Nucleotide Excision Repair Defect Influences Lethality and Mutagenicity Induced by Me-lex, a Sequence-Selective *N*3-Adenine Methylating Agent in the Absence of Base Excision Repair†

Paola Monti,‡,§ Raffaella Iannone,‡,§ Paola Campomenosi,|| Yari Ciribilli,‡ Sridhar Varadarajan,⊥ Dharini Shah,⊥ Paola Menichini,‡ Barry Gold,*,‡ and Gilberto Fronza*,‡

Mutagenesis Laboratory, National Cancer Research Institute, L.go R. Benzi, 10, 16132 Genova, Italy, Human Genetics Laboratory, University of Insubria, Varese, Italy, and Eppley Institute for Research in Cancer and Department of Pharmaceutical Sciences, University of Nebraska Medical Center, Omaha, Nebraska 69198-6805

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ABSTRACT: Using a yeast shuttle vector system, we have previously reported on the toxicity and mutagenicity of Me-lex, {1-methyl-4-[1-methyl-4-[3-(methoxysulfonyl)propanamido]pyrrole-2-carboxamido]pyrrole-2-carboxamido}propane, a compound that selectively generates 3-methyladenine (3-MeA). We observed that a mutant strain defective in Mag1, the glycosylase that excises 3-MeA in the initial step of base excision repair (BER) to generate an abasic site, is significantly more sensitive to the toxicity of Me-lex with respect to wild type but shows only a marginal increase in mutagenicity. A strain defective in AP endonuclease activity (Δapn1apn2), also required for functional BER, is equally sensitive to the toxicity as the Δmag1 mutant but showed a significantly higher mutation frequency. In the present work, we have explored the role of nucleotide excision repair (NER) in Me-lex-induced toxicity and mutagenicity since it is known that NER acts on abasic sites in vivo in yeast and in vitro assays. To accomplish this, we have deleted one of the genes essential for NER in yeast, namely, RAD14, both in the context of an otherwise DNA repair-proficient strain (Δrad14) and in a BER-defective isogenic derivative lacking the MAG1 gene (Δmag1rad14). Interestingly, no sensitivity to the treatment with Me-lex was conferred by the simple deletion of RAD14. However, a significant enhancement in toxicity and mutagenicity was observed when cells lacked both Rad14 and Mag1. The mutation spectrum induced by Me-lex in the Δmag1rad14 strain is indistinguishable from that observed in the Δapn1/Δapn2 or in the Δmag1 strains. The results indicate that in yeast NER can play a protective role against 3-MeA-mediated toxicity and mutagenicity; however, the role of NER is appreciable only in a BER-defective background.

Modification of DNA at the 3-position of purines is thought to be a potent block of DNA replication due to the loss of a required contact between this position and an arginine residue that is faithfully conserved in all known DNA polymerases (*1*−*4*). For this reason we have designed a molecule, Me-lex,[1] that methylates DNA to selectively and efficiently afford 3-MeA (*5*). As predicted, Me-lex is cytotoxic at micromolar concentrations in *Escherichia coli* (*6*, *7*) and in human cells (*8*, *9*), and cells that are defective in base excision repair (BER) show enhanced sensitivity (*8*). The compound causes cell cycle arrest in S-phase, sister chromatid exchange, p53 induction, and apoptosis in Aag null ES cells that cannot remove the lesion from the genome (*10*). Because 3-MeA should block DNA replication, which would induce cytotoxicity, it was assumed that Me-lex would not be particularly mutagenic. The mutagenicity of Me-lex was determined by use of a shuttle vector harboring the human p53 cDNA in wild-type *Saccharomyces cerevisiae* and in Mag1 glycosylase (Δmag1) and/or AP endonuclease (Δapn1apn2 and Δmag1apn1apn2) mutants (*11*, *12*). These studies showed that 3-MeA is not particularly mutagenic in wild-type yeast. However, the mutation frequency (MF) was slightly elevated in a Δmag1 background and significantly rose similarly in the Δapn1apn2 or Δmag1apn1apn2 strains (*12*). While MFs induced by Me-lex in the various mutant strains were quantitatively different, there was no qualitative difference between the mutation spectra in Δmag1 and Δapn1apn2 strains. Most of the targeted mutations were AT → TA transversions and AT → GC transitions, consistent

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* To whom correspondence should be addressed. G.F.: gilberto.fronza@istge.it. B.G.: bgold@unmc.edu.

‡ National Cancer Research Institute.

§ These authors contributed equally to this work.

|| University of Insubria.

⊥ University of Nebraska Medical Center.

[1] Abbreviations: BER, base excision repair; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; lex, lexitropsin; 3-MeA, *N*3-methyladenine; 3-MeG, *N*3-methylguanine; 7-MeG, *N*7-methylguanine; Me-lex, {1-methyl-4-[1-methyl-4-[3-(methoxysulfonyl)-propanamido]pyrrole-2-carboxamido]pyrrole-2-carboxamido}-propane; MF, mutation frequency; MMS, methyl methanesulfonate ester; NER, nucleotide excision repair; PCR, polymerase chain reaction; Tris, tris(hydroxymethyl)aminomethane.

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with the predominant formation of 3-MeA lesions. Moreover, their locations in the p53 cDNA were highly sequence-specific. To explain the common mutation pattern in the different repair backgrounds, which is consistent with a common promutagenic intermediate, we proposed that an abasic site at a blocked replication fork was involved (*12*).

In the present work, we have explored the role of nucleotide excision repair (NER) in Me-lex-induced toxicity and mutagenicity, since it is known that NER can process abasic sites in vivo in yeast as well as in vitro assays. To accomplish this goal, we have deleted one of the genes essential for NER in yeast, namely, RAD14, both in the context of an otherwise DNA repair-proficient strain (Δrad14) and in a BER-defective isogenic derivative lacking the MAG1 gene (Δmag1rad14). The results indicate that NER plays a role in the detoxification of 3-MeA, but this is only observed in a BER-defective background.

EXPERIMENTAL PROCEDURES

Hazardous Procedures. Me-lex should be considered toxic and a potential human carcinogen and was handled accordingly.

Compounds. Reagents of the highest purity were purchased from Sigma (St. Louis, MO) or Aldrich (Milwaukee, WI) unless otherwise stated. Me-lex was prepared as previously described (*5*). Restriction enzymes were obtained from New England Biolabs (Beverly, MA).

Vectors, Strain, and Media. The yeast expression vector pTS76 harbors a human wild-type *p53* cDNA under the control of an ADH1 constitutive promoter and contains the TRP1 selectable marker. The haploid *S. cerevisiae* strain yIG397 (MATa ade2-1 leu2-3,112 trp1-1 his3-11,15 can1-100 ura3-1 URA3 3xRGC::pCYC1::ADE2) and its isogenic BER- or NER-deficient derivatives were used as recipients for pTS76. yIG397 was also used for gap repair with plasmid pRDI-22 as previously described (*15*). The p53-dependent reporter ADE2 gene allowed the phenotypic selection of p53 mutants as its recombinant *cyc1* promoter contains three copies of the responsive element *RGC* (*12*). Standard yeast manipulations were performed as previously described (*16*).

DNA Modification, Analysis, and Transformation. Me-lex was dissolved in DMSO immediately before the treatment. 3.0 μg of plasmid pTS76 DNA was treated with different Me-lex concentrations (up to 12 mM) in 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 50% EtOH for 1 h at 37 °C. DNA was purified by EtOH precipitation, washed with 70% EtOH, and resuspended in sterile water. Damaged or undamaged vectors were then transformed by the LiAc method into the same number of yeast cells (measured by OD_{600}), under the same growth conditions, and transformants were plated on selective synthetic medium plates specific for each strain (see below). After 3 days of incubation at 30 °C, colonies were evaluated. The selection for the plasmid marker (TRP1) allowed an indirect determination of the lethal effect of the damaging treatment as the number of transformants scored in transfections with methylated plasmids with respect to that obtained with undamaged vector. As transformation plates contained a minimal amount of adenine, adenine auxotrophs produced small red colonies. The spontaneous (and induced) MF was defined as the number of small red colonies with respect to the total number of transformants. Phenotypic mutant clones were purified and characterized at the molecular level as previously described (*15*).

Construction of NER-Defective Yeast Strains: Disruption of the RAD14 gene. A 2-μm DNA-based marker recycling system for multiple gene disruption was used to inactivate the RAD14 gene (*17*). pGFKG, an 11.25 Kbp plasmid containing two recombinogenic FRT sequences flanking the FLP recombinase gene and the selectable marker KAN^R (a generous gift of Dr. Francesca Storici, NIEHS, Research Triangle Park, NC), was used as template to amplify the FRT::FLP::KAN::FRT cassette flanked by two short (40 bp) portions 5′ and 3′ of the RAD14 gene sequences. For this purpose, primers R14KF and R14K were prepared and used with pGFKG as template for a PCR reaction. R14KF has the sequence 5′-gAg TTT ggA TCT TCg Tag TgA Agg TAT CgA Acg TAA CgC *TAA AAA TAg gCg TAT CAC gAg*-3′, where the underlined sequence corresponds to positions −40 to −1 that is 5′ to RAD14 coding sequence and the italicized 21 bases are homologous to the 5′ region of the FRT::FLP::KAN FRT cassette in pGFKG. R14KR has the sequence 5′-TAT gAC TTT CTT gTT ATA TTC TTA TAT ACA TAA CCA ACA *TTC gAT gAT AAg CTg CTg TCA AAC*-3′, where the underlined sequence corresponds to position +40 to +1 that is 3′ to RAD14 coding sequence and the italicized 21 bases are homologous to the 3′ portion of the FRT::FLP::KAN FRT cassette in pGFKG. The PCR conditions are 94 °C for 90 s, 57 °C for 45 s, and 72 °C for 120 s, repeated for 35 cycles. Yeast strains were transformed with unpurified PCR product, and transformants were selected on YPDA plates containing 450 μg/mL G418. To control the disruption of the RAD14 gene, two primers were designed: R14 has the sequence 5′-gAA ggt TTA gAT gAg gAA Tgg-3′, corresponding to positions +937 to +957, and hybridizes in the 3′ region of the RAD14 coding sequence (i.e., it is specific for undisrupted RAD14 gene). R14K has the sequence 5′-TAT CAA TCA Atg AAC ACA ATC AA-3′, corresponding to positions +73 to +51, and hybridizes in the 3′ region of the RAD14 coding sequence (i.e., it is common to both undisrupted and KAN-disrupted RAD14 gene). K2 has the sequence 5′-AgT CgT CAC TCA Tgg TgA TT-3′ and hybridizes in the terminal portion of the KAN marker gene.

RAD14 disruption was verified by two PCR reactions with genomic DNA extracted from KAN^R clones, one with primers R14K/R14 and the other with primers R14K/K2. While DNA extracted from wild-type showed PCR product after amplification with R14K/R14 but not with R14K/K2, the opposite was observed with DNA extracted from KAN^R clones. The Δmag1rad14 strain was obtained by disrupting the RAD14 gene as described above, in the Δmag1 strain.

Statistical Analysis. The Adams and Skopek algorithm uses a Monte Carlo method to simulate a *P*-value of the standard hypergeometric test for a contingency table (*18*). Unlike the χ^2 test, which can also be applied to contingency tables and requires that all cells contain five or more events, the hypergeometric test is appropriate when applied to sparse data sets, as is often found in mutational spectra analyses. The Cariello program (*19*) uses a random number generator to produce a large number of simulated spectra based on the hypergeometric probability of the experimentally observed input spectra. The degree to which the simulated spectra differ from the input spectra is used to estimate the

Table 1: *S. cerevisiae* Strains Used in This Study

strain	genotype	ref
WT	ade2-1 leu2-3,112 trp1-1 his3-11,15 can1-100 ura3-1 URA3 3× RGC::pCYC1::ADE2	*11, 12*
Δmag1	same as WT plus mag1::LEU2 (mag1 LEU2)	*12*
Δapn2	same as WT plus apn2::KAN^R (apn2 KAN^R)	*12*
Δapn1apn2	same as WT plus apn1::HIS3 apn2::KAN^R (apn1apn2 HIS3 KAN^R)	*12*
Δmag1apn1apn2	same as Δapn1apn2 plus mag1::LEU2 (mag1apn1apn2 HIS3 KAN^R LEU2)	*12*
Δrad14	same as WT plus rad14::KAN^R (rad14 KAN^R)	this study
Δmag1rad14	same as Δmag1 plus rad14::KAN^R (mag1rad14)	this study

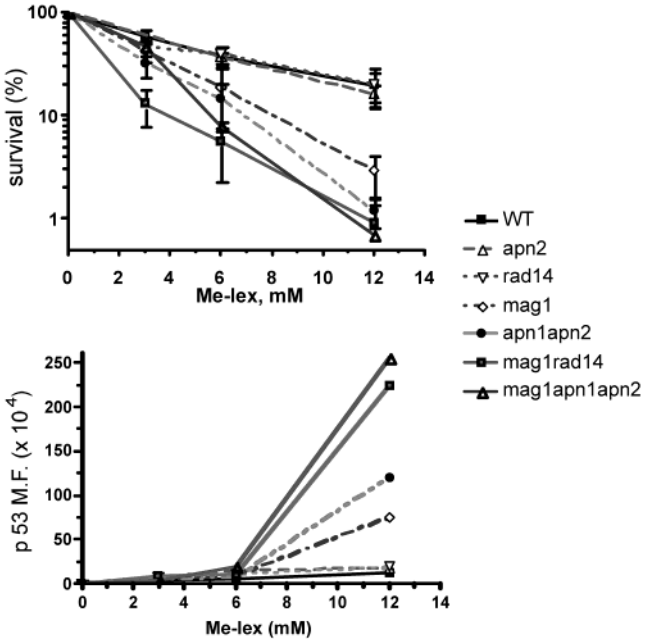

FIGURE 1: Influence of DNA repair capacities on the lethality (upper panel) and mutagenicity (lower panel) of Me-lex induced lesions in repair-proficient and repair-deficient yeast strains. The results described in this figure are completely independent from those already described in ref *12* for some strains. In the present study, WT, Δmag1, Δapn1apn2, and Δmag1apn1apn2 strains were used as internal controls for correct comparison with Δrad14 and Δmag1rad14 strains.

probability that the two input spectra were derived from the same population. A *P*-value = 0.05 leads one to reject the null hypothesis and conclude that the input spectra are different.

RESULTS

Me-lex Lethality Is Influenced by NER Defect Only in the Absence of BER. To construct NER-deficient strains, RAD14 was deleted via a PCR-based method (*17*) in two otherwise isogenic strains differing in the presence (yIG397, DNA repair wild type) or the absence of MAG1 (Δmag1), obtaining Δrad14 and Δmag1rad14 strains, respectively (Table 1). The accuracy of the RAD14 disruption was verified by PCR. To evaluate the influence of the NER defect on the lethality of Me-lex-induced lesions, plasmid pTS76 was damaged in vitro by exposure to increasing Me-lex concentrations (Figure 1, upper panel) and transformed into strains proficient or deficient in BER (used as internal control) and/or NER pathways. A Me-lex concentration-dependent decrease in survival was observed in every strain and was influenced by the DNA repair capacities of the recipient strain. Deletion of RAD14 alone has no effect on survival, suggesting that in a wild-type BER repair background NER does not confer any additonal protection to cells from the lethality of Me-lex-induced lesions. This result is similar to that obtained by disrupting only a minor AP-endonuclease gene (i.e., APN2). In contrast, and as previously shown (*12*, *14*), we confirmed that the inactivation of different steps in the BER pathway—inactivation of only MAG1 (Δmag1), inactivation of APN1 and APN2 (Δapn1apn2), or inactivation of the glycosylase and AP endonuclease steps (Δmag1apn1apn2)—causes an increased sensitivity to Me-lex. The results showed with Δmag1, Δapn1apn2, and Δmag1apn1apn2 in the present study are derived from new independent experiments performed along with those with the new constructed Δrad14 and Δmag1rad14 strains. Therefore, the data fully confirm previously published results obtained in independent experiments (*12*). Interestingly, inactivation of NER in a mag1-deficient background (Δmag1rad14) strongly increases sensitivity to Me-lex relative to Δrad14. This result suggests that, in the absence of Mag1 activity, Me-lex DNA lesions are repaired or detoxified by a protein required for NER.

Spontaneous MF Is Influenced by BER and NER Defects. To determine spontaneous MF, undamaged plasmid pTS76 was transformed into DNA repair-proficient and -deficient strains. Transformants were selected on plates lacking tryptophan but containing sufficient adenine for adenine auxotrophs to grow and turn red. While Δmag1, Δrad14, and Δapn2 strains showed MFs indistinguishable from that of the wild-type strain, the Δmag1rad14 strain revealed a significantly higher sMF ($p < 0.001$, χ^2 test) than wild type (Table 2). Taken together, these results suggest that in yeast BER is the major repair pathway for abasic sites generated by removal of spontaneous damage bases, while NER can have a back-up role in the processing of modified bases or abasic sites. Consistent with this latter hypothesis, Hendricks et al. (*20*) showed that a mag1rad4 double mutant strain has a higher spontaneous recombination frequency than a mag1 mutant strain.

Me-lex Mutagenicity Is Influenced by BER and NER Defects. MFs not only increased in a concentration-dependent way (Figure 1, lower panel) but also were strongly influenced by the nature of the DNA repair defects in each strain. MFs increased in the following order: wild type = Δapn2 = Δrad14 < Δmag1 < Δapn1apn2 < Δmag1rad14 = Δmag1apn1apn2. It is worth noting that Me-lex mutagenicity is only observed at concentrations that cause significant toxicity (less than 10% survival).

Molecular Characterization of Me-lex-Induced p53 Mutants in Δmag1rad14. The results described above show that the inactivation of NER in the absence of Mag1 activity increases both the lethality and mutagenicity of Me-lex-induced lesions. This is similar to what is observed when Apn1 and Apn2 activities are inactivated in the absence of

Table 2: Spontaneous Mutation Frequency Is Influenced by BER and NER Defects[a]

	wild-type	Δmag1	Δapn2	Δrad14	Δapn1apn2	Δmag1rad14	Δmag1apn1apn2
sMF	5.9×10^{-4} [b,c] (48/81 660)[d]	3.9×10^{-4} (46/117 092)	3.3×10^{-4} (16/48 575)	6.5×10^{-4} (21/32 491)	25.2×10^{-4} [c] (60/23 767)	11.2×10^{-4} [b] (72/64 012)	10.3×10^{-4} (6/5829)

[a] Summary of the spontaneous mutation frequency (sMF) in different yeast strains determined in the present study. [b] $p < 0.001$ χ^2 test. [c] $p < 0.0001$. [d] p53 mutant frequency, i.e., number of confirmed red colonies/total number of colonies, is shown.

Table 3: Molecular Characterization of Me-lex-Induced P53 Mutants in Δmag1rad14 Strain

mutant	position	codon	mutation	codon change	amino acid change
			base pair substitutions		
MR25[a]	246	82	G → A	CCG → CCA	Pro → Pro silent
MR43	301	101	A → T	AAA → TAA	Lys → Stop
MR47	301	101	A → T	AAA → TAA	Lys → Stop
MR174	326	109	T → C	TTC → TCC	Phe → Ser
MR128	338	113	T → C	TTC → TCC	Phe → Ser
MR33	361	121	T → C	TCT → CCT	Ser → Pro
MR33.6	361	121	T → C	TCT → CCT	Ser → Pro
MR5.6	395	132	A → C	AAG → ACG	Lys → Thr
MR19	402	134	T → G	TTT → TTG	Phe → Leu
MR95.6	403	135	T → C	TGC → CGC	Cys → Arg
MR21	403	135	T → C	TGC → CGC	Cys → Arg
MR37	416	139	A → T	AAG → ATG	Lys → Met
MR44	434	145	T → A	CTG → CAG	Leu → Gln
MR37.6	475	159	G → C	GCC → CCC	Ala → Pro
MR165	528	176	C → G	TGC → TGG	Cys → Trp
MR34	578	193	A → G	CAT → CGT	His → Arg
MR107	584	195	T → C	ATC → ACC	Ile → Thr
MR61[a]	597	199	A → G	GGA → GGG	Gly → Gly silent
MR151[a]	602	201	T → A	TTG → TAG	Leu → Stop
MR125	602	201	T → A	TTG → TAG	Leu → Stop
MR97	613	205	T → A	TAT → AAT	Tyr → Asn
MR36	617	206	T → A	TTG → TAG	Leu → Stop
MR93	734	245	G → C	GGC → GCC	Gly → Ala
MR155	737	246	T → C	ATG → ACG	Met → Thr
MR162	784	262	G → T	GGT → TGT	Gly → Lys
MR151-1[a]	823	275	T → C	TGT → CGT	Cys → Arg
MR123	840	280	A → T	AGA → AGT	Arg → Ser
MR159	843	281	C → G	GAC → GAG	Asp → Glu
MR61-1[a]	916	306	C → T	CGA → TGA	Arg → Stop
MR38.6	949	317	C → T	CAG → TAG	Gln → Stop
MR39	955	319	A → T	AAG → TAG	Lys → Stop
			deletions		
MR25-1[a]	247	83	−1G	GCG → −CG	
MR44.6	324−326	108−109	−1T	GTTTC → GGTTC	
M*R*32	597−599	199−200	−1A	GGAAAT → GGAAT	
MR159	659	220	−1A	TAT → TT	
MR96.6	509	170	−1C	ACG → AG	

[a] Indicates different mutations (e.g. MR25 and MR25-1) found in the same mutant (MR25). Note: Mutants were isolated primarily at 12 mM Me-lex; those mutants isolated at 6 mM Me-lex are indicated with a 6 extension (e.g., MR96.6).

Mag1 activity. To verify whether NER can influence at the molecular level the mutation spectrum induced by Me-lex (i.e., the type and distribution of Me-lex-induced mutations along the p53 cDNA), mutants isolated in the Δmag1rad14 strain were characterized. A total of 33 p53 mutants were sequenced. At least one molecular alteration was found in each mutant (Table 3). Thirty-three mutants evidenced 36 independent mutations (three mutants showed two mutations). The majority of mutations (31/36; 86%) were base-pair substitutions, the remaining being −1 deletions. The molecular features of the base-pair substitutions determined in this study are summarized in Table 4 along with those previously determined in three groups of mutants (wild type, Δmag1, and Δapn1apn2) (*12*). In Δmag1rad14 more than 74% of base-pair substitutions were AT-targeted, while the same class of mutations was slightly less frequent in the other three strains (approximately 60% in each). This result suggests that AT-targeted lesions normally repaired by NER are responsible for this difference. With respect to GC-targeted mutations, Δmag1rad14 showed a lower percentage of GC transversions and a higher percentage of GC → AT

Table 4: Summary of the Molecular Features of Me-lex-Induced p53 Base-Pair Substitution Mutations in Wild-Type, Δmag1, Δapn1apn2, and Δmag1rad14 Strains

	wild-type	Δmag1	Δapn1apn2	Δmag1rad14
AT-targeted	14 (61)	20 (59)	14 (61)	23 (74)
AT → TA	8 (35)	9 (26)	8 (35)	10 (32)
AT → CG	3 (13)	1 (3)	0 (0)	2 (6.5)
AT → GC	3 (13)	10 (30)	6 (26)	11 (35.5)
GC-targeted	9 (39)	14 (41)	9 (39)	8 (26)
GC → AT	4 (17)[a]	0 (0)[a−c]	4 (17)[b]	3 (10)[c]
GC → TA	3 (13)	6 (18)	2 (9)	1 (3)
GC → CG	2 (9)	8 (21)	3 (13)	4 (13)
total base pair substitutions	23 (100%)	34 (100%)	23 (100%)	31 (100%)

[a] $p < 0.03$. [b] $p < 0.02$. [c] $p < 0.04$.

transitions when compared with Δmag1 (both differences are statistically significant: $p < 0.04$, Fisher's exact test).

There were four Me-lex-induced mutation hotspots ($n = 2$, $p < 0.001$, Poisson's normal distribution) in the Δmag1rad14 mutation spectrum: position 301 (codon 101), where two AT → TA transversions were observed; position 361 (codon 121), where two AT → GC transitions were observed; position 403 (codon 135), where two AT → GC transitions were observed; and position 602 (codon 201, 5′-A_{602}AATTT$_{597}$-3′), where two AT → TA transversions were observed. While the position 602 hotspot was previously found in Δapn1apn2 (two mutations), Δmag1 (three mutations), and wild type (four mutations), the others hotspots were found in the different BER-deficient strains: position 403, present in both Δmag1 and Δapn1apn2; position 301, present in Δmag1; and position 361, present in Δmag1rad14.

DISCUSSION

Me-lex was designed to preferentially yield 3-MeA, a lesion assumed to be cytotoxic but not mutagenic. In *S. cerevisiae*, 3-MeA repair is initiated by Mag1, followed by the action of AP endonucleases (Apn1 and Apn2), DNA polymerase (δ and ϵ), and DNA ligase (III). APN1 encodes the major AP endonuclease that shares extensive homology with Endo IV in *E. coli* (*21*). A second AP endonuclease, the product of the APN2 gene, which is the *S. cerevisiae* homologue of the major human AP endonuclease *HAP1*, probably accounts for less than 10% of the total AP endonuclease activity in budding yeast (*22*). Previously, using a genetic approach in yeast, we showed that 3-MeA is critical for Me-lex cytotoxicity and that its mutagenicity is only somewhat elevated in the absence of Mag1 glycosylase activity but significantly elevated in the absence of AP endonuclease activity (*12*). The mutation hotspots did not correlate with the most intense sites of methylation but were always associated with the A/T-rich regions targeted by Me-lex (*11*, *12*). A novel finding was that the mutation spectra induced by Me-lex in the Δmag1 and Δapn1apn2 backgrounds were indistinguishable. This is consistent with (i) an unrepaired 3-MeA and an abasic site causing a similar misincorporation by DNA polymerase or (ii) a common premutagenic lesion, i.e., an abasic site, being formed (*12*). If 3-MeA was directly mutagenic through a miscoding mechanism, the MF in the Δmag1 cells would be significantly elevated, but it is not. Thus, Me-lex mutagenicity is attributed to the formation of abasic sites formed from either the enzymatic (Mag1) or hydrolytic release of 3-MeA (*12*). As will be discussed below, all abasic sites may not be equally promutagenic since in a Δmag1rad14 mutant the presence of functional APN1 and APN2 cannot rescue the cells from the toxicity or mutagenicity of Me-lex.

Although NER was originally thought to exclusively repair DNA lesions able to distort DNA structure (e.g. bulky lesions), in vivo studies (*E. coli*, *S. cerevisiae*, and *Schizosaccharomyces pombe*) revealed a role for NER in the repair of abasic sites, as well as some methylated bases that do not cause major helical distortion (*14*, *23*−*26*). Biochemical studies confirmed that DNA lesions such as AP sites, O^4-ethylthymine, O^4-methylthymine, O^6-methylguanine, and N^6-methyladenine can be substrates for NER (*24*, *27*−*29*). NER is initiated by dual incision of the adducted DNA strand, excising the lesion in the form of a short oligonucleotide. In *S. cerevisiae*, seven genes (RAD1, RAD2, RAD3, RAD4, RAD10, RAD14, and RAD25) essential for NER have been identified (*30*). Rad14 is a zinc metalloprotein with damage recognition function and forms a tertiary complex (nucleotide excision repair factor 1) with Rad1−Rad10 nuclease (*31*). The absence of one essential gene product impairs the NER reaction completely (*32*). To explore whether specific Me-lex-induced DNA lesions were repaired by NER, the toxicity and mutagenicity of Me-lex were determined in isogenic yeast strains defective only in NER (by deletion of the RAD14 gene, known to be essential for NER; Δrad14) or defective in both NER and BER pathways (Δmag1rad14).

Me-lex Lethality and Mutagenicity. The results presented in this study demonstrate that the lethality of Me-lex-induced lesions is counteracted mainly by BER (mainly via Mag1 activity), but the role of NER is appreciable in the absence of BER (Figure 1, upper panel). Indeed, the lethality of Me-lex is higher in Δmag1rad14 than in Δmag1. The mutagenicity of Me-lex is also significantly enhanced in Δmag1rad14 relative to Δmag1, especially at high Me-lex concentrations. The data indicate either that NER works on lethal and promutagenic Me-lex lesions or that its absence facilitates the transformation of Me-lex lesions into more mutagenic ones, for example, abasic sites.

Which lesion(s) induced by Me-lex is(are) processed by NER? AP sites are known substrates of NER both in vitro (*27*) and in vivo (*14*), while 3-MeA is repaired exclusively via BER, at least in *E. coli* (*7*). Recently, the repair of 3-MeA in mouse embryo fibroblasts proficient (aag$^{+/+}$) or deficient in BER (aag$^{-/-}$) and/or NER (xpa$^{-/-}$) has been reported (*33*). Me-lex was used to generate 3-MeA, and at different time points alkali-labile sites were quantified in the different cell lines. The results showed rapid repair in wild-type and NER null cells and slow and no decrease of alkali-labile sites in the BER and BER/NER nulls, respectively. Thus, there may be a role of NER in the repair of 3-MeA or abasic sites that, similar to the results reported here for the Δmag1rad14 mutant, is only evident in the BER null background.

Since Δmag1rad14 and Δmag1 strains showed different toxic and mutagenic responses to Me-lex, we reasoned that the comparison of Me-lex-induced p53 mutants isolated in Δmag1rad14 with other mutational spectra obtained in different DNA repair backgrounds could give us some hints about the nature of the promutagenic lesion(s) responsible for the observed effects. To this end, we compared the mutational spectrum induced by Me-lex in Δmag1rad14 with those previously obtained (*12*) in wild-type, Δmag1, and Δapn1apn2 strains. The comparisons of mutational spectra induced by Me-lex in different repair backgrounds were performed by Cariello's test that considers both mutation type and position (*19*). The application of this rigorous statistical test reveals that the Δmag1rad14, Δmag1, and Δapn1apn2 spectra are indistinguishable ($p = 0.74$, $p = 0.85$) (Figure 2). The fact that the Me-lex-specific mutation hotspot at position 602 is common to all strains, and is not "hotter" in any of the examined backgrounds, contributes to the similarity of the mutation spectra. If 3-MeA was directly responsible for the generation of mutations at A602, it is reasonable to expect that this hotspot would be hotter in those strains unable to repair 3-MeA and/or AP sites. However, the fact that this position was found to be equally hot in

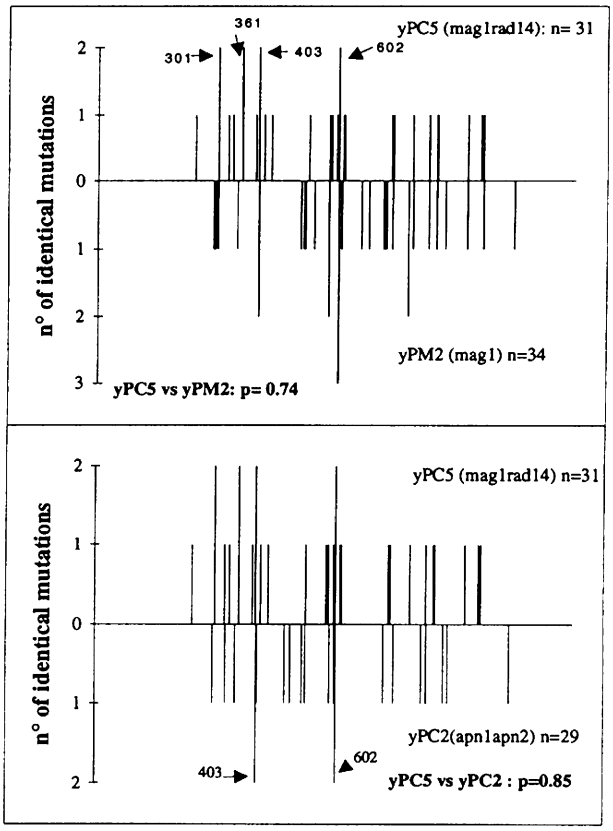

FIGURE 2: Sequence distribution of p53 mutations induced by Me-lex and isolated in Δmag1rad14, in Δmag1, and in Δapn1apn2 strains. Mutation spectrum induced by Me-lex in Δmag1rad14 is compared to those previously obtained in Δmag1 and in Δapn1apn2 strains (*12*) by use of Cariello's program (*19*). The mutation spectrum observed in Δmag1rad14 is indistinguishable from the one observed in Δmag1 (upper panel) and from the one observed in Δapn1apn2 (lower panel).

different repair backgrounds suggests that the lesion responsible for the mutations at A602 is not a substrate of Mag1, Apn1, Apn2, or Rad14. Alternatively, the lesion in this particular sequence context may be inefficiently repaired and/or efficiently bypassed by translesion polymerase(s). We previously demonstrated that 3-MeA was formed in abundant amounts at position A602 (*11*). A602 (opposite T^{602}) is in a 5′-$GA^{597}AATTT^{602}G$ palindrome, yet the mutation frequency is much lower at A597, suggesting that flanking bases (i.e., 5′-G versus 5′-C) may alter the MF. The existence of cold spots for repair is supported by the work of Ye et al. (*34*), which showed that the sequence-dependent rate of 3-MeA repair varied by 6-fold from site to site. In a similar manner, the rate of NER may vary as a function of sequence context (*35*).

A complicating factor in understanding the direct effect of 3-MeA is its potential to depurinate to an abasic site. Abasic sites lead to AT → CG or AT → TA transversions according to the work of Kunz et al. (*36*) or AT → GC or AT → TA according to Gibbs and Lawrence (*37*). While an AT → TA transversion at position 602 will be detectable in our assay (Leu^{201} → STOP), an AT → GC transition or an AT → CG transversion will cause two different amino acid substitutions (Leu → Ser or Leu → Trp) whose effects on the functionality of the p53 protein are unknown. It has been noted that, at codon 201, methionine, proline, glutamine, and valine are naturally occurring variants observed during

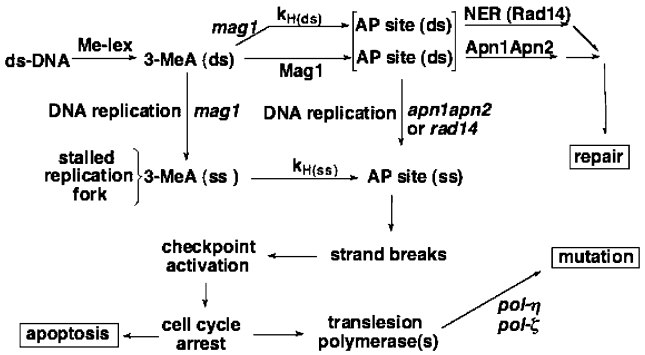

FIGURE 3: Pathways for the induction of mutations by Me-lex in different repair backgrounds. The possible role of NER (Rad14) in the toxicity and mutagenicity of 3-MeA is indicated; $k_{H(ss)}$ and $k_{H(ds)}$ are the rates of hydrolysis of 3-MeA to an abasic site in single- and double-stranded DNA, respectively (adapted from ref *12*).

evolution (*38*). This suggests that codon 201 can accommodate some amino acid substitutions without affecting transactivation function. That only AT → TA transversions are observed in all strains at position 602 may be caused by the mutant selection process in our assay.

The question remains whether 3-MeA, which so effectively prevents DNA synthesis, can also be a promutagenic lesion. We suggest the pathways outlined in Figure 3 to explain both the toxicity and mutagenicity of 3-MeA formed by Me-lex in the different genetic backgrounds. Our data show that despite the fact that Mag1 strongly protects cells against Me-lex toxicity, its deletion has little effect on mutagenicity. Therefore, Me-lex is not directly mutagenic and any AP sites that form hydrolytically are efficiently repaired. Clearly AP sites are both toxic and mutagenic since the Δapn1apn2 mutant is highly sensitive to Me-lex. Because NER is thought to inefficiently repair AP sites relative to Apn1 or Apn2, it is not surprising that the Δrad14 strain is not appreciably sensitive to Me-lex in terms of toxicity or mutagenicity. Even the deletion of Apn1, which accounts for ∼90% of AP endonuclease activity, does not confer any phenotype when cells are challenged with Me-lex-treated plasmids (*12*). This implies that Apn1 and Apn2 compensate for each other in the single mutant and presumably for Rad14 in the Δrad14 strain. The same phenomenon has been observed in *S. cerevisiae* treated with MMS: deletion of either Apn1 or Apn2 shows no increased sensitivity to MMS. Conversely, Rad14 does not compensate for the combined deletion of Apn1 and Apn2 (*14*).

What is more confusing is why Me-lex becomes more mutagenic in the Δmag1 background (where 3-MeA will persist) when AP endonuclease or NER is also defective. At high levels of toxicity, the Rad14 and Apn1/Apn2 activities do not compensate for each other, i.e., mutation frequencies are equally high in Δmag1apn1apn2 and Δmag1rad14, under conditions where the level of AP sites should be low due to lack of glycosylase excision. As previously proposed (*39*), it is possible that Apn1 and Apn2 are coupled to the glycosylase and their efficiency in finding AP sites is decreased when the lesion arises hydrolytically. Under these conditions, the ability of the cell to remove AP sites by the combination of BER and NER becomes limiting, and the deletion of RAD14 or Apn1 and Apn2 shows a phenotype. Furthermore, when 3-MeA levels remain high, as in the Δmag1 mutant, the lesions will block DNA

replication. 3-MeA will rapidly depurinate at a replication fork because the rate of hydrolysis [$k_{H(SS)}$] is approximately 40-fold faster in single-stranded than double-stranded DNA (*40*). We know that Me-lex can induce chromatid gaps and breaks and cell cycle arrest in mouse ES cells (*10*) that will create regions of single-stranded DNA. Single-stranded DNA activates the checkpoint response (*41*) that is associated with the activation of the polymerases involved in mutagenic translesion synthesis (*42*, *43*). It appears that the critical step in activation of translesion synthesis by polymerases η and ζ, which are required for AP-site-derived mutagenesis in yeast (*44*), is mediated by RAD6 monoubiquination of PCNA (*45*). If there is a threshold level of DNA damage required for the cell to turn on error-prone translesion synthesis, this will occur at lower concentrations of Me-lex in the Δapn1apn2 or Δrad14 mutants since the persistent amount of AP sites will be higher than in the WT analogues. We specifically suggest that AP sites and/or downstream lesions, rather than 3-MeA, are involved in triggering this effect because there is no significant mutagenicity in the Δmag1 strain. However, once the density of BER intermediates in the genome exceeds the threshold, the error-prone bypass polymerase pathway is turned on and causes the mutations at or near sites that are damaged. The damage that is bypassed may actually be a 3-MeA lesion or AP site. In this way, there is an on/off switch above a certain amount of damage. In the present studies, this occurs in any strain that is defective in the repair of AP sites when plasmids are exposed to Me-lex at concentrations between 6 and 12 mM. Importantly, this scheme would also explain why we see the same mutation pattern for Δapn1apn2, Δmag1apn1apn2, and Δmag1rad14 mutants at equimutagenic doses.

Figure 3 is also consistent with explanation proposed to interpret the enhanced mutagenesis of methyl methanesulfonate (MMS) in yeast defective in both BER and NER (Δapn1apn2rad14) relative to BER mutants (Δapn1apn2) (*14*). The authors suggested that an AP site was the common intermediate, although, as in our case, the AP endonucleases and NER did not completely compensate for each other, i.e., there was enhanced mutagenesis over background when either Apn1Apn2 or Rad14 was defective. Moreover, the hydrolytic instability of 3-MeA generated from Me-lex is greater than for 7-MeG, the predominant lesion formed from MMS (*46*), so more hydrolytic AP sites will be present after Me-lex exposure.

In summary, by combining a chemical and genetic approach, we have shown that in yeast both BER and NER can play a protective role against 3-MeA-mediated toxicity and mutagenicity, which may involve a common abasic site intermediate. However, the role of NER is appreciable only in a BER-defective background.

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