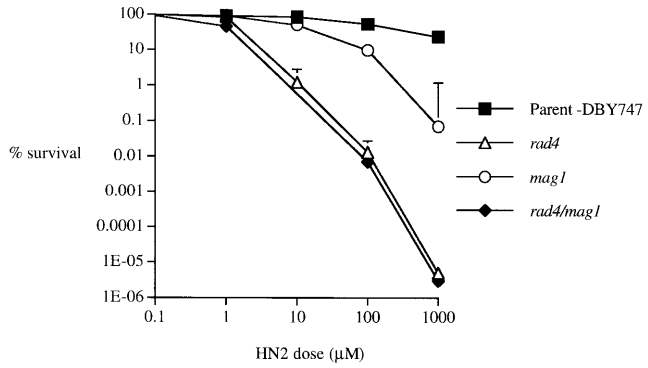

Figure 3. Epistasis between NER mutants and *mag1* for HN2 sensitivity. The sensitivities of parental (DBY747), *rad4* and *mag1* mutant strains and a *rad4*/*mag1* double mutant were determined as described for Figure 1. Results are the average of three independent experiments; error bars show the standard error of the mean.

alterations in the *ARG1* gene resulting in resistance to the antibiotic canavanine we have tested the frequency of HN2-induced events in the parent (DBY747) compared to the *rad14* and *mag1* mutants (Fig. 2). It is clear that when either gene is eliminated the frequency of HN2-induced forward mutations increases in a dose-dependent fashion. Clearly the excision repair competence of the target cell can influence the extent to which HN2 may potentiate further mutation. It is striking that although the adducts acted on by BER and NER exhibit very different cytotoxic properties both repair systems are involved in the repair of the class of adducts which induce mutations.

The sensitivity of the NER mutants examined is ~10-fold greater than the *mag1* strain at the LD_{50} where values are between 1–2 μM for the *rad4* strain and 10 μM for the *mag1* strain. This suggests that either a subset of adducts is acted on by each pathway, a less toxic lesion being removed by the BER route and the more toxic lesion by NER, or that the same set of lesions are acted upon by both pathways but with different relative efficiencies. A genetic test which can help distinguish between these possibilities was employed. Examination of the relative roles of NER and BER in the repair of nitrogen mustard adducts involved determining the epistatic relationship between the two pathways by comparing the sensitivity of *rad4* and *mag1* single mutants and a *rad4*/*mag1* double mutant strain. It is clear from Figure 3 that the sensitivity of the double mutant is identical to that of the most sensitive single mutant, i.e. the NER-deficient *rad4* strain. Taken together, the implication is that BER repairs the less toxic adducts, presumably monoadducts, and NER is required to repair the far more highly toxic lesion, i.e. the ICL, as well as monoadducts.

A modified Southern blotting assay was used to relate the sensitivity of these strains to the rate of repair of HN2 adducts in the *RPB2* gene, which encodes the second largest subunit of RNA polymerase II (24). Cells were incubated without drug or treated with 100 μM HN2 for 2 h and allowed to repair in rich medium (YEPD) for increasing times. Genomic DNA was extracted and subjected to restriction digestion with *Acc*65I and *Pvu*I which release a 4.5 kb fragment of the *RPB2* gene. Following DNA quantitation total HN2 *N*-alkylpurine adducts were converted into strand breaks by thermal depurination and alkaline hydrolysis at the resulting apurinic sites. Since ~90% of HN2–DNA adducts are monoadducts this treatment almost exclusively reveals this class of lesion. The DNA was run on a 0.8% agarose gel and subjected to Southern hybridisation using a probe specific for the TS and autoradiographed. The same blots were stripped of probe and subsequently hybridised to a probe specific for the NTS. In preliminary experiments 100 μM HN2 was found to be the lowest dose which permitted accurate quantitation of *N*-alkylpurines in this size fragment.

Figure 4A and B shows typical results obtained when parental (DBY747), *rad4*, *mag1* or *rad4*/*mag1* cells were incubated with no drug, treated with 100 μM HN2 for 2 h and DNA harvested immediately or allowed to repair for a further 60 or 120 min in rich medium (YEPD). It is striking that in the parental strain no damage can be observed immediately following treatment or during post-treatment incubation. This applies to both the TS and NTS (Fig. 4A and B, respectively). Preliminary experiments using shorter incubation times gave similar results indicating that the HN2 adducts are repaired very rapidly in this strain and that the duration of drug treatment does not influence the ability to reveal adducts in these blots (data not shown). In contrast, a significant number of adducts are present in the *rad4* strain immediately following treatment. However, these adducts appear to be partially removed during post-treatment incubation in rich medium. The rates of adduct repair were determined by densitometric scanning of autoradiographs and expressed as the fraction of undamaged DNA present prior to and immediately after HN2 treatment and following 60 and 120 min recovery in rich medium (Fig. 5). Immediately following treatment the fraction of undamaged DNA in the TS of the *RPB2* gene of *rad4* cells is 0.1. However, following 60 min post-treatment incubation this increases to 0.4, but does not continue to increase further at 120 min. Clearly there is a defect in HN2 adduct repair in *rad4* cells, but it appears to be compensated for by other repair activities

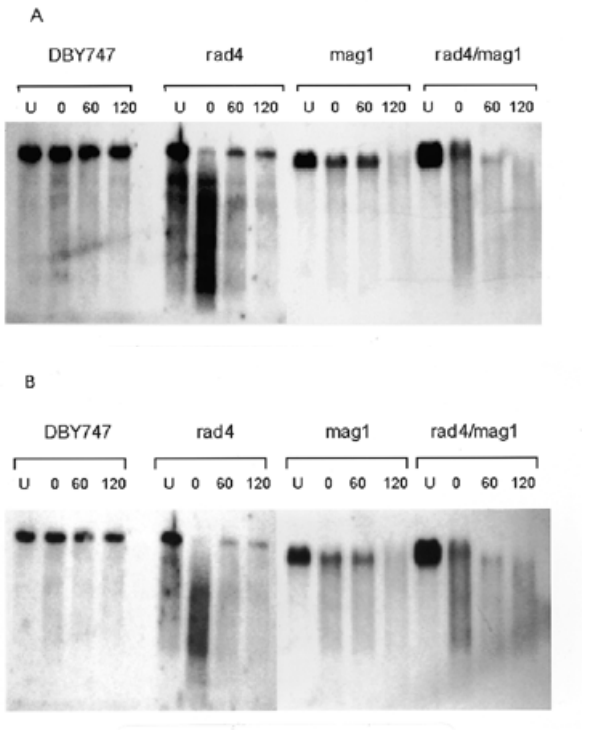

Figure 4. HN2 adduct formation and repair in the *RPB2* gene of the parental strain (DBY747) and *rad4*, *mag1* and *mag1*/*rad4* mutants determined by Southern blotting of DNA cleaved at sites of *N*-alkylpurines. (**A**) Adduct formation and repair on the TS prior to treatment (U), immediately following treatment (0) or following post-treatment recovery in rich medium for 60 and 120 min. (**B**) As (A) but employing a probe specific for the NTS.

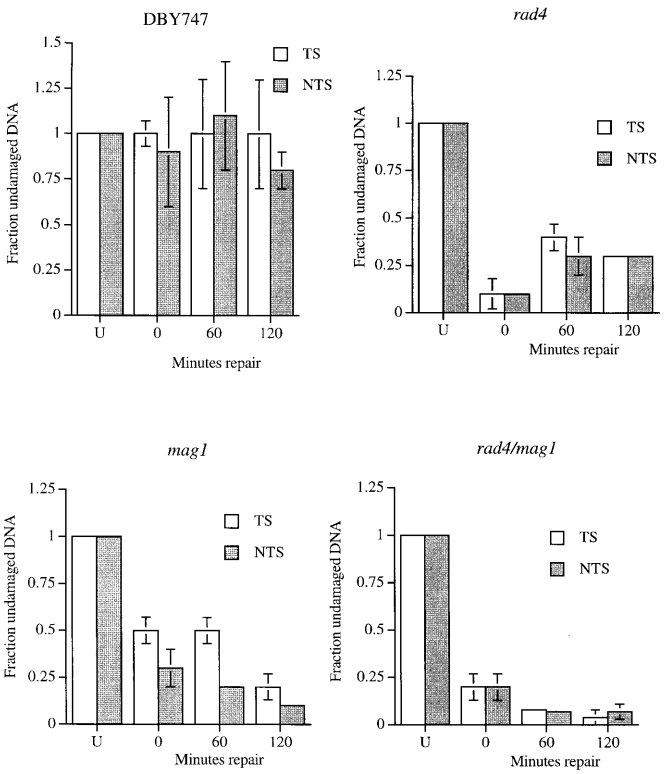

Figure 5. Repair profiles obtained from densitometric scans of autoradiographs as shown in Figure 4 expressed as the fraction of undamaged DNA present prior to HN2 treatment (U), immediately following treatment (0) or following 60 and 120 min recovery in rich medium. The data shown are from two independent experiments; the error bars show the standard error.

to some extent. The *rad14* mutant shows a repair profile which is indistinguishable from the *rad4* strain (data not shown). The NTS of the *RPB2* gene demonstrates a very similar defective repair profile to the TS (Figs 4B and 5).

Also shown in Figure 4A and B is the repair of HN2 adducts in the *mag1* strain prior to and following HN2 treatment and also following 60 and 120 min post-treatment incubation in rich medium. Immediately after HN2 treatment the fraction of undamaged DNA of the TS of the *RPB2* gene is 0.5 (Fig. 5, *mag1*). In contrast to the *rad4* strain the fraction of undamaged DNA does not then increase during post-treatment incubation in rich medium, but instead continues to fall to 0.2 after 120 min. Clearly the *mag1* strain also suffers from a defect in repairing total HN2 adducts, but the defect is kinetically distinct from that seen in a NER defective strain in this gene. The NTS of the *mag1* strain shows a very similar profile in qualitative terms but here the defect appears to be quantitatively greater, with the fraction of undamaged DNA being 0.3 immediately following treatment and falling to 0.1 at 120 min (Fig 5). Consistent with the data obtained for the single mutants the *rad4*/*mag1* double mutant shows a very severe defect in repair. Adduct levels reach a near maximum level immediately following treatment and there is no removal of adducts during the 120 min post-treatment recovery period on either the TS or NTS.

To examine whether the defects observed are specific to highly transcribed genes or are more general in nature the Southern blot assay was applied to a 4.3 kb *Hae*II restriction fragment of chromosome XIV surrounding the *MFA2* gene which encompasses five genes and intergenic regions (shown schematically in Fig. 6A) and is of a similar size to the *RPB2* fragment. The results of a typical experiment conducted on this region are shown in Figure 6B. These experiments were performed in an identical manner to those on the *RPB2* gene except for the region-specific nature of the restriction digest and probe used. Quantitation of these autoradiographs demonstrates that again the parental strain (DBY747) repairs adducts so efficiently that it is not possible to observe damage immediately following treatment (Figs 6B and 7). In contrast to the results obtained with the *RPB2* gene the *rad14* strain now appears to demonstrate only a slight defect in the removal of HN2 adducts. Densitometric analysis of the autoradiographs confirmed this and the results are displayed graphically in Figure 7. In contrast, the *mag1* strain does demonstrate a defect in the repair of this region which is initially more severe than that seen on either strand of the *RPB2* gene. In addition, this analysis shows that the kinetics of adduct induction and repair are different from those observed in the *RPB2* gene (Figs 6B and 7). In the *RPB2* gene adducts continued to accumulate during post-treatment incubation, whereas in this region the

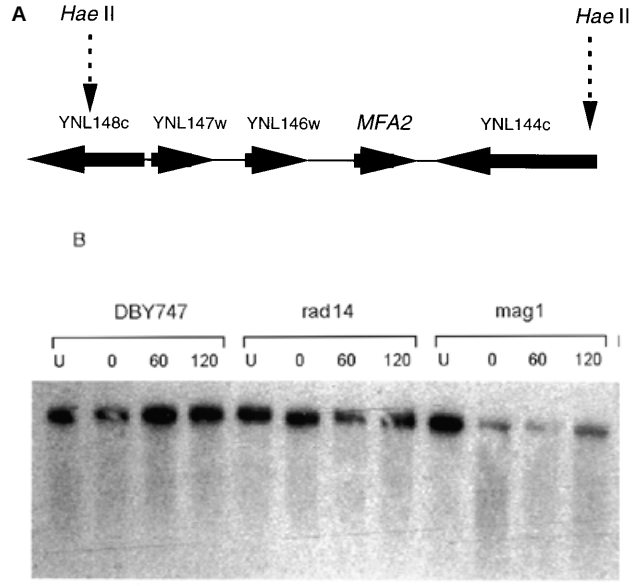

Figure 6. HN2 adduct formation and repair in the *MFA2* gene of the parental strain (DBY747) and *rad14* and *mag1* mutants determined by Southern blotting of DNA cleaved at sites of *N*-alkylpurines. (**A**) Schematic map of region analysed showing *Hae*II sites and relative size and orientation of genes. (**B**) Adduct formation and repair in this region prior to HN2 treatment (U), immediately following treatment (0) or following post-treatment recovery in rich medium for 60 and 120 min.

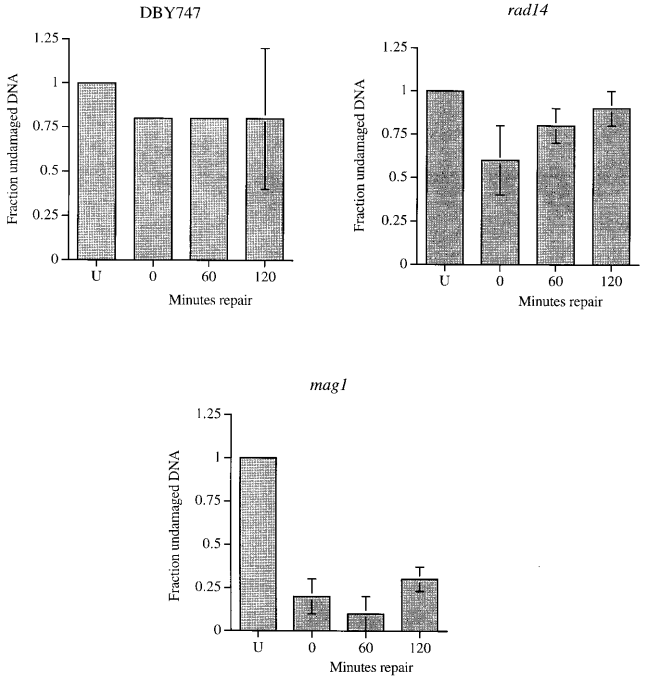

Figure 7. Repair profiles obtained from densitometric scans of autoradiographs as shown in Figure 6 and expressed as the fraction of undamaged DNA remaining prior to HN2 treatment (U), immediately following treatment (0) or following 60 and 120 min recovery in rich medium. The data shown are from two independent experiments; the error bars show the standard error.

peak level of adducts (fraction of undamaged DNA is 0.1) is reached 60 min following treatment and declines during incubation in rich medium with the fraction of undamaged DNA having risen to 0.3 after 120 min. Clearly the requirement for NER and BER in the repair of HN2 adducts is region specific and rapidly transcribed genes may be repaired differently from the genome overall.

DISCUSSION

Although it is well established (5,9) that NER plays a crucial role in determining the sensitivity of yeast cells to HN2, little is known about the contribution of BER. By screening the sensitivity of isogenic BER mutant *S.cerevisiae* strains to HN2 it was possible to demonstrate that cells lacking 3-methyladenine glycosylase are hypersensitive to the drug. None of the other BER mutants examined displayed any hypersensitivity, including the *apn1* strain. Since an apurinic endonuclease activity is required to complete the repair initiated by the *MAG1*-encoded 3-methyladenine glycosylase, it was initially surprising that the *apn1* strain does not demonstrate a similar level of sensitivity to HN2 as the *mag1* strain. However, the very recent finding that there is another major apurinic endonuclease in *S.cerevisiae* (*APN2*) accounts for the lack of sensitivity of the *apn1* strain, since it seems likely that the two enzymes are able to functionally complement each other (23).

These results are not entirely unprecedented since *in vitro* and *in vivo* studies of the *E.coli* AlkA enzyme indicate that this 3-methyladenine glycosylase acts on HN2 and other nitrogen mustard-induced damage (13,32). The purified protein is able to excise HN2 adducts from DNA fragments and *alkA* mutant *E.coli* cells display hypersensitivity to the drug. One of these studies (13) also demonstrated that the human and rat 3-methyladenine glycosylases have *in vitro* activity against HN2 adducts. Additionally, it has recently been shown that mouse cells with a null mutation in the 3-methyladenine glycosylase gene (*Aag–/–* cells) display hypersensitivity to several alkylating drugs, including mitomycin C, although not to HN2 (14).

The hypersensitivity of the *mag1* cells is ~10-fold less than that exhibited by isogenic NER (*rad14* and *rad4*) mutants at the LD_{50}. This suggests that the adducts acted upon by the two pathways differ, with the adducts eliminated by NER being more toxic. However, epistasis analysis (Fig. 3) indicated that the *rad4* and *mag1* strains belong to the same group for HN2 sensitivity. A likely explanation is that both these pathways functionally overlap for the repair of relatively non-toxic HN2 monoadducts, with NER also acting on the far more toxic ICL. These results are contradictory to those obtained using *Aag–/–*mouse cells which display no hypersensitivity to HN2 (14). It is possible that the relative contributions of NER and BER differ in these two organisms due to different 3-methyladenine glycosylase substrate specificities or that repair in mammalian cells depends on the origin of the cell and that the embryonic stem

cells employed in this study are not representative. It is also possible that additional glycosylase activities are present in murine cells which are able to act on HN2 monoadducts. In this respect it will be interesting to examine the range of DNA interacting drugs to which a *S.cerevisiae mag1* mutation confers sensitivity and compare these to the murine study.

It has previously been demonstrated that the failure to repair endogenous alkylation damage leads to an increase in spontaneous forward mutation rates in *mag1* cells (15). Measurements of the HN2-induced forward mutation frequency (canavanine^s to canavanine^r) indicate that the frequency of these events is substantially increased in *mag1* and *rad14* mutant cells. This raises the possibility that loss of genes in these pathways could confer a selective advantage to cells during HN2-induced secondary malignancy formation in treated individuals. The results presented here also suggest that the HN2 monoadducts are the primary mutagenic lesion induced by HN2, since in *rad14* cells ICL and monadduct repair is defective but induced mutagenicity occurs at very similar levels to the *mag1* strain where only monoadduct repair appears defective.

A Southern blotting-based assay was employed to follow the fate of total HN2 *N*-alkylpurine adducts in treated cells. Since the vast majority (>90%) of HN2 adducts are in this class this assay essentially provides a measure of monoadduct levels. We initially examined the *RPB2* gene (Figs 4A and B and 5) since this gene is sufficiently large to permit detection of adducts using HN2 doses (100 μM) at which a significant number of repair-proficient cells survive (55% of DBY747). The repair of HN2 adducts was extremely efficient in the parental, repair-proficient strain, such that it was not possible to detect formation of any DNA adducts either during treatment or post-incubation in rich medium. This applied to both the TS and NTS. In contrast, the *rad4* and *rad14* strains exhibited a repair defect on both strands, although *N*-alkylpurine repair was only delayed in these strains and not eliminated. The *mag1* strain also demonstrated a defect, but this was associated with an accumulation of adducts at longer time points, during post-treatment incubation. A possible explanation for these observations is that in an actively transcribed gene NER is able to initiate repair of monoadducts due to the shared physical identity between core TFIIH components and the NER apparatus (10). Thus NER could be a rapidly mobilised response. However, following the cessation of transcription due to the DNA damage, NER may not be the primary repair response and instead BER may now dominate. This could explain the gradual accumulation of HN2 adducts in the *mag1* cells and the gradual adduct removal in *rad4* and *rad14* cells. Although the *MAG1* gene is inducible (25), the kinetics of this response are unlikely to account for the kinetic differences seen here as this induction is rapid, for example peaking 30 min after treatment with MMS (26).

Small differences in the rates of repair in the TS versus the NTS in the *RPB2* gene in cells with functional NER were observed in the study presented here. Repair rates were so rapid in the parental strain (DBY747) that adduct levels could not be determined. However, in the *mag1* strain NER is still functional and here an increased removal of adducts was observed in the TS versus NTS after 60 min recovery suggesting that the NER component of this repair may be transcription coupled. Consistent with the results obtained with single mutants the *rad4*/*mag1* double mutant is completely defective in both classes of repair.

Studies with mammalian cells have demonstrated a great deal of genomic heterogeneity in both the formation and repair of HN2 adducts (22,27,28). In CHO cells actively transcribed genes appeared to be subject to more damage than bulk genomic DNA and repair was more rapid in these active regions (27). Further, α-amanitin (an RNA polymerase II inhibitor) was demonstrated to slow repair in these active genes consistent with the notion that transcription coupling is involved in the repair of HN2 adducts (28). There have been reports of coupling BER to transcription in both yeast and mammalian cells, although it is not clear if this is a general phenomenon which involves active targeting of BER enzymes or simply reflects heterogeneity of access to lesions of the BER apparatus (29,30). The removal of HN2 adducts by BER did not show any transcription coupling in the study presented here. A recent study demonstrated that the heterogeneity of adduct formation may be very dependent upon which gene is studied and can also differ significantly amongst human cell lines (22), emphasising the value of yeast studies where an isogenic background can be achieved. A further advantage of this approach is that it is possible to explore the contribution of BER since defined mutants are available. A recent report examining the repair of melphalan *N*-alkylpurines demonstrated a role for the mammalian NER, but here BER was not examined (31).

In further support of a key role for BER in HN2 monoadduct repair is data obtained examining the kinetics of adduct removal in a 4.3 kb chromosome XIV fragment (Fig. 6A and B). This region contains genes transcribed in both directions and intergenic regions. Here only a very small defect is observed in the *rad14* strain, but a more severe defect is observed in the *mag1* strain. Notably this *mag1* defect is apparent at earlier time points (is maximal immediately following 120 min HN2 treatment) suggesting that BER is the primary repair pathway acting on adducts in this region. Some repair occurs in the *mag1* strain following 120 min post-treatment incubation, which may be the result of gradual monoadduct removal by NER.

The formation and repair of HN2 monoadducts is clearly complex, involving both NER and BER, and heterogeneous within the genome. Despite the decreased clinical importance of HN2 in recent years, it is important to establish which repair pathways act on the DNA adducts formed by this archetypal alkylating drug since related compounds are currently widely used in the clinic and several novel compounds containing nitrogen mustard functionalities are being evaluated for their chemotherapeutic potential (33). Alkylating agents are likely to continue to play an important clinical role in the future and the modulation of repair pathways acting on the adducts produced by these drugs may represent a possible route for improving their clinical efficacy.

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REFERENCES

1. Povirk,L.F. and Shuker,D.E. (1994) *Mutat. Res.*, **318**, 205–226.
2. Ojwang,J.O., Greuneberg,D.A. and Loechler,E.L. (1989) *Cancer Res.*, **49**, 6529–6537.
3. Hartley,J.A. (1993) In Neidle,S.N. and Waring,M.J. (eds) *Molecular Aspects of Anticancer Drug–DNA Interactions*. Macmillan, Basingstoke, UK, pp. 1–31.
4. Prakash,S., Sung,P. and Prakash,L. (1993) *Annu. Rev. Genet.*, **27**, 33–70.
5. Brendel,M. and Haynes,R.H. (1973) *Mol. Gen. Genet.*, **125**, 197–216.
6. Ruhland,A. and Brendel,M. (1979) *Genetics*, **92**, 83–97.
7. Cassier,C. and Moustacchi,E. (1981) *Mutat. Res.*, **84**, 37–47.
8. Ruhland,A., Haase,E., Siede,W. and Brendel,M. (1981) *Mol. Gen. Genet.*, **181**, 346–351.
9. Siede,W. and Brendel,M. (1982) *Curr. Genet.*, **5**, 33–38.
10. Friedberg,E.C., Walker,G.C. and Siede,W. (1995) *DNA Repair and Mutagenesis*. ASM Press, Washington, DC.
11. Wu,X. and Wang,Z. (1999) *Nucleic Acids Res.*, **27**, 956–962.
12. Cooper,P.K., Nouspikel,T., Clarkson,S.G. and Leadon,S.A. (1997) *Science*, **275**, 990–993.
13. Mattes,W.B., Lee,C.-S., Laval,J. and O'Connor,T.R. (1996) *Carcinogenesis*, **17**, 643–648.
14. Allan,J.M., Engelward,B.P., Dreslin,A.J., Wyatt,M.D., Tomasz,M. and Samson,L.D. (1998) *Cancer Res.*, **58**, 3965–3973.
15. Xiao,W. and Samson,L. (1993) *Proc. Natl Acad. Sci. USA*, **90**, 2117–2121.
16. Chen,J., Derfler,B. and Samson,L. (1990) *EMBO J.*, **9**, 4569–4575.
17. Xiao,W., Chow,B.L. and Rathgeber,L. (1996) *Curr. Genet.*, **30**, 461–468.
18. Reed,S.H., McCready,S., Boiteux,S. and Waters,R. (1996) *Mol. Gen. Genet.*, **250**, 515–522.
19. Van der Kemp,P.A., Thomas,D., Barbey,R., de Olivera,R. and Boiteux,S. (1996) *Proc. Natl Acad. Sci. USA*, **93**, 5197–5202.
20. Guthrie,C. and Fink,G.R. (eds) (1991) *Guide to Yeast Genetics and Molecular Biology*. Academic Press, San Diego, CA.
21. Kowalski,D. (1979) *Anal. Biochem.*, **93**, 346–354.
22. Sunters,A., Grimaldi,K.A., Souhami,R.L. and Hartley,J.A. (1998) *Nucleic Acids Res.*, **26**, 5617–5623.
23. Johnson,R.E., Torres-Ramos,C.A., Izumi,T., Mitra,S., Prakash,S. and Prakash,L. (1998) *Genes Dev.*, **12**, 3137–3143.
24. Kolodziej,P.A. and Young,R.A. (1991) *Mol. Cell. Biol.*, **11**, 4669–4678.
25. Chen,J. and Samson,L. (1991) *Nucleic Acids Res.*, **19**, 6427–6432.
26. Zhu,Y. and Xiao,W. (1997) *Mol. Microbiol.*, **23**, 777–789.
27. Wasserman,K., Kohn,K.W. and Bohr,V.A. (1990) *J. Biol. Chem.*, **265**, 13906–13913.
28. Wasserman,K. and Damgaard,J. (1994) *Cancer Res.*, **54**, 175–181.
29. Leadon,S.A. and Cooper,P.K. (1993) *Proc. Natl Acad. Sci. USA*, **90**, 10499–10503.
30. Leadon,S.A., Barbee,S.L. and Dunn,A.B. (1995) *Mutat. Res.*, **337**, 169–178.
31. Grant,D.F., Bessho,T. and Reardon,J.T. (1998) *Cancer Res.*, **58**, 5196–5200.
32. Brooks,N., McHugh,P.J., Lee,M. and Hartley,J.A. (1999) *Anti-Cancer Drug Des.*, **14**, 11–18.
33. Weiss,G.R, Poggesi,I., Rocchetti,M., DeMaria,D., Mooneyham,T., Reilly,D., Vitek,L.V., Whaley,F., Patricia,E., Van Hoff,D.D. and O'Dwyer,P. (1998) *Clin. Cancer Res.*, **4**, 53–59.