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Separation of the SOS-Dependent and SOS-Independent Components of Alkylating-Agent Mutagenesis

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***Escherichia coli* plasmids containing the *rpsL*$^+$ gene (Str^s phenotype) as the target for mutation were treated in vitro with *N*-methyl-*N*-nitrosourea. Following fixation of mutations in *E. coli* MM294A cells (*recA*$^+$ Str^s), an unselected population of mutant and wild-type plasmids was isolated and transferred into a second host, *E. coli* 6451 (*recA* Str^r). Strains carrying plasmid-encoded forward mutations were then selected as Str^r isolates, while *rpsL*$^+$ plasmids conferred the dominant Str^s phenotype in the second host. Mutation induction and reduced survival of *N*-methyl-*N*-nitrosourea-treated plasmids were shown to be dose dependent. Because this system permitted analysis and manipulation of the levels of certain methylated bases produced in vitro by *N*-methyl-*N*-nitrosourea, it afforded the opportunity to assess directly the relative roles of these bases and of SOS functions in mutagenesis. The methylated plasmid DNA gave a mutation frequency of 6×10^{-5} (a 40-fold increase over background) in physiologically normal cells. When the same methylated plasmid was repaired in vitro by using purified *O*^{6}-methylguanine DNA methyltransferase (to correct *O*^{6}-methylguanine and *O*^{4}-methylthymine), no mutations were detected above background levels. In contrast, when the methylated plasmid DNA was introduced into host cells induced by UV light for the SOS functions, *rpsL* mutagenesis was enhanced eightfold over the level seen without SOS induction. This enhancement of mutagenesis by SOS was unaffected by prior treatment of the DNA with *O*^{6}-methylguanine DNA methyltransferase. These results demonstrate a predominant mutagenic role for alkylation lesions other than *O*^{6}-methylguanine or *O*^{4}-methylthymine when SOS functions are induced. The mutation spectrum of *N*-methyl-*N*-nitrosourea under conditions of induced SOS functions revealed a majority of mutagenic events at A · T base pairs.**

Alkylating agents such as *N*-methyl-*N'*-nitro-*N*-nitrosoguanidineand *N*-methyl-*N*-nitrosourea (MNU) form reaction products at DNA oxygen and nitrogen atoms (22). Among the oxygen adducts formed by these agents are O^6-methylguanine (O^{6}MeGua) and O^4-methylthymine (O^{4}MeThy), which are widely believed to be the major sites of alkylation mutagenesis. For both DNA adducts, the mutational mechanism is thought to involve passive mispairing with incorrect nucleotides during replication (25). MNU and related methylating agents produce G · C→A · T transitions nearly exclusively (9, 37), and this change is also the dominant mutation induced by synthetic O^{6}MeGua in DNA in vitro and in vivo (3, 5, 24). Alkylating agents with longer alkyl chains additionally show A · T→G · C transitions, implicating O^4-alkylthymidines in DNA as possible premutagenic lesions (36, 37).

In contrast to the comparatively well understood pattern of mutagenesis with S_N1 alkylating agents, the mutational specificity of agents reacting by an S_N2 mechanism (e.g., methylmethane sulfonate [MMS]) differs because they yield a significant proportion of transversions in addition to G · C→A · T transitions (43) and require a functional *umuC* gene product for mutagenesis (40, 42). The *umuC* and *umuD* genes are part of the SOS network (4), encoding a diverse set of cellular functions induced in *Escherichia coli* following DNA damage (49). The SOS network is negatively controlled by the product of the *lexA* gene (4).

The existence of two types of methylating agents, those requiring the *umuCD* gene products for mutagenesis and those independent of this system, implies that these agents may produce two distinct types of premutagenic lesions. Consequently, methylated bases other than or in addition to O^{6}MeGua and O^{4}MeThy may be mutagenic when the error-prone (*umuCD*) functions are expressed. In this study, we have investigated the involvement of O^{6}MeGua in both SOS-dependent and SOS-independent mutagenesis. As the target for mutation, we used a plasmid with which it is possible to (i) detect chemically induced forward mutations, (ii) select directly for the mutant phenotype, and (iii) analyze chemically the products of DNA damage before and after treatments that alter the profile of the adducts present and also alter the pattern of mutagenesis. This plasmid was treated in vitro with MNU and then repaired with the 19-kilodalton (kDa) Ada O^{6}MeGua DNA methyltransferase (MT), a repair protein for O^{6}MeGua and O^{4}MeThy (13, 31). We show here that when the SOS system was not already induced, virtually all of the premutagenic damage could be repaired by MT. However, MT-resistant DNA modifications were found to be strikingly mutagenic when SOS functions had been induced. In addition, the mutational specificity of MNU under SOS conditions was very different from that under non-SOS-induced conditions.

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* K-12 strain MM294A (*recA*$^+$ *Str*^{s} *pro thi endA hsr*$_K$ *hsm*$_K$$^+$) and *E. coli* 6451 (*recA* Str^r) were obtained from K. Backman and D. Baltimore, respectively, both of the Massachusetts Institute

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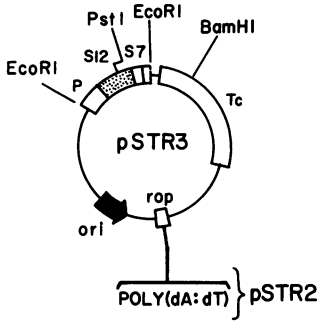

FIG. 1. Physical maps of pSTR2 and pSTR3.

of Technology (MIT). Plasmid pKH47 was purchased from Bethesda Research Laboratories, and plasmid pNO1523 was obtained from D. Dean and M. Nomura, University of Wisconsin. Plasmid pSE117 was obtained from G. Walker, MIT.

Enzymes and chemical reagents. MNU was the gift of G. Buchi, MIT. *N*-[^{3}H]methyl-*N*-nitrosourea ([^{3}H]MNU) (2 Ci/mmol) was purchased from Amersham. The following base standards were purchased from commercial sources: guanine (Gua) and 7-methylguanine (7-MeGua) from Sigma Chemical Co.; 1-methyladenine and 6-methyladenine from Fluka; 3-methyladenine from Cyclo Chemicals; 3-methylguanine from Vega Biochemicals; adenine (Ade) from P-L Biochemicals. 7-Methyladenine was provided by A. Chin, Battelle Memorial Institute; O^{6}MeGua was the gift of P. Newberne, MIT. The MT was produced as described before (13).

Construction of plasmids pSTR2 and pSTR3. Plasmid pSTR2 was constructed from plasmids pKH47 (19) and pNO1523 (10) (Fig. 1). A 1-kilobase (kb) fragment from pNO1523 containing a portion of the *E. coli* S12 operon encoding ribosomal protein S12 (the *rpsL* gene), a portion of the ribosomal protein S7 gene, and their natural promoter was cloned into a portion of pKH47, a plasmid that contains the tetracycline resistance (Tc^r) gene and a poly(dA):poly(dT) DNA tract (~100 base pairs [bp]) inserted into the *rop* gene.

Plasmid pSTR3 was identical to pSTR2 except that it contained a functional *rop* gene.

Methylation of plasmid DNA, in vitro repair of methylated DNA, and HPLC analysis. Plasmid DNA (~90 μg) was treated with MNU (0, 15, 25, and 50 mM) in 0.2 M sodium cacodylate buffer, pH 6.9, at 37°C for 1 h at a DNA concentration of 0.3 μg/μl. Following the reaction, 1/10 volume of 5 M NaCl in 50 mM Tris hydrochloride buffer, pH 7.5, was added, the DNA was ethanol precipitated and collected by centrifugation. DNA pellets were washed twice with 80% ethanol and suspended in sterile H_2O. When radiolabeled MNU was used to methylate pSTR2, the specific activity of MNU (25 mM) in the reaction was 0.16 μCi/nmol. Following a 1-h incubation at 37°C, the DNA was precipitated as described above. The DNA pellet was suspended in 10 mM sodium cacodylate buffer, pH 7.0, at a concentration of 1 μg/μl and drop dialyzed as described before (44). Following dialysis, the DNA was ethanol precipitated.

The in vitro repair of methylated plasmid involved incubating 200 μg of pSTR2 (15 ng/μl) that had been methylated with 25 mM [^{3}H]MNU with 9,300 U of the MT at 37°C for 30 min in 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-KOH buffer (pH 7.8)–20 mM dithiothreitol–1 mM EDTA–50 μM spermidine hydrochloride–5% glycerol (13). In parallel, 245 μg of methylated pSTR2 DNA was incubated in the absence of MT. The reaction mixtures were extracted sequentially with equal volumes of phenol (saturated with 10 mM Tris hydrochloride buffer [pH 8.0] and 1 mM EDTA), chloroform-isoamyl alcohol (24:1), and ether; after ethanol precipitation, the samples were stored at –80°C. DNA samples used for high-pressure liquid chromatography (HPLC) analysis were dialyzed against H_2O (44) prior to acid hydrolysis. Dialysis removed ammonium sulfate, which prevented complete acid hydrolysis of DNA.

Methylated plasmid DNA was analyzed for the presence of methylated and unmodified purines by hydrolyzing the DNA (0.15 μg/μl) with 0.1 N HCl at 70°C for 20 min (22). The hydrolysate was made 0.1 M in ammonium formate and brought to a pH of ~5 by the addition of 2 M ammonium formate and 2 N KOH. The hydrolysate was injected onto a Durrum DC4A cation-exchange HPLC column (Dionex Chemical Corp.; 0.2 by 25 cm) and analyzed with a Beckman Instruments model 100A chromatograph equipped with a Waters Associates model 440 UV absorbance detector (254 nm). Hydrolysates from nonlabeled DNA samples were eluted isocratically at 0.3 ml/min with 0.1 M ammonium formate, pH 5.1, at 50°C and analyzed for the presence of Gua, Ade, and 7-MeGua. Hydrolysates from labeled DNA samples were coinjected with authentic base standards, and after 30 min of isocratic elution with 0.1 M ammonium formate, pH 5.1, the composition of the mobile phase was programmed over 20 min to 0.1 M ammonium phosphate, pH 7.7, and maintained at that composition for 50 min. Radioactivity coeluting at the retention volumes of the respective methylated bases was used to determine the level of each base in MNU-treated DNA.

Use of *rpsL* gene to detect chemically induced mutation. *E. coli* MM294A (Str^s *recA*$^+$) was grown in LB to a density of ~2 × 10^8 cells per ml. In experiments with MM294A (pSE117), the cells were grown to a cell density of 7 × 10^7/ml. A portion of the suspension was UV irradiated (15 J/m^2 in 0.85% saline, 10 mM $MgSO_4$) with a General Electric 15-W germicidal lamp (maximum output at 254 nm), which had been calibrated by using an International Light IL 782 spectroradiometer. (This dose of UV resulted in 60 to 70% survival of cells.) The cells were centrifuged, resuspended in LB, and incubated at 37°C for 50 min in the dark to allow induction of SOS functions (11). Both irradiated and unirradiated cells were made competent for transformation by a modification of the procedure of Mandel and Higa (28). The transformation mixture consisted of 2 ml of competent cells and 10 μg of MNU-treated or untreated pSTR2. Following heat shock, the mixture was added to 20 ml of LB and incubated for 90 min. A portion of the transformation mixture was removed for plating on LB plates containing tetracycline (20 μg/ml). The number of colonies arising from this plating was used to determine the efficiency of transformation and to measure survival of methylated plasmid relative to that of untreated plasmid DNA. The remainder of the mixture was incubated for 1 to 4 h, at which time tetracycline was added to a final concentration of 20 μg/ml. Following a 30-min incubation period, 80 ml of LB containing tetracycline (20 μg/ml) was added, and the culture was incubated overnight (18 to 20 h). Plasmid was isolated from the 100-ml overnight culture by a modification of the protocol of Maniatis et al. (29). This DNA was used to transform *E. coli* 6451; the transformation mixture was added to 20 ml of LB and incubated at 37°C for 90 min. One portion of the cell suspension was plated in the presence of tetracycline

and a second in the presence of both tetracycline and streptomycin. The mutation frequency was calculated by dividing the number of cells growing in the presence of both antibiotics by the number of cells growing in the presence of tetracycline alone.

Reconstruction experiments. To determine whether there was bias either for or against the mutant phenotype during the course of the mutation assay, wild-type (WT) plasmid (either pSTR2 or pSTR3) was mixed with known amounts of individual mutant plasmids at ratios of $1:10^1$ to $1:10^5$ (mutant DNA to WT DNA), and the mixed DNAs were carried through all steps of the mutation assay (except the mutagen treatment).

Depurination of plasmid DNA and enumeration of apurinic sites. Plasmid DNA was depurinated by the protocol described by Bichara and Fuchs (7). pSTR3 DNA (1 μg) was incubated in 10 μl of 10 mM sodium citrate–100 mM KCl (pH 4.0) buffer at 70°C for times ranging from 2 to 10 min. The number of apurinic sites formed per plasmid molecule was estimated by treatment with exonuclease III in buffer containing $CaCl_2$, conditions under which exonuclease III functions mainly as an apurinic endonuclease. The DNA was ethanol precipitated and incubated for 15 min at 37°C in 10 μl of buffer (50 mM Tris hydrochloride [pH 8], 0.05 μM dithiothreitol, 0.1 mg of bovine serum albumin per ml, 5 mM $CaCl_2$) with 10 U of exonuclease III. The supercoiled and nicked forms were separated by agarose gel electrophoresis in the presence of ethidium bromide and quantified by densitometric scanning of the gel photograph. Assuming a Poisson distribution, the number of apurinic sites (A) per plasmid molecule was calculated by using the relation $A = -\ln(B)$, where B is the relative amount of supercoiled DNA (14). Under these conditions, ~4 apurinic sites per plasmid molecule were generated in 10 min of acid-heat treatment. For the mutation assays, pSTR3 DNA (50 μg) was acid depurinated for 10 min or treated with 25 mM MNU.

Isolation and DNA sequencing of mutant plasmids. To ensure the independence of each mutant, only one mutant colony was picked from each transformation reaction and grown in medium containing tetracycline (20 μg/ml) and streptomycin (100 μg/ml). Plasmid DNA was extracted and used to transform *E. coli* MM294A cells to the tetracycline-resistant phenotype; MM294A cells lack endonuclease A, and plasmids isolated from cells of this genotype are good substrates for enzymatic DNA sequence analysis. Plasmid DNA was isolated and sequenced by the chain-termination method (38). The three synthetic oligonucleotide primers used for sequencing the ~450-bp promoter and coding regions of the *rpsL* gene were d(CAGCCAGATGGCCTG) (purchased from the Whitehead Institute, MIT), d(ACGCA AACCACGTGC), and d(TTCGTCTGACTAACG) (purchased from the DNA Synthesis Facility, University of Massachusetts Medical School).

RESULTS

Experimental design. The principle underlying the forward selection of mutants is illustrated in Fig. 2. Plasmids pSTR2 and pSTR3 (Fig. 1) both contain the *E. coli rpsL* gene as the mutation target and the Tc^r gene to enable determination of the total number of plasmid-transformed cells. The *rpsL* gene encodes the small ribosomal protein S12. When present at high concentrations, streptomycin binds to the small subunit of ribosomes that contain WT S12 proteins (35), forming abortive translation initiation complexes (27, 50). Some strains, however, such as *E. coli* 6451, have selective *rpsL* mutations that render them resistant to the effects of streptomycin.

TABLE 1. Effect of in vitro repair by MT on MNU-induced mutagenesis in MM294A cells[a]

DNA treatment		Survival (%)	No. of mutants (per 10^7 survivors) ± 1 SD	No. of adducts/pSTR2 genome						
MNU (mM)	MT			7G	O6G	7A	3G	3A	1A	AP
0	−	100	9 ± 3							
25	−	22	380 ± 28	100	13	1.5	1.2	10.6	0.3	19
25	+	22	8 ± 2	112	0.14	2.1	1.3	10	0.2	21

[a] pSTR2 was treated with 25 mM MNU, and a portion of the methylated DNA was treated with MT. Both MT-treated and untreated plasmids were subjected to methylated base analyses. Analytical data are the average of duplicate chromatographic analyses. The adducts observed were 7-methylguanine (7G), O^6MeGua (O6G), 7-methyladenine (7A), 3-methylguanine (3G), 3-methyladenine (3A), and 1-methyladenine (1A); AP is depurinated DNA (apurinic acid) eluting in the void volume of the HPLC column. In parallel, MT-treated and untreated plasmids were used in a mutation assay as described in Materials and Methods. Mutation data are the average of duplicate (0 mM MNU) or triplicate (25 mM MNU) mutation assays and have been corrected downward for a bias (13-fold) in favor of the mutant plasmids (see text).

The Str^s phenotype is dominant to Str^r (8, 23, 46), presumably because streptomycin-inhibited ribosomes block the attachment to mRNA of ribosomes not directly inhibited by the drug (27, 50) (Fig. 2B). Hence, cells synthesizing only WT S12 proteins, or a mixture of both WT and mutant S12 proteins, were sensitive to streptomycin (Fig. 2B, i to iii). By contrast, cells synthesizing only mutant S12 proteins were resistant to the drug (Fig. 2B, iv).

Plasmid DNA was treated in vitro with MNU and introduced into the *E. coli* host strain MM294A or UV-irradiated MM294A(pSE117) (to study mutagenesis under SOS-induced conditions). These cells were chosen as hosts in which to fix mutations because they are efficiently transformed with plasmid DNA and encode a WT *rpsL* gene. In $recA^+$ cells, the genotype of the chromosomal *rpsL* gene must be identical to that of the plasmid-encoded *rpsL* gene to avoid the isolation of Str^r recombinants (1, 26). In another laboratory, the use of an Str^r *E. coli* host (i.e., one encoding a mutant *rpsL* gene) to examine the mutagenic effects of chemical damage in a plasmid-encoded *rpsL* gene was limited by this problem (1).

To select for mutations inactivating the WT *rpsL* gene, plasmid DNA was isolated from MM294A cells and then introduced into the second host, *E. coli* 6451, which carries *recA* and is Str^r. Following transformation, mutant fractions were determined by comparing the number of colonies arising on LB plates containing tetracycline and streptomycin with the number arising in the presence of tetracycline alone.

Reconstruction experiments were conducted to investigate the possibility of bias distorting the ratio of mutant and WT *rpsL* transformants obtained in the multistep process of amplification and selection. Mutant and WT plasmids were mixed at various ratios and carried through the manipulations (except chemical treatment) used to detect chemically induced mutations. The mutants used in the reconstruction experiments had been induced by MNU as described in Materials and Methods. The results of a reconstruction experiment with contrived mutant fractions of 10^{-1} to 10^{-5} (ratio of mutant plasmid DNA to pSTR2 DNA) are shown in Fig. 3. The final mutant fractions were consistently observed

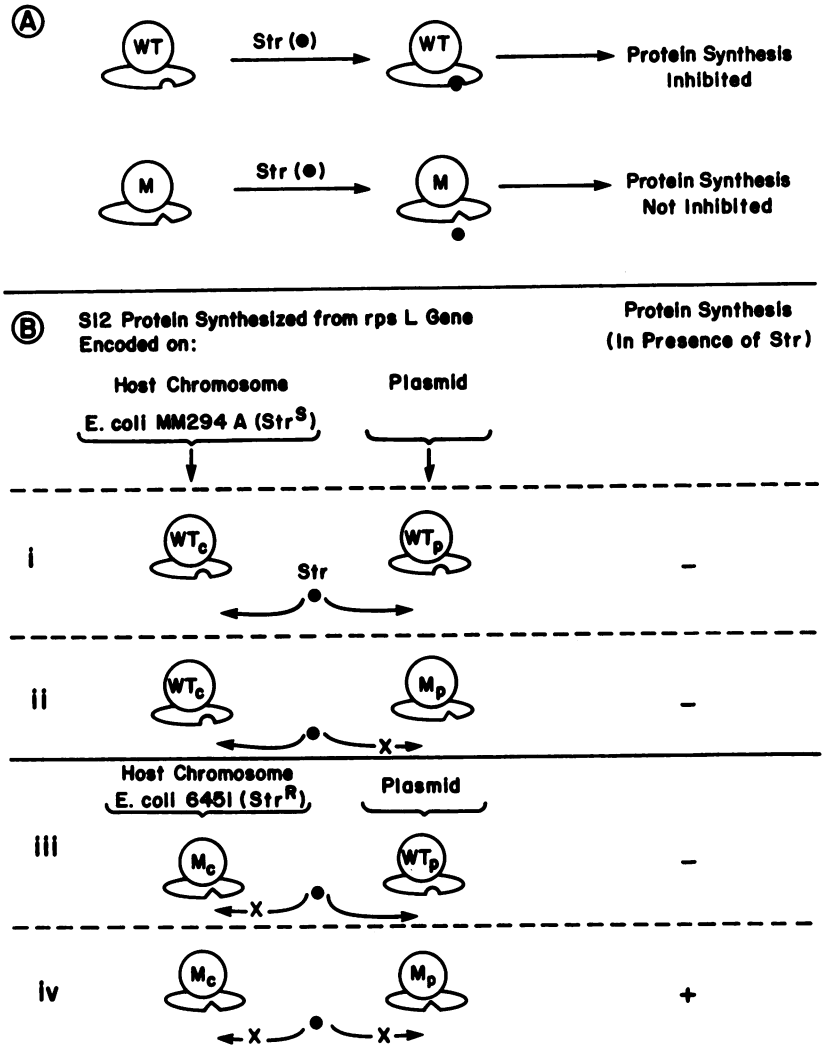

FIG. 2. Principle by which plasmid-encoded rpsL gene is used as target for mutation. (A) Mechanism of action of streptomycin (●, Str). (B) Dominance of Str^s phenotype (see text). WT refers to a ribosome containing WT S12 protein, and M indicates a ribosome that contains mutant (Str^r) S12 protein. WT_c refers to a ribosome containing WT S12 protein that was synthesized from an *E. coli* chromosomal *rpsL* gene, and WT_p refers to a ribosome containing WT S12 protein that was synthesized from a plasmid-encoded *rpsL* gene. M_c designates a ribosome containing mutant S12 protein that was synthesized from an *E. coli* chromosomal *rpsL* gene, and M_p refers to a ribosome containing mutant S12 protein that was synthesized from a plasmid-encoded *rpsL* gene. X indicates the inability of streptomycin to bind to the ribosome.

to be 13.4 ± 3.5-fold higher than the starting ratio with MM294A cells as the host and 5.2 ± 1-fold higher when MM294A cells containing pSE117 were used. These biases were used as downward correction factors in calculating the actual mutant fractions reported in Tables 1 and 2 (discussed below).

Since the bias in favor of selecting the mutant phenotype was constant over 5 orders of magnitude, the interpretation of the mutagenesis results obtained with plasmid pSTR2 should not have been affected. However, it was found that the observed bias resulted from insertion of the poly(dA):poly(dT) sequence into the *rop* gene of pSTR2. The *rop* gene encodes a protein that has a role in controlling the plasmid copy number in the cell (48). Apparently, cells harboring *rpsL* mutant *rop* plasmids outgrow by a uniform margin cells containing WT plasmids (data not shown). This discovery led to the construction of pSTR3, which contains a functional *rop* gene (Fig. 1). Reconstruction experiments established that the bias was largely eliminated in pSTR3 (Fig. 3). The SOS-dependent increase in the mutagenicity of alkylated DNA (see below) was confirmed by using pSTR3 (data not shown).

Detection of chemically induced mutagenesis in the plasmid-encoded *rpsL* gene. Plasmid pSTR2 was treated with doses of MNU ranging from 0 to 50 mM, and the effect of the resultant DNA damage on mutation of the *rpsL* gene was evaluated. In order to estimate the level of DNA methylation, samples of MNU-treated DNA were acid hydrolyzed and analyzed for the presence of Gua, Ade, and 7-MeGua by HPLC. In experiments with radiolabeled MNU, it was determined that 7-MeGua accounted for 69% of the radioactive methyl groups incorporated into DNA (Table 1), as expected (6, 22). With this value, in experiments with nonradioactive MNU, the level of 7-MeGua was determined by HPLC and used to compute the total amount of genome modification.

A nearly linear increase in mutation frequency was observed with increasing MNU methylation of plasmid DNA

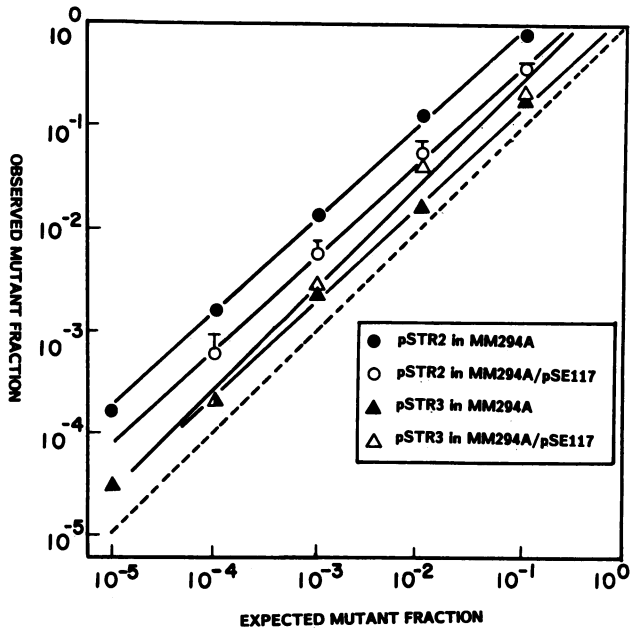

FIG. 3. Reconstruction experiment. Mutant and WT pSTR2 or mutant and WT pSTR3 plasmid DNAs were mixed at different ratios to generate starting mutant fractions ranging from 10^{-1} to 10^{-5}. Competent MM294A or MM294A(pSE117) cells (200 μl) were transformed with 1 μg of the DNA mixtures, and mutation assays were performed. The observed mutant fractions determined from plating were plotted against the initial mutant fractions. Data represent the average of duplicate or triplicate experiments; typical standard deviations are shown for one set of data points. Dashed line indicates expected values if there were no bias.

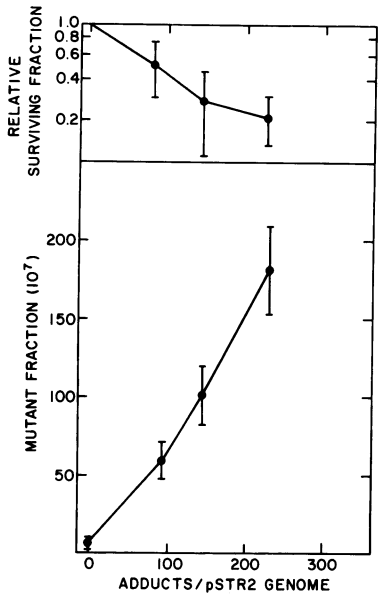

FIG. 4. Evaluation of plasmid pSTR2 as target for mutation. Plasmid pSTR2 was treated with increasing concentrations of MNU, and mutation assays were performed. The number of DNA adducts per genome was calculated from the amount of 7-MeGua as determined by HPLC; this was 69% of the total level of DNA modification. Survival of MNU-treated plasmid in MM294A cells was measured relative to survival of untreated plasmid. Mutant fractions were determined by the ratio of Tc^r Str^r colonies to Tc^r colonies following transformation of *E. coli* 6451 cells. Triplicate mutation assays were performed for each dose of MNU used. Mutation data represent mean ± 2 SD.

(Fig. 4). A dose-dependent decrease in plasmid survival was also observed. A level of DNA modification of 81 alkyl adducts per pSTR2 genome resulted in 37% survival, which thus represented ~1 lethal hit. With other plasmid or phage mutation assays, others have estimated one lethal hit for *N*-acetoxy-*N*-acetylaminoflourine, benzo[a]pyrene diol epoxide, and apurinic acid lesions as, respectively, 18, 3, and 3.5 adducts per duplex genome of comparable size to pSTR2 (21, 33, 39). This comparison indicates that methylated DNA bases are collectively much less lethal than the above-mentioned lesions or that they are repaired more efficiently.

Role of O^6-MeGua DNA MT-sensitive lesions in mutagenesis. O^6MeGua and O^4MeThy are the lesions thought to be principally responsible for methylating-agent mutagenesis, but direct evidence for this has been lacking (2, 3, 18, 25, 41, 45). To investigate the role of these base adducts in mutagenesis, methylated plasmid DNA was treated with the purified 19-kDa MT protein (13), which is capable of removing the methyl groups from O^6MeGua and O^4MeThy (13, 31, 34, 47). This treatment resulted in the detectable loss of only one methylated purine, O^6MeGua, of which >99% was repaired (Fig. 5; Table 1). It is expected that the MT protein also removed methyl groups from O^4MeThy, although the levels of this base were below the limits of detection in our experiments. When methylated plasmid (25 mM MNU) was introduced into *E. coli* MM294A cells, a mutation frequency of 380×10^{-7} was observed (Table 1). However, prior treatment of this same DNA with the MT protein completely eliminated alkylation mutagenesis. The decrease in mutation frequency (98%) with MT treatment was consistent with the decrease in the level of O^6MeGua in the DNA (99%). The 19-kDa MT fragment used in these studies is unable to repair methyl-phosphotriesters (32), ruling out any significant role of these adducts in mutagenesis.

Role of O^6MeGua DNA MT-insensitive lesions in SOS-dependent mutagenesis. Mutagenesis by MNU is generally independent of SOS (i.e., *umuC*$^+$) functions, while that produced by MMS is largely dependent on a functional *umuC* gene product (42). A possible explanation of this difference comes from a comparison of the spectra of methylated bases produced by each agent. MNU reacts efficiently at both oxygen and nitrogen atoms in DNA, whereas MMS reacts primarily at base nitrogens (6). The data suggest, albeit indirectly, that the nitrogen-containing adducts require a functional *umuC* gene product to yield mutations. To test this hypothesis, the pSTR2 system was modified so that mutation of alkylated, MT-treated plasmid could be studied in SOS-induced cells. This modification involved the use of MM294A host cells harboring the plasmid pSE117 (30), a plasmid encoding the *E. coli umuCD* gene products required for the mutagenic component of the SOS response (16, 20). UV irradiation of these cells induced the SOS response.

When samples of the methylated plasmid that had been either treated or not treated with the MT protein were introduced into nonirradiated MM294A(pSE117) cells, the results on mutagenesis were similar to those obtained with the same hosts without pSE117 (Table 2). However, when mutations were fixed in UV-irradiated hosts, the mutation results were strikingly different. With or without prior MT treatment, very high induced mutant fractions of $\sim 4{,}200 \times 10^{-7}$ were observed. These data indicate that MNU-induced

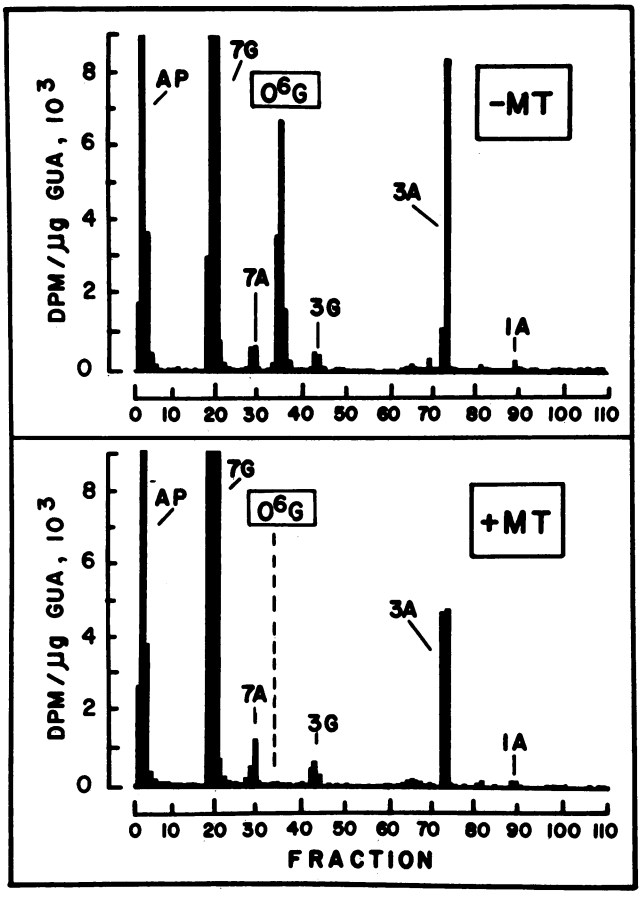

FIG. 5. Analysis of methylated pSTR2 DNA before and after treatment with the MT protein. Plasmid pSTR2 DNA (600 μg) was methylated with [^{3}H]MNU (25 mM, 0.16 Ci/mmol) in 0.2 M sodium cacodylate, pH 6.9, at 37°C for 1 h. Following the reaction, the DNA was dialyzed and ethanol precipitated. Then, 200 μg of methylated DNA (0.015 μg/μl) was incubated with 9,300 U of MT (+MT), and in a parallel reaction, 245 μg of methylated DNA was incubated in the absence of MT (−MT). Portions of the DNA were hydrolyzed in 0.1 N HCl at 70°C for 20 min, and the hydrolysates were analyzed for the presence of methylated purines by cation-exchange chromatography. Abbreviations for DNA lesions are as described in Table 1, footnote *a*.

lesions other than those removed by the MT protein are highly mutagenic in an SOS-induced cell.

Possible involvement of abasic sites formed in vitro in SOS-dependent mutagenesis. The large enhancement of mutagenesis observed in SOS-induced cells was not due to abasic sites formed in alkylated pSTR2 before its entry into cells. Plasmids treated with 25 mM MNU contained ~3 apurinic sites per molecule, a number 10-fold higher than the value obtained for a buffer control (Table 3). The MNU-treated DNA had a mutant fraction of $1,500 \times 10^{-7}$, whereas acid-depurinated genomes with about 4 abasic sites per genome had a mutant fraction of only 140×10^{-7}. These data show that apurinic sites formed during in vitro treatment of the DNA with MNU could account for only ~7% of the total mutation frequency. This experiment of course did not rule out the possibility that mutations may be caused by apurinic sites being formed inside the cell.

Mutational specificity of MNU under conditions of induced SOS functions. A total of four mutants were sequenced from cells not induced for SOS functions, and all were, as expected (9, 37), G · C→A · T transitions. By contrast, the mutational spectrum of MNU-damaged DNA in SOS-induced cells showed a marked preference for mutagenic events at A · T base pairs; 42 mutations were sequenced, 28 of which were at A · T sites. The most frequent class of mutations was the A · T→G · C transition (17 of 42). The A · T→T · A transversions (6 of 42) and (−1) A · T deletions (5 of 42) were also prominent features of the mutational spectrum. The G · C→A · T transition occurred in 8 of 42 mutants. The remaining mutations were a G · C→T · A, an insertion, and four deletions.

TABLE 2. Effect of in vitro repair by MT on MNU-induced mutagenesis in UV-irradiated and nonirradiated MM294A(pSE117) cells[a]

DNA treatment		UV treatment	Survival (%)	No. of mutants (per 10^7 survivors) ± 1 SD
MNU (mM)	MT			
0	−	−	100	14
25	−	−	30	530 ± 56
25	+	−	46	23 ± 13
0	−	+	100	29
25	−	+	33	4,130 ± 350
25	+	+	62	4,290 ± 320

[a] pSTR2 was introduced into nonirradiated or UV-irradiated MM294A (pSE117) cells, and mutation assays were performed as described in Materials and Methods. Data are the average of two to six separate mutation assays and have been corrected downward for a bias (fivefold) in favor of mutant plasmids (see text).

DISCUSSION

This work has examined MNU mutagenesis with and without induced SOS processing. Chemical analysis of MT-treated methylated plasmid indicated that 99% of the O^{6}MeGua residues present had been removed by the repair protein, consistent with a 98% reduction in the mutation frequency in non-SOS-induced cells. These data confirm a dominant and direct role for O^{6}MeGua and/or O^{4}MeThy in MNU-induced mutagenesis in the absence of SOS functions.

MNU mutagenesis under conditions of induced SOS processing differed from that in the normal physiological state in two ways. First, there was an eightfold increase in mutation frequency. This result was unexpected since, in previous genetic studies, it was concluded that MNU is a *umuC*$^+$-independent mutagen (40). This conclusion was based on the observation that there was no quantitative difference in MNU-induced mutagenesis between *umuC*$^+$ and *umuC E.*

TABLE 3. Comparative mutation frequencies for MNU- and acid-treated DNA[a]

DNA treatment		No. of AP sites/plasmid	Survival (%)	No. of mutants (per 10^7 survivors) ± 1 SD	Avg. mutagenic efficiency of detectable lesions (%)
MNU	Acid				
−	−	0.3	100	25 ± 2	
+	−	3	70	1,500 ± 400	0.0015
−	+	~4	100	140 ± 70	0.0035

[a] pSTR3 DNA was treated with 25 mM MNU for 1 h at 37°C or with acid (pH 4) for 10 min at 70°C. Mutation data are the average of triplicate experiments. The mutagenic efficiency of a lesion is defined as the mutation frequency divided by the lesions in the target area times 100. The target area is 10% of the total plasmid. AP, Apurinic.

coli strains. The present results may differ because we used host cells that were already induced for the SOS response. It is possible that MNU itself is only a weak inducer of the SOS system but that one or more of the lesions it forms are strongly mutagenic once the system is induced. Supporting this contention is the observation by others of an SOS-dependent component of mutagenesis for alkylating agents that are also mutagenic under non-SOS-induced conditions. Foster and Eisenstadt (17) demonstrated that *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine can also induce *umuC*$^+$-dependent mutations, especially in *alkA* strains. In those experiments, O^{6}MeGua and O^{4}MeThy lesions were presumably removed by prior induction of the adaptive response, suggesting that one or more of the methylated bases removed by the *alkA* gene product are responsible for *umuC*$^+$-dependent mutagenesis. In other studies, Eckert and Drinkwater (15) showed a significantly greater mutation frequency for *N*-ethyl-*N*-nitrosourea in *recA*$^+$ bacteria preirradiated with UV.

A second piece of evidence suggesting an SOS-dependent pathway for MNU mutagenesis was the failure of MT treatment of plasmid DNA to reduce the level of mutagenesis in SOS-induced bacteria. This result also showed directly that O^{6}MeGua and O^{4}MeThy were not the dominant premutagenic lesions under conditions of SOS induction. The preference for A · T mutations observed in the mutation spectrum of MNU under induced SOS conditions suggested that an A or T adduct (or adduct breakdown product) was responsible for most of the SOS-induced mutations. Previous genetic studies aimed at determining the premutagenic lesions of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine in *alkA umuC*$^+$ *E. coli* strains revealed that 30% of all targeted mutations were A · T→T · A transversions (17). On the basis of this result, it was suggested that the abasic site formed by removal of 3-methyladenine is the major *umuC*$^+$-dependent premutagenic lesion in *alkA* bacteria. Although some A · T→T · A transversions were observed in our study, the majority of the A · T mutations were A · T→G · C transitions. While O^{4}MeThy has been shown to cause A · T→G · C transitions (36), a mutagenic role for this adduct may be ruled out on the basis of the observation that MT repair of MNU-treated DNA in vitro did not lower the mutation frequency under SOS conditions. Other possibilities for the precursors to these A · T mutations are 1-methyladenine, 3-methyladenine, 7-methyladenine, *O*^{2}-methylthymine, and an abasic site. Further studies are being conducted to identify the lesion(s) responsible for MNU mutagenesis under conditions of SOS induction.

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