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Relationship between DNA Methylation and Mutational Patterns Induced by a Sequence Selective Minor Groove Methylating Agent*

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Me-lex, a methyl sulfonate ester appended to a neutral *N*-methylpyrrolecarboxamide-based dipeptide, was synthesized to preferentially generate N^3-methyladenine (3-MeA) adducts which are expected to be cytotoxic rather than mutagenic DNA lesions. In the present study, the sequence specificity for DNA alkylation by Me-lex was determined in the p53 cDNA through the conversion of the adducted sites into single strand breaks and sequencing gel analysis. In order to establish the mutagenic and lethal properties of Me-lex lesions, a yeast expression vector harboring the human wild-type p53 cDNA was treated *in vitro* with Me-lex, and transfected into a yeast strain containing the *ADE2* gene regulated by a p53-responsive promoter. The results showed that: 1) more than 99% of the lesions induced by Me-lex are 3-MeA; 2) the co-addition of distamycin quantitatively inhibited methylation at all minor groove sites; 3) Me-lex selectively methylated A's that are in, or immediately adjacent to, the lex equilibrium binding sites; 4) all but 6 of the 33 independent mutations were base pair substitutions, the majority of which (17/33; 52%) were AT-targeted; 5) AT → TA transversions were the predominant mutations observed (13/33; 39%); 6) 13 out of 33 (39%) independent mutations involved a single lex-binding site encompassing positions $A^{600-602}$ and 9 occurred at position 602 which is a real Me-lex mutation hotspot ($n = 9, p < 10^{-6}$, Poisson's normal distribution). A hypothetical model for the interpretation of mutational events at this site is proposed. The present work is the first report on mutational properties of Me-lex. Our results suggest that 3-MeA is not only a cytotoxic but also a premutagenic lesion which exerts this unexpected property in a strict sequence-dependent manner.

Most carcinogens and alkylating antineoplastic agents react with DNA to afford a diverse mixture of DNA lesions (1, 2). This complexity is a barrier to being able to quantitatively and qualitatively dissect out the biological role(s) of the individual DNA lesions relative to the mutagenic and/or toxic potency of the agent. Most methylating agents, for example, transfer methyl groups to as many as 10 nucleophilic nitrogens and oxygens in DNA (2). O^6-Methylguanine represents one of the few lesions extensively studied for which the mutagenic and toxic potential have been documented in detail (3).

In order to exercise some regulation over the alkylation pattern, we have prepared groove and sequence selective DNA damaging agents that provide significant control over the types of DNA lesions that are generated (4–7). One such compound is Me-lex[1] (Fig. 1), which is a methyl sulfonate ester appended to a neutral *N*-methylpyrrolecarboxamide-based dipeptide (6). The dipeptide equilibrium binds in the minor groove of DNA at $(A/T)_n$ sequences (5, 6). Based on empirical observation, Me-lex has the following binding preference: 5′-ATT > TTA > TAA (6). Reactivity at those sites is consistent with the mode of lex-DNA interaction determined by a series of structural studies (cited in Ref. 6). While such information on lex binding to DNA provides a basis to rationalize experimentally verified binding domains, it is not possible to predict *a priori* which A/T-rich regions will be preferred binding sites within large DNA fragments. As a consequence of the dipeptide's binding affinity, the Me-lex efficiently methylates DNA in the minor groove at the N^3 position of adenine (3-A) (6, 8). This contrasts with the methylation pattern induced by simple methylating agents such as methyl methanesulfonate ester (MMS), dimethyl sulfate, and *N*-methyl-*N*-nitrosourea (MNU), which predominantly yield the major groove N^7-methylguanine (7-MeG) adduct (2). For comparison, the ratio of 3-MeA to 7-MeG is approximately 1:10 for MMS based on *in vivo* and *in vitro* assays (1, 2), while it changes to 100:1 for Me-lex (8). The strong methylation preference of Me-lex for 3-A is also observed when it is incubated with cells in culture: under these conditions the only lesion detected is 3-MeA while MMS gives the traditional 3-MeA to 7-MeG 1:10 ratio (9). The significant increase in 3-MeA formation closely parallels the increased cytotoxicity of Me-lex *versus* MMS. This is consistent with the

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[1] The abbreviations used are: Me-lex, {1-methyl-4-[1-*methyl*-4-(3-methoxysulfonylpropanamido)pyrrole-2-carboxamido]-pyrrole-2-carboxamido}propane; 3-A, N^3 position of adenine; MMS, methyl methanesulfonate ester; MNU, *N*-methyl-*N*-nitrosourea; CENU(s), *N*-chloroethyl-*N*-nitrosourea(s); lex, lexitropsin; N^3-Alkyl-A, N^3-alkyladenine; CCNU, *N*-(2-chloroethyl-*N*-cyclohexyl-*N*-nitrosourea; O^6-Alkyl-G, O^6-alkylguanine; 3-MeA, N^3-methyladenine; 3-MeG, N^3-methylguanine; 7-MeG, N^7-methylguanine; EC, electrochemical; HPLC, high performance liquid chromatography; Me, methyl; PCR, polymerase chain reaction.

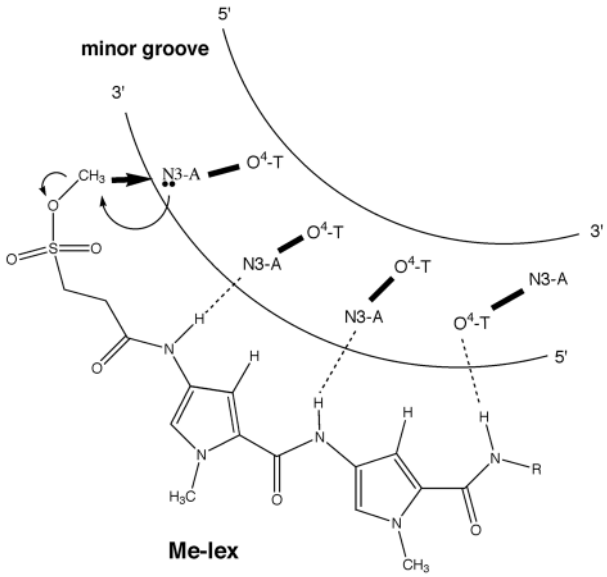

FIG. 1. **Equilibrium binding of Me-lex to DNA and formation of N^3-methyladenine.**

observation that Me-lex cytotoxicity is magnified in alkyladenine-DNA glycosylase (*Aag*) null ES cells (10) and in repair-deficient (tagA) bacteria (11). The Aag protein repairs a variety of DNA lesions including 3-alkyladenine (12) while tagA is specific for 3-alkyladenine. Recently, by combining a chemical and a genetic approach it has been shown that unrepaired 3-MeA have the potential to induce sister chromatid exchanges, chromosome aberrations, cell cycle arrest at the G_1/S boundary, p53 induction, and apoptosis (9), but exactly how this is achieved is not yet clear. Those combined results point toward 3-MeA being mainly a lethal lesion (10, 13). If this were true the 3-MeA specific inducer Me-lex can be considered a new potential antineoplastic agent. Furthermore, it might be expected that such a compound would combine a high cytotoxicity and a low mutagenicity, avoiding (or diminishing) the undesired and well documented carcinogenic property of alkylating agents used in cancer therapy (14, 15). Therefore, Me-lex may potentially have a higher therapeutic index with respect to other antineoplastic compounds. In this light, we wanted to investigate the relationship between A-specific DNA lesions and mutations induced by Me-lex at the nucleotide level. Using the human p53 tumor suppressor gene cDNA inserted in a yeast expression vector as a target (16), the sequence specificity for DNA alkylation by Me-lex was determined through the conversion of the adducted sites into single strand breaks by sequential neutral thermal hydrolysis and exposure to base. These results are compared with the lethal and mutagenic effects of Me-lex using an *in vitro* mutagenesis protocol for plasmid DNA modification, and exploiting a DNA repair-proficient haploid *Saccharomyces cerevisiae* strain for the processing of DNA lesions into mutations (17).

MATERIALS AND METHODS

Compounds—Unless stated otherwise, reagents of the highest purity were purchased from Sigma or Aldrich (Milwaukee, WI). Me-lex was prepared as described previously (6). Restriction enzymes, phosphatases, kinases, and DNA polymerases were obtained from New England Biolabs (Beverly, MA).

Quantitation of Adducts from Me-lex and MMS—Calf thymus DNA (1 mM) was incubated with 100 μM Me-lex or 5 mM MMS in 10 mM buffer Tris-HCl buffer (pH 7.6) for 24 h at room temperature, in the presence or absence of 100 μM distamycin. The DNA was precipitated, washed, and dissolved in Tris-HCl buffer (pH 7.0). The solution was heated at 100 °C for 30 min to selectively release *N*-alkylpurines from DNA. The solution was then treated with ice-cold 0.1 N HCl to precipitate the DNA, and the supernatant collected and analyzed for 3-MeG, 7-MeG, and 3-MeA using reverse phase HPLC: column, ODS C18 YMC (5 mm × 25 cm); column temperature, 40 °C; solvent: 95% 0.1 M NaOAc (pH 5.2), 5% MeOH; UV (λ, 270 nm) and electrochemical (EC) (3-MeG and 7-MeG) detection.

Reaction of Methylating Agents with DNA Restriction Fragments—The preparation of the 5′-^{32}P-labeled restriction fragments from the plasmid DNA was performed as described previously (18). Briefly, plasmid pLS76 containing the p53 cDNA, was restricted to obtain fragments including positions 169 to 1037 (*Psh*AI site (position 169), *Nco*I site (position 476), *Bsu*36I (position 667), *Stu*I (position 1037)). The *Psh*AI/*Nco*I DNA fragment (5′-^{32}P-labeled on the transcribed (noncoding) strand at the *Nco*I site) was obtained using *Nco*I restriction endonuclease, phosphatase, and kinase treatments, followed by a final *Psh*AI digestion and polyacrylamide gel purification. The 5′-^{32}P-end-labeled *Nco*I/*Bsu*36I DNA fragment (5′-^{32}P-labeled on the nontranscribed (coding) strand at the *Nco*I site) was obtained by initial endonuclease restriction with *Nco*I, followed by sequential treatment with calf intestine alkaline phosphatase, phosphorylation with T4 kinase in the presence of [γ-^{32}P]ATP. The DNA was then restricted with *Bsu*36I and the labeled fragment isolated from a 6% polyacrylamide gel. The same procedure was used to prepare the 5′-^{32}P-end-labeled *Bsu*36I/*Nco*I DNA fragment (5′-^{32}P-labeled on the transcribed (noncoding) strand at the *Bsu*36I site), except that the order of enzyme digestion was reversed. The 5′-^{32}P-end-labeled *Stu*I/*Bsu*36I DNA fragment (5′-^{32}P-labeled on the transcribed (noncoding) strand at the *Stu*I site) was obtained by initial endonuclease restriction with *Stu*I, followed by sequential treatment with calf intestine alkaline phosphatase, phosphorylation with T4 kinase in the presence of [γ-^{32}P]ATP. The DNA was then restricted with *Bsu*36I and the labeled fragment isolated from a 6% polyacrylamide gel.

The p53 5′-labeled restriction fragment (200,000 cpm) and sonicated calf thymus DNA (83 μM final concentration) in 10 mM Tris, 1 mM EDTA buffer (pH 7.6) were incubated with Me-lex (final concentrations in figure legends) in the presence or absence of 100 μM distamycin for 2 h at 37 °C. The DNA was EtOH/NaOAc precipitated, and washed with cold 70% EtOH. The DNA was dissolved in 30 μl of 10 mM Tris buffer (pH 7.0), and heated at 90 °C for 15 min. The DNA was then re-precipitated, washed as described above, and dried under vacuum. The pellet was resuspended in 100 μl of 1 M piperidine and heated at 90 °C for 30 min. The piperidine was removed by repeated lyophilization and the DNA resuspended in 10 μl of 80% formamide (v/v), 50 mM Tris borate buffer (pH 8.3), 1 mM EDTA, 0.1% xylene cyanol (w/v), and 0.1% bromphenol blue (w/v). The radioactivity of an aliquot from each sample was determined; the remaining sample was denatured (3 min at 90 °C) and then chilled in ice-water. An equivalent amount of radioactivity for each reaction was loaded on a 12% polyacrylamide denaturing gel (7.78 M urea) and run at 65 W constant power. For sequence markers, lanes containing Maxam-Gilbert G and G + A (and in some cases C and C + T) reactions were included in each gel. The gels were dried and analyzed on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). In all cases cleavage bands were assigned by underexposing the gel so that the identification of the site was unequivocal.

Vectors, Strain and Media—The yeast expression vector pLS76 harboring a human wild-type *p53* cDNA under the control of an ADH1 promoter and containing the LEU2 selectable marker, the plasmid pRDI-22 used for gap repair assay, and 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) used as recipient of pLS76 and for gap repair assays were previously described (16). The p53-dependent reporter *ADE2* gene allowed the phenotypic selection of p53 mutants (17). Standard yeast manipulations were performed as described (19).

DNA Modification, Analysis, and Transfection—Me-lex was dissolved in absolute EtOH immediately before the treatment. 1.5 μg of plasmid pLS76 DNA was treated with different Me-lex concentrations in 10 mM Tris HCl (pH 7.4), 1 mM EDTA, 50% EtOH for 1 h at 37 °C. DNA was purified by 3 EtOH precipitations, washed with 70% EtOH, and resuspended in sterile water. Damaged or undamaged vectors were then transfected into yIG397 cells by electroporation and transformants were plated on selective synthetic medium containing 1 M sorbitol. After 3 days of incubation at 30 °C colonies appeared. The selection for the plasmid marker (LEU2) allowed an indirect determination of the lethal effect of the damaging treatment as the number of transformants scored in transfections with damaged plasmids with respect to that obtained with undamaged vector. As transformation plates contained a minimal amount of adenine, adenine auxotrophs were able to achieve only a few cell divisions and thus produced smaller red colonies. Spontaneous and induced mutant frequency were defined as the number of small red

TABLE I
In vitro methylation of calf thymus DNA

Compound	Adduct yields[a]			Adduct ratio	
	3-mA	7-mG	3-mG	3-mA/7-mG	3-mA/3-mG
mM					
$MeOSO_2(CH_2)_2$-lex[b]					
0.1	20.35 ± 1.71	0.16 ± 0.03	0.05 ± 0.01	127.2	407.0
0.1 + 0.1 Distamycin	ND[c]	0.24 ± 0.16	ND		
MMS[b]					
5.0	0.68 ± 0.08	3.47 ± 0.34	0.08 ± 0.02	0.2	8.5
5.0 + 0.1 Distamycin	ND	3.54 ± 0.24	ND		

[a] Picomoles of adduct/μg of DNA.
[b] Incubation time, 24 h at ambient temperature.
[c] ND, not detected, *i.e.* less than, 0.008 and 0.005 pmol of adduct/μg of DNA for 3-mA and 3-mG, respectively.

colonies with respect to the total number of transformants. The fold of mutant induction was defined as the ratio between the mutant frequency observed with damaged vector with respect to the spontaneous frequency. Phenotypic mutant clones were purified and characterized at the molecular level as described previously (16). Only independent mutants were included in the mutation spectrum; those carrying the same genetic alterations were considered independent only if they were isolated from different transfections.

RESULTS

Me-lex Induces Almost Exclusively 3-MeA—To study the biological consequences of 3-MeA, a synthetic compound was developed to target alkylation to the N^3 position of adenine (3-A). Me-lex is composed of an S_N2 methyl donating domain and a lexitropsin dipeptide, which equilibrium binds in the minor groove at A:T-rich regions in double-stranded DNA. Specific minor groove binding at A:T base pairs limits alkylation to the minor groove atoms and enhances alkylation at 3-A that are in a lex-binding domain. After *in vitro* DNA alkylation with Me-lex (100 μM) and MMS (5 mM), the yields of the major (7-MeG) and the minor groove (3-MeG and 3-MeA) adducts were determined using reverse phase HPLC coupled with UV and EC detection. EC detection was required to measure the 3-MeG and 7-MeG adducts when formed in very low yields. More than 99% of the lesions induced by Me-lex were 3-MeA, while 7-MeG represents the major adduct induced by MMS (82%) (Table I). Similar results were obtained with MNU (data not shown). The absolute amount of 3-MeG was the same for the two methylating agents. The specificity for alkylation at adenines by Me-lex was judged by determining the relative abundance of 3-MeA *versus* 7-MeG. As expected, the ratio of 3-MeA to 7-MeG was >120:1 for Me-lex while it was 1:5 for MMS. Thus, the relative abundance of minor to major groove adduction is ~600-fold increased with Me-lex compared with MMS. Assuming a linear dose/lesion relationship, it can be calculated that at equimolar concentration Me-lex caused a 1500-fold increase in the absolute yield of 3-MeA and approximately a 30-fold increase in minor groove G-lesion compared with MMS. Therefore, it appears that while Me-lex may enhance the delivery of methyl groups to potential nucleophilic atoms in the minor groove, there is an overwhelming selection for the 3-A site. Interestingly, the co-addition of distamycin, which binds to the same recognition sites as the lex peptide (5), quantitatively inhibited methylation by both Me-lex and MMS at all minor groove sites, *i.e.* 3-MeA and 3-MeG (Table I).

Me-lex Induces 3-MeA at Lex-binding Sites—The sequence-dependent DNA alkylation by Me-lex in different restriction fragments is shown in Figs. 2–5. The assignment of the cleavage sites was done using gels analyzed under conditions of lower exposure. In order to generate strand breaks at potential 7-MeG and 3-MeA adduction sites, the DNA was subjected to neutral thermal hydrolysis. This procedure to release *N*-alkylpurine lesions is identical to that described for the quantitative

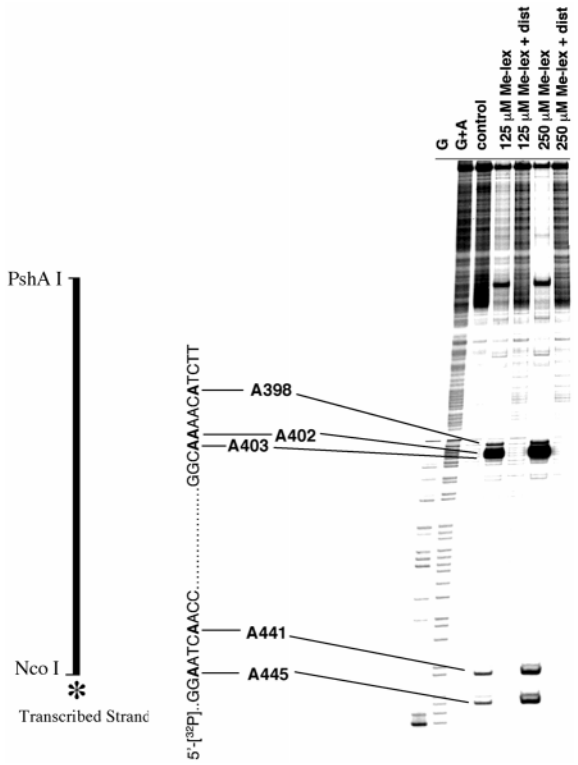

FIG. 2. **Cleavage of the *Psh*AI/*Nco*I DNA fragment (5′-^{32}P-labeled (*) on transcribed (noncoding) strand at the *Nco*I site) of the p53 cDNA, induced by Me-lex at the indicated concentrations in the presence or in the absence of distamycin (*dist*), by sequential heating at neutral pH and exposure to hot piperidine.** *Control,* undamaged DNA subjected to sequential heating at neutral pH and exposure to hot piperidine; *G* and *G + A*, lanes containing Maxam-Gilbert G and G + A sequence markers. The assignment of the cleavage sites was done using gels analyzed under conditions of lower exposure.

HPLC adduct analysis. Consistent with the HPLC-based adduct studies, the sequencing gels demonstrate that DNA methylation by Me-lex was almost exclusively at A, with virtually no G bands being observed.

The methylation at A is not random: only A within (or in the vicinity of) A/T-rich lex equilibrium-binding sites (≥3 A/T base pairs) are targeted (Figs. 2–5). The strongest adduction sites in the restriction fragments are at A^{398}, A^{402}, A^{403}, A^{441}, A^{445} (Fig. 2), A^{601}, A^{602}, A^{616}, A^{617}, A^{635}, and A^{636} (Fig. 3), A^{598}, A^{583}, A^{553} (Fig. 4), and A^{982} (Fig. 5). In addition to the preference for A/T-rich regions, there is a strong preference for specific A's within the binding sites. For example, the bands at A^{635} and A^{636} are of equal intensity, while the two adjacent A's (A^{633} and A^{634}) are not methylated at all.

Me-lex Treatment Is Lethal and Mutagenic: Induction of p53

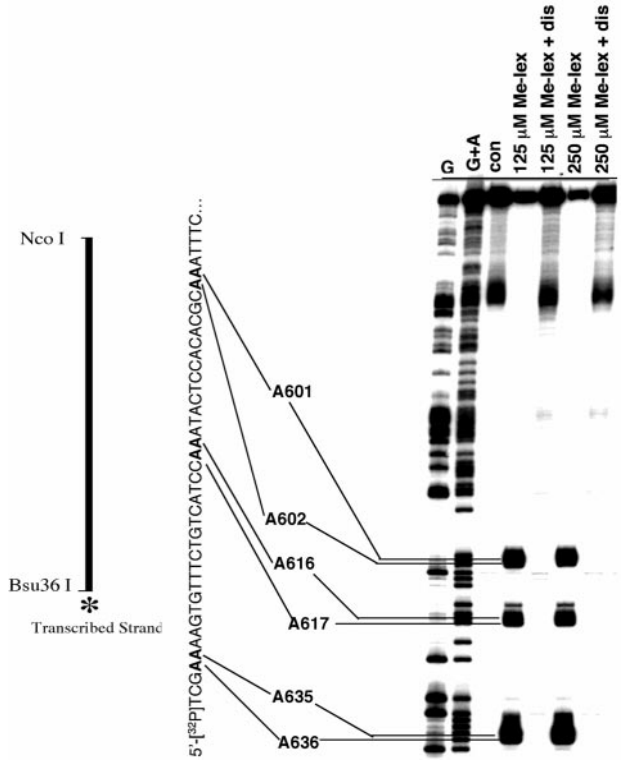

FIG. 3. **Cleavage of the 5′-^{32}P-end-labeled *Nco*I/*Bsu*36I DNA fragment (5′-^{32}P-labeled (*) on the nontranscribed (coding) strand at the *Bsu*36I site) induced by Me-lex at the indicated concentrations in the presence or in the absence of distamycin (*dis*), by sequential heating at neutral pH and exposure to hot piperidine.** *Control,* undamaged DNA subjected to sequential heating at neutral pH and exposure to hot piperidine; *G* and *G* + *A*, lanes containing Maxam-Gilbert G and G + A sequence markers.

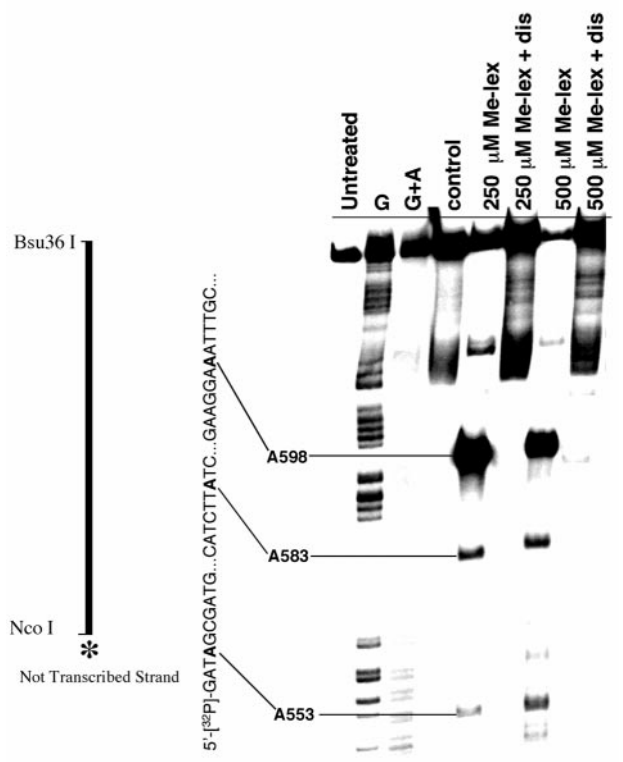

FIG. 4. **Cleavage of the 5′-^{32}P-end-labeled *Bsu*36I/*Nco*I DNA fragment (5′-^{32}P-labeled (*) on the nontranscribed (coding) strand at the *Nco*I site) of the p53 cDNA, induced by Me-lex at the indicated concentrations in the presence or in the absence of distamycin (*dis*), by sequential heating at neutral pH and exposure to hot piperidine.** *Control,* undamaged DNA subjected to sequential heating at neutral pH and exposure to hot piperidine; *G* and *G* + *A*, lanes containing Maxam-Gilbert G and G + A sequence markers.

Mutants—Plasmid pLS76 was damaged *in vitro* with increasing Me-lex concentrations (Table II). Damaged or undamaged plasmids were transfected into *yIG397*. Transformants were selected on plates lacking leucine but containing sufficient adenine for adenine auxotrophs to grow and turn red. Survival showed a Me-lex concentration-dependent decrease while the mutant frequency increased in a concentration dependent way (Table II). Only 5 mM induced mutants were purified for the molecular analysis as the level of induction (47-fold above background) guaranteed that 97% of the mutants were actually drug-induced.

Me-lex Induced AT-targeted p53 Mutations at Lex-binding Sites—After purification by successive platings, 90 Me-lex-induced mutants were cultured for plasmid recovery. Twenty-eight mutant clones were negative for p53 cDNA amplification. To determine whether adenine auxotrophy was due to p53 mutations rather than, for example, mutations in the promoter, the p53 open reading frame from nucleotides 125 to 1122 was PCR amplified from the remaining 62 ade$^-$ leu$^+$ clones and tested by gap repair. Unpurified PCR product and *Hin*dIII-*Stu*I linearized pRDI-22 (16) were co-transfected by electroporation into yIG397. The *Hin*dIII-*Stu*I digested pRDI-22 gapped plasmid has two regions homologous to the terminal regions of the PCR product. After co-transfection and followed by homologous recombination *in vivo* (gap repair) a p53 expression vector is reconstituted having the wild-type promoter region (derived from pRDI-22) and the core region of the p53 open reading frame (derived from the PCR product). If the clone was initially ade$^-$ due to a mutation in the promoter region, the gap repaired transformants will originate almost exclusively white normal size colonies on limiting adenine plates (no small red colonies, gap repair negative), since the promoter in the gap-repaired plasmid, derived from pRDI-22, is wild-type. On the contrary, in case the PCR product contains a single mutation, approximately 100% of the derived transformed clones will give rise to small red colonies (gap repair positive) (see Fig. 1 in Ref. 16). PCR fragments from 27 clones gave a low percentage of red colonies, suggesting that the mutation causing adenine auxotrophy lay outside the region tested by gap repair. The remaining 35 gave 100% red colonies after gap repair, and p53 mutations were found by DNA sequencing in every case. However, two mutants could not be considered independent because both derived from the same damaging treatment and showed the same mutation. Only one of these two mutants was considered for further analysis. All of the remaining 34 independent mutants but one (X272) contained a single mutation (Table III).

To be conservative, clone X272 was not considered for further analysis since the distance between the two base pair substitutions observed (less than 10 base pairs apart) did not guarantee their independent origin. Thirty-four mutants evidenced 33 independent mutations (Table IV). Except for 6 frameshifts, all mutations were base pair substitutions, the majority of which (17/33; 52%) were AT-targeted. AT → TA transversions represented the predominant class of base pair substitutions (13 out of 33, 39%). Thirteen out of 33 (39%) independent mutations (base pair substitutions and deletions) involved a single lex-binding site encompassing positions A$^{600-602}$.

The number of sites in the p53 cDNA where a mutation is phenotypically selectable was calculated to be 542 base pairs according to a yeast-based functional assay (20), and about 450 considering the mutations found in tumors and cell lines (21).

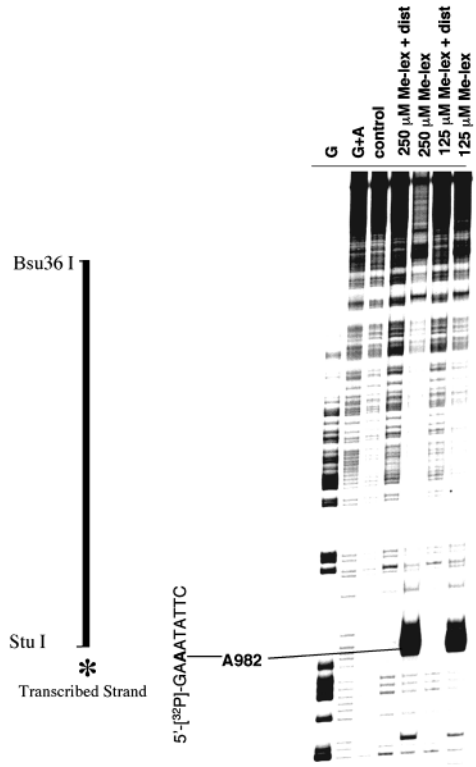

FIG. 5. **Cleavage of the 5′-^{32}P-end-labeled *Stu*I/*Bsu*36I fragment (5′-^{32}P-labeled (*) transcribed (noncoding) strand at the *Stu*I site) induced by Me-lex at the indicated concentrations in the presence or absence of distamycin (*dist*), by sequential heating at neutral pH and exposure to hot piperidine.** *Control,* undamaged DNA subjected to sequential heating at neutral pH and exposure to hot piperidine; *G* and *G* + *A*, lanes containing Maxam-Gilbert G and G + A sequence markers.

TABLE II
Survival and mutation induction in undamaged and Mc-lex damaged pLS76 after passage through yIG397 strain

Me-lex	Survival	p53 mutant frequency	F^a
mM	%		
0	100 ($n = 6$)[b]	1.8×10^{-4} (6/32,601)	1
0.5	57 ± 22 ($n = 10$)[b]	3.3×10^{-4} (15/45,669)	1.8
2	29 ± 6 ($n = 4$)[b]	22×10^{-4} (36/16,366)	12
4	14 ± 4 ($n = 5$)[b]	60×10^{-4} (99/16,466)	33
5	8.5 ± 6.3 ($n = 9$)[b]	84×10^{-4} (121/14,397)	47

[a] *F*, mutant frequency of Me-lex damaged/mutant frequency of undamaged DNA.
[b] *n*, number of independent transfections, *i.e.* number of transfections performed with DNA from different damaging treatments.

Hence, finding 9 identical independent mutations affecting the same nucleotide (position 602) among a collection of 27 base pairs substitutions is an extremely rare event on the basis of random distribution ($n = 9$, $p < 10^{-6}$, Poisson's normal distribution). Position 602 (codon 201, 5′-A^{602}AATTT^{597}-3′), should be considered a real Me-lex-induced mutation hotspot.

DISCUSSION

Me-lex has been designed to be preferentially targeted at DNA sites (N^3 of adenine) whose alkylation is expected to form cytotoxic rather than mutagenic DNA lesions. In the present work, we determined both the localization of lesions at the nucleotide level and the mutation spectrum induced by Me-lex in human p53 cDNA. The data allow us to determine whether the adduct frequency is predictive of the mutation spectrum and to obtain insights into the mutagenic and/or toxic properties of Me-lex specific lesions, *i.e.* A-targeted lesions at lex-binding sites (3-MeA).

Based on sequencing gel studies, the lex-based methylating sulfonate ester selectively methylates A that are in, or immediately adjacent to, the dipeptide's minor groove equilibrium-binding sites (6). This process involves initial association of the lex moiety with its binding site followed by transfer of the methyl group from the sulfonate ester to accessible nucleophilic atoms in the minor groove. If Me-lex was quantitatively associated with the minor groove, little methylation at major groove sites would be expected. However, the quantitative data in Table I show that Me-lex does methylate DNA in both grooves, although the normal dominance of major groove alkylation, *i.e.* 7-G (2), is clearly surmounted. The relative yields of 3-MeA to 7-MeG is 600-fold higher with Me-lex than with other methylating agents (MMS, see Table I; MNU data not shown). At the nucleotide level, however, the gel studies provide no evidence that Me-lex reacts at 7-G, which is located in the major groove. This discrepancy is likely due to the different sensitivities of the two approaches (HPLC/UV-EC *versus* sequencing gel). Indeed, less than 1% of the lesion induced by Me-lex was characterized as 7-MeG by HPLC/UV-EC detection, a level which would be not appreciable with the sequencing gel approach. The co-addition of distamycin (a competitive inhibitor of lex binding) does not affect the yield of 7-MeG, but quantitatively inhibits 3-MeA and 3-MeG formation, confirming that Me-lex reactivity is almost totally dependent by the lex portion of the molecule.

Me-lex has a pronounced orientational preference for A within its binding site (5). Based on empirical observation, Me-lex has the following binding preference: 5′-ATT > TTA > TAA (6). In fact, the preferred methylation sites in Figs. 2–5 result from lex binding to such sites (Fig. 6). The 2-base pair offset between the coding (nontranscribed) and noncoding (transcribed) strands in Fig. 6 depicts the three-dimensional topology of the minor groove and illustrates that lex does not necessarily associate with both Watson-Crick base pairs (6). In addition, at A ≥ 4 sequences, the methylation takes place toward the 5′ terminus of the A-tract as previously observed in other A ≥ 4 runs (5). The orientational preference in this case results from the asymmetry of A ≥ 4 runs (22).

The alkylation pattern observed with Me-lex is very different from that observed for CENU-lex (5, 18), another minor groove binding alkylating agent. Unlike Me-lex, CENU-lex does not directly transfer the methyl group to DNA. CENU-lex undergoes hydrolysis and generates a diffusible alkylating agent (5). Comparison of the two compounds in the same restriction fragments shows that CENU-lex, but not Me-lex, still induces G targeted lesions clearly observed at the nucleotide level (18). Although both agents often alkylate the same A/T-rich region, there is almost no overlap at the nucleotide level and they show an opposite orientational preference within the same lex-binding site: Me-lex preferentially methylates the 5′-A, while CENU-lex alkylates the 3′-A (this work; Ref. 18) in a run of 3 or 4 A's. An example of the difference between the two compounds is at the A$^{633-636}$-binding site: Me-lex methylates at A^{635} and A^{636}, while CENU-lex generates lesions specifically at A^{633}. The origin of the difference between the two compounds may reflect the preference of the different alkylating agents (methyl sulfonate *versus* alkanediazonium ion), and/or the affinity binding preferences of Me-lex and CENU-lex (5, 6).

The localization of A lesions and mutations at the p53 cDNA

TABLE III
Me-lex induced mutation spectra at the p53 cDNA after in vitro treatment of plasmid pLS76 and passage into yIG397 cells

Mutant number[a]	Mutation type[b]	Nucleotide position	5′ → 3′[c] (NT strand)	Amino acid change
Single base pair substitutions				
X183	GC → CG	**256**	CCCT*gCA* → cCA	**Ala^{86}→ Pro**
X47	AT → GC	403	GTTT*tGC* → *cGC*	Cys^{135}→ Arg
X42	GC → AT	438	GCTG*TGg* → *TGa*	Trp^{146}→STOP
X18	GC → AT	455	ACCC*CcG* → *CtG*	Pro^{152}→ Leu
X169	GC → TA	595	GGAA*gGa* → *tGA*	**Gly^{199}→STOP**
X17	AT → TA	**602**	AAAT*TtG* → *TaG*	Leu^{201}→STOP
X24	AT → TA	**602**	AAAT*TtG* → *TaG*	**Leu^{201}→STOP**
X101	AT → TA	**602**	AAAT*TtG* → *TaG*	**Leu^{201}→STOP**
X148	AT → TA	**602**	AAAT*TtG* → *TaG*	**Leu^{201}→STOP**
X158	AT → TA	**602**	AAAT*TtG* → *TaG*	**Leu^{201}→STOP**
X177	AT → TA	**602**	AAAT*TtG* → *TaG*	**Leu^{201}→STOP**
X209	AT → TA	**602**	AAAT*TtG* → *TaG*	**Leu^{201}→STOP**
X251	AT → TA	**602**	AAAT*TtG* → *TaG*	**Leu^{201}→STOP**
X274	AT → TA	**602**	AAAT*TtG* → *TaG*	**Leu^{201}→STOP**
X370	GC → TA	610	*gAG*TATTTT → *tAG*	Glu^{204}→STOP
X132	AT → TA	625	TGAC*aGA* → *tGA*	Arg^{209}→STOP
X227	AT → GC	712	CATG*tGT* → *cGT*	Cys^{238}→ Arg
X136	AT → TA	714	CATG*TGt* → *TGa*	Cys^{238}→STOP
X43	AT → GC	716	TGT*AaC* → *AgC*	Asn^{239}→ Ser
X172	AT → TA	721	CAGT*tCC* → aCC	Ser^{241}→ Thr
X318	AT → CG	736	CGGC*aTG* → *cTG*	Met^{246}→ Leu
X215	AT → TA	770	CACA*CtG* → *CaG*	Leu^{257}→ Gln
X302	GC → TA	818	GGTG*CgT* → *CtT*	Arg^{273}→ Leu
X145	GC → CG	827	TTGT*GcC* → *GgC*	Ala^{276}→ Gly
X151	GC → AT	835	TCCT*gGG* → *aGG*	Gly^{279}→ Arg
X155	GC → TA	856	AGAG*gAA* → *tAA*	Glu^{286}→STOP
X94	GC → CG	1009	TGAG*cGC* → *gGC*	**Arg^{337}→ Gly**
Other mutations				
X272	AT → CG	590	CCGA*GtG* → *GgG*	Val^{197}→ Gly
	AT → GC	598	AGGA*aAT* → *gAT*	Asn^{200}→ Asp
X146	−1C	171–173	AGACCCA → AGACCA	Frameshift
X152	−1T	600–602	AAATTTG → AAATTG	Frameshift
X228	−1T	600–602	AAATTTG → AAATTG	Frameshift
X273	−1T	600–602	AAATTTG → AAATTG	Frameshift
X286	−1T	600–602	AAATTTG → AAATTG	Frameshift
X319	−1T	633–636	CACTTTC → CACTTC	Frameshift

[a] Independent mutants showing the same mutations are identified by their number.
[b] Mutations at the GC (or AT) base pair are reported as induced by G- (or A-) targeted lesions.
[c] Lower case; mutated base; underlined, p53 codon. Bold: site or type of mutations not found thus far in human tumours or cell lines (9330 mutations, Ref. 20).

TABLE IV
Molecular features of Me-lex-induced p53 mutations selected in yIG397

Independent mutations[a]	33 (100%)
Base pair substitutions	27 (82%)
GC targeted	10 (30%)
GC → AT	3 (9%)
GC → TA	4 (12%)
GC → CG	3 (9%)
AT targeted	17 (52%)
AT → TA	13 (40%)
AT → CG	1 (3%)
AT → GC	3 (9%)
Frameshift	6 (18%)
−1 A/T	5 (15%)
−1 G/C	1 (3%)

[a] The 2-base pair substitutions found in mutant number X272 were not considered independent because of their distance (less than 10 base pairs).

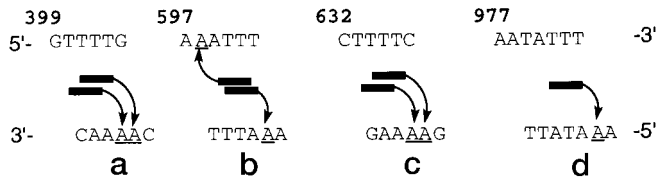

FIG. 6. **Me-lex orientational binding at four specific equilibrium binding sites (*a–d*) and indication of alkylation positions.**

sequence is displayed in Fig. 7. With the exception of the A^{597}AATTT^{602} lex-binding site, where 39% of all independent mutations occurred (13/33), all the other lesion hotspots are cold spots for mutations, *e.g.* T^{400}TTT^{403}. At the nucleotide level, there is also no correlation between the abundance of adduction at the A^{597}AATTT^{602} sequence and the frequency of mutations: A^{598} was abundantly damaged by Me-lex, but only a single base pair substitution was found at this position. Apart from the 4 frameshifts (−1 T) occurring in the T^{600}TT^{602} sequence, 9 independent mutations occurred at nucleotide A^{602}, although position 601 is the most heavily damaged of the two A's. In the same experimental system, also CENU-lex showed no correlation between the abundance of adduction and the frequency of mutations (18). It should be pointed out that the mutational spectrum induced by Me-lex is in sharp contrast to that previously induced by a nonequilibrium binding methylating agent, MMS, at the URA3 locus in yeast: MMS induces mostly GC → AT transitions (23).

There is strong evidence that 3-MeA can be considered a lethal lesion. First, unrepaired 3-MeA lesions are toxic in *Escherichia coli* (11, 13). In particular, different DNA repair-deficient *E. coli* strains (the *tagA*, the *tagAalkA* double mutant, and the *nfo*) were extremely sensitive to Me-lex. Furthermore, the fact that the *nfo* mutant strain appeared at least an order of magnitude less sensitive than *tagA* (or *tagAalkA* double mutant) to Me-lex, may suggest that the 3-MeA adduct rather than an AP site derived from the primary lesion is responsible

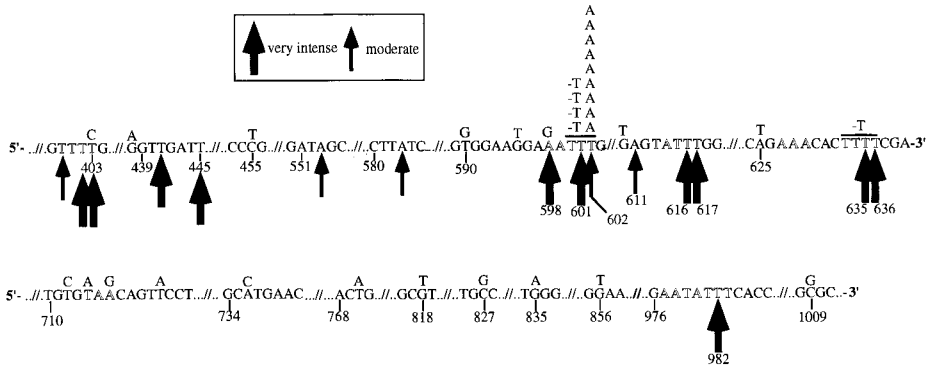

FIG. 7. **Localization and band intensity of Me-lex A-specific cleavage sites and induced mutations in the *Psh*AI/*Stu*I DNA fragment of the p53 cDNA sequence (nontranscribed (coding) strand) are reported above and below the sequence, respectively.** For simplicity G lesions are not reported.

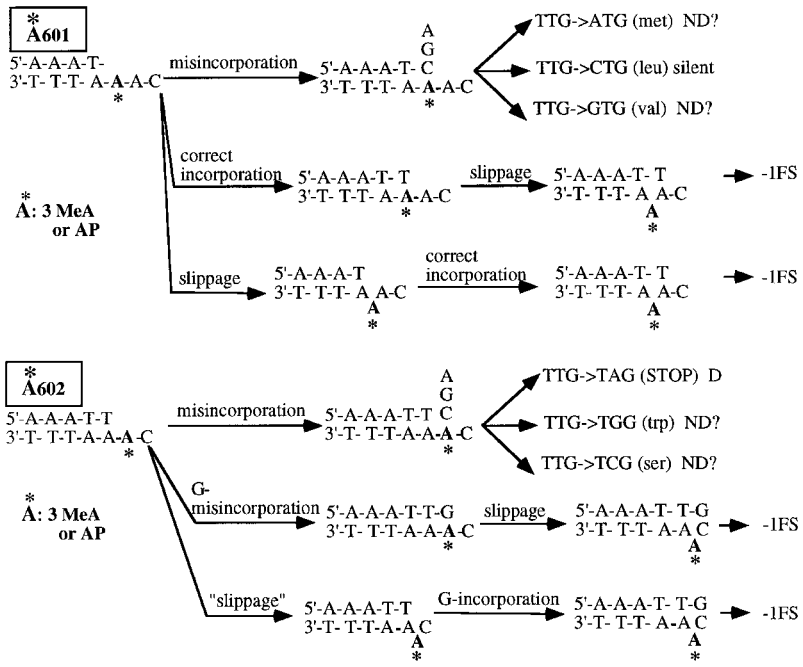

FIG. 8. **Hypothetical model explaining mutational events at the lex-binding site encompassing positions $A^{597}AATTT^{602}$ where 13 out of 33 (39%) independent Me-lex induced mutations occurred.** *ND,* not detectable; ? indicates that it is not certain that such amino acid substitution can inactivate p53 transactivation function; *−1FS,* −1 frameshift.

for toxicity in bacteria.[2] Consistently, Xiao and Samson showed that in *S. cerevisiae* MMS toxicity is orders of magnitude higher in 3-methyl-adenine-DNA glycosylase strain deficient for 3-MeA repair compared with apn1 mutant strain (24). In addition, mouse embryonic stem cells deficient in the DNA repair enzyme responsible for the first step of the base excision removal of 3-MeA (3-alkyladenine-DNA glycosylase, *Aag*) proved very sensitive to killing by Me-lex (10). Second, it has been shown that unrepaired 3-MeA have the potential to induce SCEs, chromosome aberrations, G_1/S phase arrest, p53 induction, and apoptosis (9). Third, 3-MeA is a replication blocking lesion since different DNA polymerase were shown to terminate one nucleotide 3′ to the site of the adenine residue in the template (25). Fourth, 3-MeA (possibly through a AP site intermediate) can be mutagenic under conditions in which replication is blocked (SOS conditions) in *E. coli* (26). Finally, recent structural information on DNA polymerases provides a plausible mechanism for the biological and biochemical effects of 3-MeA. For different DNA polymerases, contacts between key amino acid residues and the N^3 atom of purines in the template strand have been established (27–29). Furthermore, for polymerase β, such contact has been shown to be crucial for catalytic activity (30). All these results are consistent with 3-MeA being a lethal noncoding lesion. At the nucleotide level, this fact would explain why heavily adducted sites were found cold spot for mutation in the present study. This can be true for all lex-binding sites with the notable exception of the $A^{597}AATTT^{602}$ site. If 3-MeA is a lethal lesion, why is it mutagenic at that particular site and almost exclusively there? Abundantly alkylated A sites could appear as cold spot for mutation because a change at the DNA level compatible with the mutational specificity of such lesion(s) will not produce any amino acid or phenotypic change. The insertion of an A opposite 3-MeA during DNA replication ("A rule") or a G opposite an abasic (AP) site (31) generated by 3-MeA will eventually produce AT → TA or AT → CG transversions. Many of the amino acid substitutions deduced from AT-targeted mutations at hotspots for 3-MeA formation have been found in tumors or cell lines (IARC data base, 9330 mutations) (positions 400 and 402, codon 134; position 403, codon 135; position 617, codon 206; position 636 codon 212) suggesting that these mutations are inactivating p53 function. No amino acid substitutions deduced from AT-targeted transversions was found at 3 hotspots for 3-MeA formation (positions 601 and 602, codon 201; position 616, codon 206). Position 441 (codon 147) is the only position that despite being heavily damaged had to be cold for mutation because all possible base pair substitutions will invariably produce synonymous codons. Therefore, it is possible that some (but not all) of the heavily damaged sites appear as cold spots for mutation because the mutation generated at the DNA level will cause no (or marginal) phenotypic change and will therefore escape the screening.

[2] B. Gold, unpublished data.

How can we explain the presence of a mutational hotspot at the $A^{597}AATTT^{602}$ lex-binding site? The coexistence of both −1 T frameshifts and AT → TA transversions suggests that the 3-MeA formed mainly at A^{601}, but to a significant extent also at A^{602}, dramatically impairs DNA replication at those sites. This would be in keeping with the notion that 3-MeA is a replication blocking lesion. Fig. 8 shows a model for the interpretation of our mutagenesis results. Mutations can derive from 3-MeA at A^{601} (*upper panel*) or at A^{602} (*lower panel*) by direct miscoding or after depurination of 3-MeA to give an abasic site. Miscoding events during DNA replication may occur at both sites. All missense mutations at A^{601} and 2 out of three at A^{602} will code for amino acid substitutions not reported so far in the IARC data bank or will cause no amino acid substitution. This might suggest that such mutations are not able to functionally inactivate p53. On the contrary the noncoding AT → TA transversion at A^{602} (Leu → STOP) is selectable since the truncated p53 is certainly functionally inactive. This would probably explain why only base pair substitutions at 602 were detected. At both positions, −1 frameshift (−1 FS in Fig. 8) could be derived from a misincorporation event followed by a slippage event (or vice versa). Such a mechanism, involving transient misalignment, has been proposed previously to interpret the fidelity of different DNA polymerases (32–34). It is worth noting that all the other lex-binding sites which are hotspots for DNA damage (Fig. 7) but that are shorter than the $A^{597}AATTT^{602}$ sequence, or do not have a palindromic A/T sequence, appeared to be invariably cold spots for mutation. The sequence context must extend beyond the single codon. This is illustrated by the fact that codons 201 and 206 that have the same TTG (Leu) sequence and are methylated to a similar extent were found to behave extremely different as far as mutability is concerned. Alternative explanations for the absence of correlation between the abundance of adduction and the frequency of mutations that we cannot exclude at present are: 1) the possible involvement of sequence-dependent repair of 3-MeA or abasic sites; and 2) that (premutagenic) lesions other than 3-MeA are preferentially formed at this sequence.

The major classes of mutations induced by (endogenous) abasic sites in yeast are transversions to CG base pairs (in particular AT → CG) (31). These type of mutations are relatively rare in our collection of mutants. These results are consistent with the hypothesis that the mutation spectrum induced by Me-lex is likely 3-MeA induced, and only marginally due to the mutagenic processing of 3-MeA derived abasic sites.

The present work represents the first report on the mutational properties of Me-lex, a methylating agent whose reactivity toward DNA is absolutely dependent on the lex portion of the molecule. As expected, Me-lex appeared much more toxic than mutagenic when compared with MNU. For example, MNU treatments inducing 94% survival can increase mutation frequency 100-fold above background, while Me-lex treatments inducing 57% survival induce only a 2-fold increase on the mutation frequency above background (MNU[3]; Me-lex: this work). These results are consistent with the finding that Me-lex produces almost exclusively toxic lesions (3-MeA), while only 9% of MNU-induced lesions are 3-MeA (2, 35). The mainly cytotoxic nature of 3-MeA is also consistent with the observation that heavily adducted sites are, in general, extremely cold spots for mutagenesis. On the other hand, our results clearly showed that Me-lex is, unexpectedly, also mutagenic albeit in an exquisitely sequence-dependent manner. If our results could be extended to other gene targets, one might predict that the absence of such specific sequences could render such genes invariably insensitive to Me-lex mutagenicity. Further studies are planned to verify the hypothesis advanced in the interpretation of the mutation results.

[3] Burns, unpublished results.

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Relationship between DNA Methylation and Mutational Patterns Induced by a Sequence Selective Minor Groove Methylating Agent

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