Alkylating Agents Induce UVM, a *recA*-Independent Inducible Mutagenic Phenomenon in *Escherichia coli*

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ABSTRACT

Noninstructive DNA damage in *Escherichia coli* induces SOS functions hypothesized to be required for mutagenesis and translesion DNA synthesis at noncoding DNA lesions. We have recently demonstrated that in *E. coli* cells incapable of SOS induction, prior UV-irradiation nevertheless strongly enhances mutagenesis at a noninstructive lesion borne on M13 DNA. Here, we address the question whether this effect, named UVM for UV modulation of mutagenesis, can be induced by other DNA damaging agents. Exponentially growing Δ*recA* cells were pretreated with alkylating agents before transfection with M13 single-stranded DNA bearing a site-specific ethenocytosine residue. Effect of cell pretreatment on survival of the transfected DNA was determined as transfection efficiency. Mutagenesis at the ethenocytosine site in pretreated or untreated cells was analyzed by multiplex DNA sequencing, a phenotype-independent technology. Our data show that 1-methyl-3-nitro-1-nitrosoguanidine, N-nitroso-N-methylurea and dimethylsulfate, but not methyl iodide, are potent inducers of UVM. Because alkylating agents induce the adaptive response to defend against DNA alkylation, we asked if the genes constituting the adaptive response are required for UVM. Our data show that MNNG induction of UVM is independent of *ada*, *alkA* and *alkB* genes and define UVM as an inducible mutagenic phenomenon distinct from the *E. coli* adaptive and SOS responses.

EVEN though mutagens are known to act by damaging DNA, the mechanisms by which they induce mutations are not well understood. Because DNA damage is effectively removed by multiple DNA repair pathways, mutation enhancement by mutagens is attributed to residual DNA damage that has escaped repair. Many mutagens can alter the mutation-fixation environment of the cell such as to enhance not only targeted mutagenesis at damage inflicted by the mutagen but also untargeted mutagenesis at undamaged DNA sites and "cotargeted" mutagenesis at damage induced by other (heterologous) mutagens.

Mutagenic DNA damage has been traditionally classified into two broad categories. *Mispairing* lesions, such as O^6-methylguanine or DNA uracil produced by deamination of DNA cytosine, can serve as templates for DNA replication, but their templating activity is "wrong" in a biological sense. *Noninstructive* lesions such as abasic (AP) sites and bulky DNA lesions represent the second category, so-named because they lack template information accessible to the DNA polymerase. Such lesions act as blocks to DNA replication and as such are believed to have high genotoxic potential. This class of lesions represents an overwhelming proportion of DNA damage and encompasses an enormous variety of chemical modifications ranging from simple abasic sites to DNA-protein crosslinks.

Much of our current knowledge on how noninstructive lesions induce mutations derives from the *Escherichia coli* SOS hypothesis. A basic postulate of the SOS hypothesis is that replication past blocking lesions requires DNA damage-inducible functions not required for normal DNA replication (DEFAIS *et al.* 1971; RADMAN 1975; WITKIN 1976; WALKER 1984, 1987; ECHOLS and GOODMAN 1991). It is now well established that DNA damage activates the *E. coli* RecA protein, leading to proteolytic inactivation of the LexA repressor, which in turn leads to a coordinated derepression of the SOS regulon (WALKER 1984, 1987). *E. coli* has additional DNA damage-inducible responses that protect cells against the mutagenic and toxic effects of oxygen radicals (GREENBERG and DEMPLE 1988, 1989; STORZ *et al.* 1990) and alkylating agents (LINDAHL *et al.* 1988; VOLKERT 1988; SHEVELL *et al.* 1990; SAGET and WALKER 1994), but these are not known to promote error-prone replication. A well-known example is the *E. coli* adaptive response in which a low dose of an alkylating agent can induce enzymes capable of repairing DNA alkylation damage. The *ada* gene has two important roles in the adaptive response: it is a methyl transferase that repairs O^6-methylguanine and other alkylated bases, and it plays a central role in the regulation of the four adaptive response genes (*ada*, *alkA*, *alkB* and *aidB*; see LINDAHL *et al.* 1988; VOLKERT 1988; SHEVELL *et al.* 1990; SAGET and WALKER 1994).

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TABLE 1

***E. coli* and M13 phage strains**

Strain	Genotype	Source
KH2R	Sup^0 Δ*lac-pro trpE9777* Δ*(srlR-recA)306*::Tn*10*(Tet^R) (F′ *lacI^q*ZΔ*M15 pro$^+$*)	K. SAMBAMURTI[a]
JH43	*lac*Δ*U169 dinD2*::Mud(Amp^R *lac*) *recA$^+$ srl?* Tet^S (F′ *lacI^q lacZ*::Tn*5*(Kan^R))	P. MODEL (HEITMAN and MODEL 1987)
CJ251	*lacY galK galT metB trpR supE supF hsdR* (F′ pOX38:Cm^R)	C. JOYCE (JOYCE and GRINDLEY, 1984)
GW7101	Δ*ada-25*(Cm^R) in AB1157[b]	L. SAMSON (REBECK and SAMSON, 1991)
AR1	As in GW7101, but Δ*(srlR-recA)306*::Tn*10*(Tet^R)	This laboratory[c]
AR2	As in AR1, but (F′ *lacI^q lacZ*::Tn*5*(Kan^R))	This laboratory[d]
GWR107	*ogt-1*::Kan^R in AB1157[b]	L. SAMSON (REBECK and SAMSON, 1991)
OR1	As in GWR107, but Δ*(srlR-recA)306*::Tn*10*(Tet^R)	This laboratory[c]
OR2	As in OR1, but (F′ pOX38:Cm^R)	This laboratory[e]
MV1571	*alkA51*::Mu-dl(Amp^R *lac*) in MV1161[f]	M. VOLKERT (VOLKERT *et al.* 1986)
MV1601	*alkB52*::Mu-dl(Amp^R *lac*) in MV1161[f]	M. VOLKERT (VOLKERT *et al.* 1986)
MV2176	*aidB1*::Mu-dl(Amp^R *lac*)Δ*(argF-lacZ)205*(u169) in MV1161[f]	M. VOLKERT (VOLKERT *et al.* 1994)
M13 mp7L2	Derivative of phage M13 mp7[g]	C. LAWRENCE (HORSFALL and LAWRENCE 1994)

Parenthetical data in last column are references.

[a] Constructed in this laboratory by K. SAMBAMURTI as described by PALEJWALA *et al.* (1991). This strain has the UV-sensitive phenotype expected of a Δ*recA* strain (PALEJWALA *et al.* 1993b).

[b] AB1157 *argE3 hisG4 leuB6 proA2 thr-1 ara-14 galK2 lacY1 mtl-1 xyl-1 thi-1 ara-1 rpsL31 supE44 tsx-33.*

[c] Constructed by P1 transduction of the Δ*(srlR-recA)*306::Tn*10*(Tet^R) allele by the procedures of STERNBERG and MAURER (1991) from KH2R. The resulting strain had the UV-sensitivity expected of a Δ*recA* strain.

[d] Constructed by introducing the F′ *lacI^q lacZ*::Tn*5*(Kan^R) factor from JH43 by the procedures described by MILLER (1972). AR2 had the MNNG-sensitivity expected of an *ada* mutant.

[e] Constructed by introducing the F′pOX38:Cm^R factor from CJ251 by the procedures described by MILLER (1972).

[f] MV1161 *thr-1 ara-14 leuB6* Δ*(pgt-proA)62 lacY1 tsx-33 supE44 galK2 hisG4 rfbD1 mgl-51 rpsL31 kdgK31 xyl-5 mtl-1 argE3 thi-1 rfa-550.*

[g] To create M13 mp7L2, the 5′-[AC]GAATTC- sequence at the 5′ boundary of the mp7 polylinker sequence was changed to 5′-[CAGT]GAATTC-. This change extends the stem of the polylinker hairpin by 4 bp, and introduces a genetic −1 frameshift in the *lacZ*(α) gene segment.

We have recently demonstrated that prior UV irradiation of *recA*$^+$ as well as of Δ*recA* cells results in a significant enhancement of mutation fixation at a site-specific noncoding lesion (3,N^4-ethenocytosine; εC) borne on M13 vector DNA molecules (PALEJWALA *et al.* 1993a,b, 1994). The existence of this phenomenon, termed UVM for UV modulation of mutagenesis in Δ*recA* cells suggests the possibility that inducible mutagenesis previously attributed to the SOS system may in fact be the result of more than one inducible phenomenon. Here, we have addressed the question whether DNA damaging agents other than UV can induce UVM mutagenesis. Our results demonstrate that DNA alkylating agents are strong inducers of UVM and that UVM is distinct from the SOS as well as the adaptive responses of *E. coli.*

MATERIALS AND METHODS

Bacterial and M13 phage strains: Table 1 lists the bacterial and phage strains used in this study.

Construction of M13 single-stranded DNA bearing site-specific ethenocytosine: Construction of M13 ssDNA bearing a site-specific εC residue (εC-DNA) or the corresponding control construct (C-DNA) bearing normal cytosine in the place of the εC residue have been described previously (PALEJWALA *et al.* 1994) and are summarized in Figure 1. DNA constructs were made freshly before transfection or were stored frozen at −20° for no longer than 2 weeks. The DNA was denatured as described (PALEJWALA *et al.* 1994) immediately before transfection to ensure that it was in ssDNA form at the time of transfection.

Mutagen treatment and transfection: In a typical experiment, a 500-ml culture flask containing 150 ml of LB medium was inoculated with 1.5 ml of fresh overnight culture of the appropriate strain, and the cells were allowed to grow to an optical density at 600 nm (OD_{600}) of 0.3–0.4 (5×10^7 to 1×10^8 cells/ml). The culture was divided into 30-ml aliquots in sterile 125-ml culture flasks for exposure to inducing agents. 1-methyl-3-nitro-1-nitrosoguanidine (MNNG; Sigma) was dissolved in acetone at a concentration of 10 mg/ml and was diluted into 0.1 M sodium citrate, pH 5.5, to obtain a 1 mg/ml working stock solution. N-nitroso-N-methylurea (NMU; Sigma) was freshly dissolved in 10% ethanol in LB medium to obtain a working concentration of 25 mg/ml. Working 1 M stock solutions for dimethylsulfate (DMS; Kodak Chemicals) and methyl iodide (MeI; Sigma) were made in dimethylsulfoxide. After the inducing agents (or appropriate solvents for controls) were added to indicated final concentrations, the cultures were allowed to continue incubation at 37° with vigorous aeration for the indicated period of time. To remove the inducing agent after the exposure period, cells were pelleted by centrifugation at 4° for 10 min in a Sorvall SS-34 rotor at 2500 rpm, washed by resuspension in an equal volume of LB medium followed by centrifugation as above. The final cell pellet was resus-

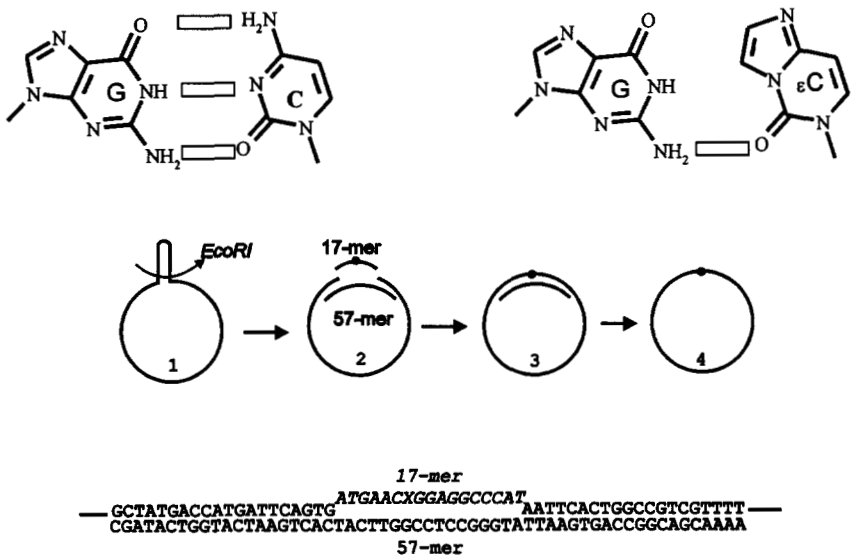

FIGURE 1.—Structure of εC and the strategy used for constructing M13 ssDNA bearing a site-specific εC residue. (Top) A normal G:C pair with three hydrogen-bonds, and a G:εC "pair" are shown to depict the effect of etheno bridging on base pairing. Whether a hydrogen bond involving guanine N2 can form *in vivo* is not known. *In vitro* primer elongation experiments by *E. coli* DNA polymerase I large Klenow fragment on an oligonucleotide template bearing a site-specific εC residue does not reveal an ability to code for guanine. Instead, in this system, εC residues display the template characteristics a classical noninstructive lesions in that there is inefficient base misinsertion that appears to follow the adenine rule (SIMHA *et al.* 1991, 1994). (Center) Strategy used for incorporating a site-specific εC residue into M13 mp7L2 DNA. Experimental procedures have been described in detail by PALEJWALA *et al.* 1994. Note that a major part of the polylinker sequence is removed during the construction and is replaced with a 17-mer bearing the εC residue. (Bottom) A part of the DNA sequence of molecule 2 (before ligation) is shown to indicate the sequence context of the εC residue. In the sequence, X represents εC, or, in the case of the control construct, normal cytosine.

pended in transfection medium and made transfection-competent as described (PALEJWALA *et al.* 1991).

Transfection, determination of survival and pooled progeny phage preparation: In a typical transfection, 1 ml of competent cells (~1 × 10^9 cells/ml) were incubated with 50 ng of the appropriate DNA construct and incubated on ice for 30 min as described (PALEJWALA *et al.* 1991). To determine phage DNA survival, two 0.1-ml aliquots of the transfected competent cells were plated with 0.2 ml of the indicator culture on LB agar plates, and the number of plaque forming units was determined after overnight incubation. The remainder of the transfection mix (~0.8 ml) was transferred to a culture tube containing 8 ml of fresh LB medium at 22°, followed by the addition of 0.2 ml of a fresh saturated KH2R ΔrecA "supporter" bacterial culture. The culture tube was incubated at 37° with vigorous aeration overnight. The pooled progeny phage were recovered from the culture as described (PALEJWALA *et al.* 1993a,b, 1994) and subjected to sequence analysis. Note that KH2R "supporter" cells are used to improve phage yield at the overnight amplification step since some strains such as AR2 and OR2 give low phage titers. We have experimentally verified that DNA uptake, as well as mutation fixation, occur only in the indicated (competent) transfected AR2 or OR2 cells as expected and not in the (noncompetent) KH2R supporter cells under these conditions. KH2R supporter cells were also used for the *alkA*, *alkB*, and *aidB* mutants.

Quantitative multiplex DNA sequence analysis: The frequency and specificity of mutations at the εC site were determined by the strategy shown in Figure 2 and summarized below (details in PALEJWALA *et al.* 1993a,b, 1994). Five micrograms of pooled progeny phage DNA (~2 pmol) were annealed to ~1 pmol of 5′-^{32}P-end-labeled 19-mer primer. Approximately 0.2 pmol of the annealed template was incubated with ~0.5 units of T7 DNA polymerase devoid of 3′-to-5′ exonuclease activity (Sequenase 2.0; U.S. Biochemicals) in the presence of 1 μM each of dCTP and dGTP, 10 μM dideoxythymidine-5′-triphosphate (ddTTP) and 20 mM $MgCl_2$ in buffer (40 mM Tris-HCl, pH 7.6, 50 mM NaCl, 10 mM dithiothreitol). Under these conditions, limited primer extension occurs such that elongation on each of the four species of template DNA (*i.e.*, wild type, C-to-T transitions, C-to-A transversions, and 1-nt deletions) results in a product of different length (see Figure 2). The elongation products are fractionated on high-resolution 16% polyacrylamide-urea gels, and the proportion of each product is determined from densitometric analyses of autoradiographs as described (PALEJWALA *et al.* 1993a,b, 1994). Every elongation assay is monitored by parallel elongation of standard template DNA mixes containing known proportions of authentic mutant and wild-type DNAs.

RESULTS

The experimental system (Figure 2): εC, the model mutagenic lesion used here, is an exocyclic DNA lesion inflicted by metabolites of vinyl chloride and urethane (briefly reviewed in PALEJWALA *et al.* 1993b) as well as by certain endogenous mutagens (CHEN and CHUNG 1994). As predicted by the etheno bridging over two base pairing positions (Figure 1), εC has been shown to have the *in vitro* (SIMHA *et al.* 1991, 1994) and *in vivo* (JACOBSEN *et al.* 1989; JACOBSEN and HUMAYUN 1990; PALEJWALA *et al.* 1991, 1993b, 1994; BASU *et al.* 1993; MORIYA *et al.* 1994) template characteristics of a noninstructive lesion. In the experiments described here, the

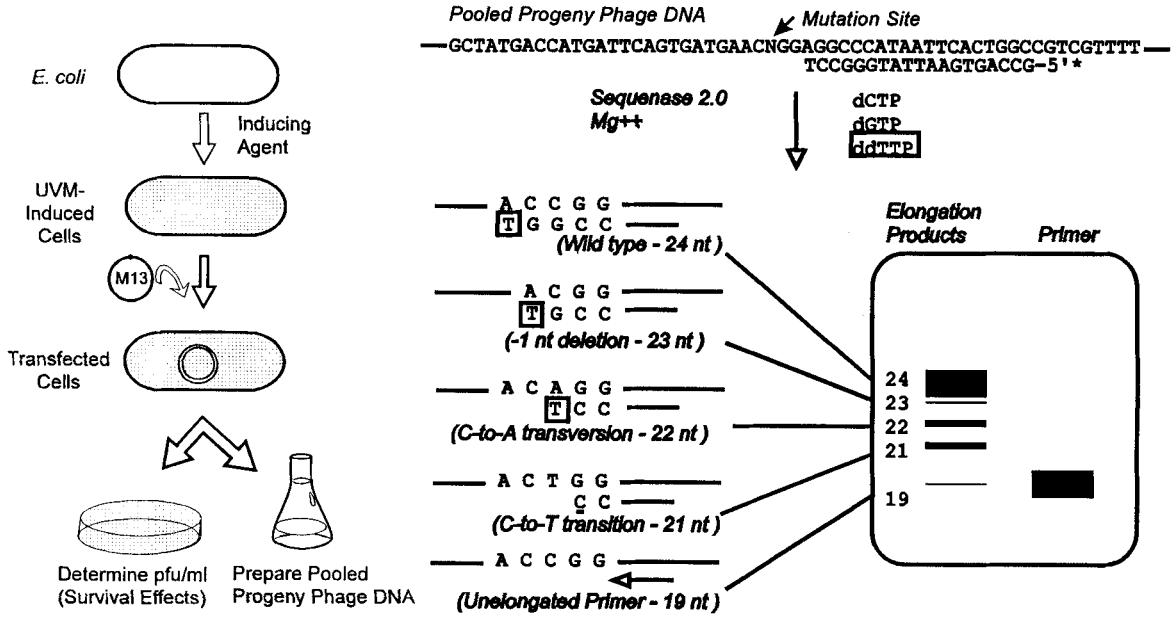

FIGURE 2.—The experimental strategy used to detect UVM mutagenesis at a site-specific ϵC residue borne on M13 ssDNA. (Left) Midlog *E. coli* cells are pretreated with an inducing agent for a defined period of time followed by removal of the agent and transfection of M13 ssDNA bearing a site-specific ϵC lesion. A portion of the transfected cells is plated to determine plaque forming units (infectious centers). The major portion of the transfected cells (infectious centers) is used for the preparation of pooled progeny phage DNA for multiplex sequence analysis. (Right) Sequence analysis is carried out by annealing a 5′ ^{32}P-end-labeled 19-mer primer to the pooled DNA, and elongation in the presence of dCTP, dGTP and dideoxythymidine triphosphate (ddTTP) such that each of four species of DNA shown will give a limit elongation product of unique length. The mutation frequency and specificity at the ϵC site is calculated from the normalized signal in the mutant (21–23 nt) and wild-type (24 nt) bands determined by computing densitometry as described in detail elsewhere (PALEJWALA *et al.* 1993a). By reconstruction experiments using known proportions of wild-type and mutant DNAs, we have rigorously established that mass phage culture does not distort the relative proportions of mutant and wild-type DNAs (PALEJWALA *et al.* 1993a) and that the sequence assay determines mutation frequency with a reasonably high accuracy for frequency values in the range of 3–95% (PALEJWALA *et al.* 1993a, 1994).

phenomenon observed is mutation fixation at a single, site-specific ϵC residue borne on single-stranded DNA of M13 mp7L2, a derivative of phage M13, that is introduced into bacterial cells by transfection. Before transfection, bacterial host cells are exposed to a UVM-inducing chemical for a period of time. Subsequently, the chemical is removed from the medium by cell pelleting and washing steps followed by procedures to induce transfection competence. Any modulation of mutagenesis at the ϵC residue is therefore an *indirect* consequence of pretreatment of the cells with the chemical and does not represent direct mutagenic modification of the M13 ssDNA by the chemical. A key feature of the experimental system used here is that it is a high-resolution system in which a site-specific noninstructive lesion in a transfected vector DNA molecule is used as a probe to monitor the mutation fixing environment of the cell. The mutational events are defined at the sequence level without dependence on phenotype-based screening or selection. We have previously described in detail the construction of ϵC-bearing M13 ssDNA (ϵC-DNA; PALEJWALA *et al.* 1994; also see Figure 1) as well as the development and validation of the multiplex DNA sequencing technology (PALEJWALA *et al.* 1993a, 1994; see Figure 2).

Exposure of *E. coli* cells to alkylating agents stimulates survival of M13 ssDNA bearing an ϵC residue: Table 2 shows that pretreatment of *E. coli* cells with alkylating agents results in a 2–12-fold stimulation of survival for subsequently transfected M13 ssDNA bearing a site-specific ϵC residue. The effect is observed in Δ*recA* cells and in cells defective for *ada*, the gene encoding a protein (Ada) that functions as a regulator of the adaptive response and as a DNA methylguanine methyltransferase. A similar effect is observed in cells defective for *alkA*, an adaptive response gene encoding the base excision repair enzyme 3-methyladenine DNA glycosylase II, and in cells defective for *alkB*, an adaptive response gene of unknown function. Finally, a similar effect is also observed in cells defective for *ogt*, the gene encoding the second, constitutive *E. coli* DNA methylguanine methyltransferase. These data suggest that the stimulation of survival does not depend on *recA*, or the genes constituting the adaptive response, or the *ogt* gene. As considered further in DISCUSSION, this *recA*-independent phenomenon (UVM reactivation) is reminiscent of Weigle reactivation (WEIGLE 1953). The observed stimulation in survival is not observed in control experiments in which ssDNA constructs bearing normal cytosine in place of ϵC are transfected (data not shown).

TABLE 2

The effect of pretreatment of various *E. coli* strains with alkylating agents on the survival and mutagenesis of transfected M13 ssDNA bearing a site-specific εC residue

Host strain	Pretreatment[a]	pfu[b]/50 ng of DNA		Percent mutation frequency[c]			
		Experiment 1	Experiment 2	Total	C → T	C → A	−1 nt
A. KH2R Δ*recA*	None (control)	920	210	7 ± 3	2 ± 1	2 ± 1	3 ± 1
	MNNG						
	1.0 μg/ml	1720	1620	14 ± 1	5 ± 1	7 ± 3	2 ± 2
	5.0 μg/ml	4120	1550	31 ± 7	6 ± 1	23 ± 8	3 ± 2
	10.0 μg/ml	3680	2500	50 ± 3	8 ± 2	41 ± 6	2 ± 2
B. KH2R Δ*recA*	None	1200		9 ± 3	3 ± 1	4 ± 2	2 ± 1
	NMU						
	0.1 mg/ml	2660		15 ± 2	7 ± 1	6 ± 1	2 ± 1
	1.0 mg/ml	4260		33 ± 6	6 ± 3	25 ± 5	1 ± 1
	5.0 mg/ml	5400		64 ± 6	12 ± 2	51 ± 6	1 ± 1
C. KH2R Δ*recA*	None (control)	620		5 ± 2	2 ± 1	2 ± 1	1 ± 1
	DMS						
	0.1 mM	1320		12 ± 3	3 ± 2	7 ± 2	1 ± 1
	1.0 mM	620		61 ± 3	9 ± 1	51 ± 3	1 ± 0
D. KH2R Δ*recA*	None (control)	840		6 ± 1	2 ± 1	2 ± 0	2 ± 0
	MeI						
	0.1 mM	1230		4 ± 1	1 ± 0	2 ± 1	1 ± 0
	1.0 mM	1780		5 ± 1	2 ± 1	2 ± 0	1 ± 0
	10.0 mM	770		5 ± 1	1 ± 0	2 ± 0	2 ± 1
E. AR2 Δ*ada* Δ*recA*	None (control)	320	210	8 ± 2	2 ± 1	3 ± 1	2 ± 2
	MNNG						
	1.0 μg/ml	580	420	46 ± 3	16 ± 6	28 ± 6	2 ± 2
	5.0 μg/ml	820	770	58 ± 7	10 ± 5	46 ± 4	2 ± 2
	10.0 μg/ml	2880	2240	70 ± 3	16 ± 2	53 ± 2	1 ± 1
F. OR2 *ogt* Δ*recA*	None (control)	340	290	7 ± 3	2 ± 0	3 ± 1	2 ± 2
	MNNG						
	1.0 μg/ml	440	1360	17 ± 3	4 ± 1	11 ± 4	2 ± 2
	5.0 μg/ml	2300	1840	52 ± 7	6 ± 2	44 ± 4	2 ± 2
	10.0 μg/ml	1420	1480	58 ± 4	8 ± 4	48 ± 3	2 ± 1
G. MV1571 *alkA*	None (control)	1100		5 ± 3	1 ± 1	2 ± 1	2 ± 2
	MNNG						
	1.0 μg/ml	6200		17 ± 5	2 ± 1	14 ± 4	1 ± 0
	5.0 μg/ml	7300		26 ± 2	2 ± 1	23 ± 3	1 ± 1
	10.0 μg/ml	2010		53 ± 2	4 ± 1	48 ± 1	1 ± 1
H. MV1601 *alkB*	None (control)	180		9 ± 2	3 ± 1	4 ± 1	2 ± 1
	MNNG						
	1.0 μg/ml	480		21 ± 1	11 ± 1	9 ± 1	2 ± 1
	5.0 μg/ml	940		33 ± 1	12 ± 1	20 ± 2	2 ± 1
	10.0 μg/ml	920		47 ± 3	16 ± 2	27 ± 1	4 ± 1

[a] Cells were pretreated for 10 min (MNNG and NMU) or 30 min (dimethylsulfate, DMS; and methyl iodide, MeI) at 37° with the indicated concentrations of the alkylating agent as described in MATERIALS AND METHODS.

[b] pfu, plaque forming units. Note that day-to-day variation in transfection efficiency for the Δ*recA* strain is about twofold, but is known to be as high as fivefold for the M13 ssDNA transfection system (*e.g.*, KUNKEL 1984). In addition, strain-to-strain variability in transfection efficiency is also observed, as reflected in the data on strains defective for alkylation repair (*cf. alkA* and *alkB* mutants). Reactivation curves are typically bell-shaped presumably because of interference from the toxic effects of high doses of inducing agents.

[c] Averages ± SD of three (parts B–D, G and H) or six (parts A, E and F) sequence analyses (see Figure 2 and MATERIALS AND METHODS) of progeny DNA pools, with numbers rounded to the nearest integer.

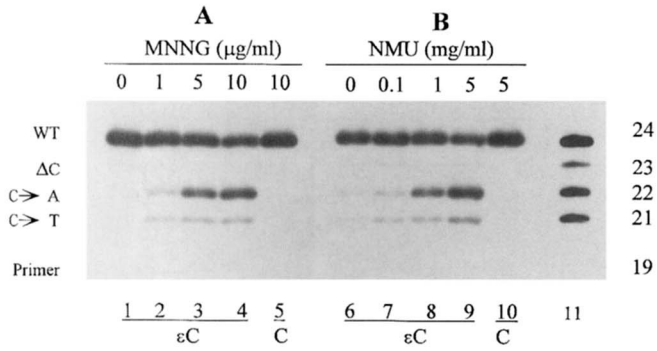

FIGURE 3.—Multiplex DNA sequence analysis of pooled progeny phage DNA obtained by transfection of M13 ssDNA constructs either bearing a site-specific εC lesion (lanes labeled εC) or normal cytosine in place of εC (lanes labeled C) into *E. coli* KH2R Δ*recA* cells pretreated with the alkylating agents. Exponentially growing *E. coli* cells (~5 × 10^7 cells/ml in LB medium) were exposed to MNNG (A) or NMU (B) at the indicated concentrations for 10 min, followed by transfection. Pooled progeny phage DNA were isolated from each transfection and subjected to multiplex sequence assay as described in Figure 2 and in MATERIALS AND METHODS. Lane 11 is a control for the assay, and shows elongation products from a standard mix containing 40% of wild-type DNA, 10% of −1 deletion mutant DNA, and 25% each of C → T transition and C → A transversion mutant DNAs. Each elongation product is labeled (left of Lane 1), and the length indicated (numbers to the right of Lane 11; also see Figure 2). Note that each lane (Lanes 1–10) represents results from an independent transfection experiment and yields data that are equivalent in principle to those obtained by sequencing a large number of independently isolated mutants in conventional experimental approaches. As expected, all progeny DNA obtained by transfection of the control construct is wild type (Lanes 5 and 10). In contrast, transfection of the εC-containing ssDNA (Lanes 1–4 and 6–9) results in wild type (24-nt product) as well as mutant (21–23-nt products) progeny. The mutation frequency at εC in untreated cells (Lanes 1 and 6) is low, but increases as a function of pretreatment of host cells. These results are expressed quantitatively in Table 2, A and B.

Exposure of E. coli cells to MNNG and NMU dramatically enhances mutagenesis at a site-specific εC residue borne on M13 ssDNA: Figure 3 shows the results of multiplex DNA sequence analyses of pooled progeny phage DNA arising from transfection of εC-bearing M13 ssDNA into *E. coli* KH2R Δ*recA* cells that have been subjected to a 10-min exposure to the indicated concentrations of the alkylating agents MNNG or NMU *before* transfection. Figure 3A shows that in uninduced cells (0 μg/ml MNNG; lane 1), mutation fixation at εC is relatively low, as has been previously shown (BASU *et al.* 1993; MORIYA *et al.* 1994; PALEJWALA *et al.* 1994). There is a strong enhancement of mutagenesis at increasing doses of MNNG as indicated by the increasing signal in the 21-mer (C → T) transition band as well as the 22-mer (C → A) transversion band, with a concomitant decrease in the signal corresponding to the 24-mer wild-type band (Figure 3, lanes 2–4). As expected, transfection of control M13 ssDNA containing normal cytosine in the place of εC into MNNG-pretreated cells does not result in detectable mutagenesis (Figure 3, lane 5). Figure 3B similarly shows that pretreatment of cells for 10 min with NMU also results in a strong induction of mutagenesis (compare lane 6 with lanes 7–9) at the εC residue borne on M13 DNA, but not in the control DNA (lane 10). A quantitative summary of data collected from two separate series of transfections in MNNG-pretreated cells, with progeny phage DNAs from each series subjected to three independent sequencing assays for a total of six sets of sequence analyses, is given in Table 2A. Data averaged from three sequence assays of progeny phage DNAs from NMU-pretreated cells are similarly summarized in Table 2B. These data demonstrate efficient UVM induction in Δ*recA* cells by the alkylating agents MNNG and NMU.

Table 3 shows that exposure of KH2R Δ*recA* cells to 10 μg/ml MNNG for 5 min is sufficient to strongly induce UVM. Increasing the length of exposure to 10- or 15-min increases the mutagenic response. It is interesting to note that C→A transversions account for most mutations in UVM-induced cells, especially at higher doses of the inducing agent.

UVM induction by the S_N2 alkylating agent dimethylsulfate, but not by the nonmutagenic agent methyl iodide: Both S_N1 and S_N2 alkylating agents predominantly alkylate nitrogen atoms in DNA bases. However, S_N1 agents such as MNNG and NMU also induce significant levels O-alkylation, an activity believed to be significant in the direct mutagenic effectiveness of S_N1 agents. S_N2 alkylating agents such as dimethylsulfate induce little O-alkylation and generally have a weaker mutagenic activity than S_N1 agents. Figure 4A shows that dimethylsulfate, an S_N2 agent, can effectively induce UVM. The magnitude of the UVM effect at the highest level tested (~60% at 1 mM) is comparable with that induced by NMU. In contrast, methyl iodide, a nonmutagenic methylating agent, does not cause appreciable UVM induction under the same experimental conditions (Figure 4B). A quantitative summary of the data on UVM induction by dimethylsulfate and methyl iodide is given in Table 2, C and D, respectively.

UVM induction by MNNG occurs in *E. coli* cells lacking functional *ada* or *ogt* genes: DNA alkylating agents such as MNNG, NMU, dimethylsulfate and methyl iodide are known to induce the *E. coli* adaptive response (*e.g.*, OTSUKA *et al.* 1985; VOLKERT *et al.* 1986; LINDAHL *et al.* 1988), thereby raising the possibility that UVM may be an unrecognized facet of this inducible response. To test whether the adaptive response is required for UVM, we asked if UVM is observable in *ada* mutants in which the adaptive response is uninducible. The data in Table 2E demonstrate that exposure of Δ*recA* Δ*ada* cells for 10 min with various concentrations of MNNG results in a strong induction of UVM mutagenesis as compared with uninduced cells. Therefore, UVM is independent of both *recA* and *ada* genes. Similarly, MNNG also in-

TABLE 3

Effect of MNNG pretreatment (time course) of *E. coli* KH2R Δ*recA* cells of mutagenesis at a site-specific ϵC residue in M13 ssDNA

MNNG (μg/ml)	Exposure time (min)	Total mutation frequency[a]	Mutation specificity		
			C → T	C → A	−1 nt
0	—	4 ± 1	1 ± 0	1 ± 1	1 ± 0
10	5	61 ± 2	13 ± 3	44 ± 3	4 ± 1
	10	61 ± 2	6 ± 1	53 ± 2	2 ± 1
	15	73 ± 8	7 ± 0	65 ± 8	1 ± 0
50	5	74 ± 3	9 ± 1	64 ± 4	1 ± 1
	10	84 ± 5	9 ± 2	74 ± 6	1 ± 1
	15	96 ± 1	2 ± 1	93 ± 2	2 ± 1

[a] Averages ± SD derived by analyzing progeny DNA pools obtained in one transfection experiment subjected to four multiplex sequencing assays. Numbers were in percentages rounded to the nearest integer.

duces UVM in a Δ*recA ogt* mutant strain, showing that *ogt,* the gene encoding an *ada*-independent DNA methyltransferase is also not required for UVM (Table 2F).

A comparison of UVM induction in KH2R Δ*recA* (Table 2A) and in AR2 Δ*recA* Δ*ada* (Table 2E) cells shows that the UVM response is significantly stronger in Δ*ada* cells than in the *ada*$^+$ cells, especially for lower MNNG doses. Because Δ*ada* cells are defective for repair of DNA alkylation damage, this observation suggests that UVM induction by MNNG is mediated through DNA damage.

UVM induction in *alkA, alkB* and *aidB* mutants: Even though *ada, alkA, alkB* and *aidB* genes are coordinately induced as a part of the adaptive response, the precise functions of *alkB* and *aidB* genes are not yet known. As briefly considered in DISCUSSION, loss of a hypothetical repair function can account for the apparent inducibility of UVM mutagenesis. To test if loss of alkylation repair is involved, we examined UVM induction in a strain defective for *alkA,* the gene that encodes 3-methyladenine DNA glycosylase II, an enzyme known to act at a variety of base modifications including etheno lesions (MATIJASEVIC *et al.* 1992; SAPARBAEV and LAVAL 1994). (It may be noted that base excision repair can be initiated, but cannot be completed, in ssDNA due to the lack of a complementary strand to template gap-filling; furthermore, cleavage of the abasic site intermediate will result in lethal linearization of the ssDNA molecule).

The data in Table 2, G and H, show that UVM induction occurs in *alkA* mutant cells and in *alkB* mutant cells. In addition, we have observed that UVM induction is also independent of the *aidB* gene (data not shown). Finally, because the *aidB* gene is also induced by an alternative *rpoS*-mediated mechanism (VOLKERT *et al.* 1994), it is interesting to ask if *aidB* induction by this mechanism is involved in UVM. To address this question, we exploited the recent finding that treatment of exponential cultures with 50 mM acetate, pH 6.5, can induce *aidB* together with a number of stationary phase genes (VOLKERT *et al.* 1994). However, our preliminary data indicate that treatment of exponential phase *aidB*$^+$ cells to 50 mM acetate (pH 6.5) for ≤3 hr at 37° does not result in significant UVM induction (data not shown).

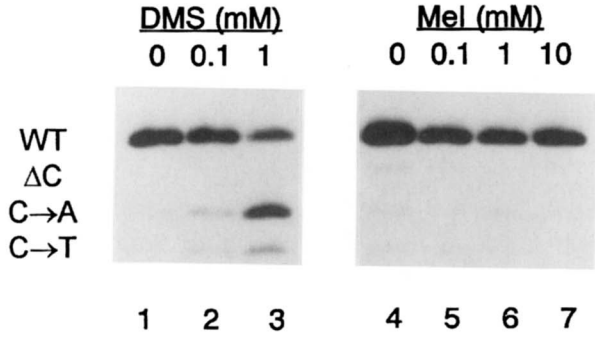

FIGURE 4.—UVM induction in *E. coli* KH2R Δ*recA* by pretreatment of cells with dimethylsulfate (DMS) or methyl iodide (MeI) for 30 min at 37°. The experiments were carried out as described in the legends for Figure 2 and Figure 3 except for substituting MNNG (or NMU) with indicated concentrations of dimethylsulfate (0, 0.1 or 1 mM) or methyl iodide (0, 0.1, 1 or 10 mM). It can be seen that mutation frequency increases as a function of dimethylsulfate dose (A). No appreciable induction is seen with up to 10 mM methyl iodide (B). These results are expressed quantitatively in Table 2, C and D.

DISCUSSION

The experiments described in this paper demonstrate that the UVM effect, originally observed in UV-irradiated *E. coli* cells, is also induced by the S_N1 alkylating agents MNNG and NMU, by the S_N2 agent dimethylsulfate, but not by methyl iodide, a nonmutagenic alkylating agent. Our analysis shows that UVM induction by alkylating agents requires neither the *E. coli recA* gene nor the five genes known to be actually or potentially involved in alkylation repair, namely, the *ada, alkA, alkB, aidB* and *ogt* genes. Furthermore, results described elsewhere show that UVM induction does not require *umuD/C* genes and occurs under conditions

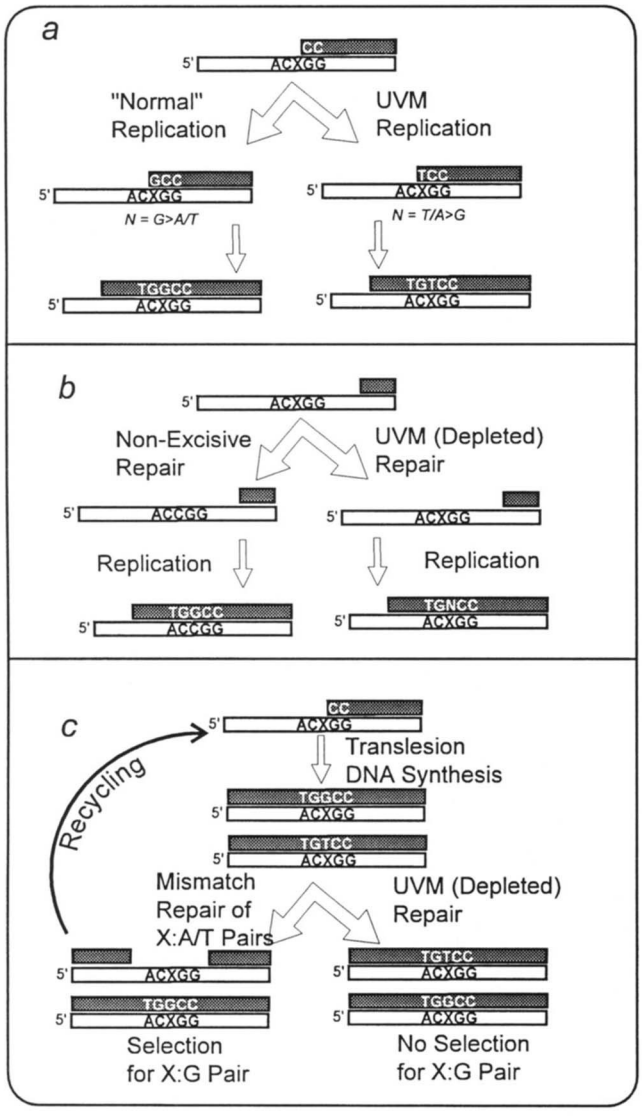

FIGURE 5.—Possible mechanisms for UVM mutagenesis. (a) Mechanisms are depicted that act at the base insertion step (only ϵC:T misinsertion is shown for the sake of simplicity). Here one hypothesizes an induced alteration of DNA replication such as to make the process more error prone. The alteration can be in the form of a factor that modifies the DNA polymerase III (pol-III) holoenzyme. It can be a transient substitution of pol-III by pol-I or pol-II (a "polymerase switch"). Finally, the alteration may be mediated through altered nucleotide precursor synthesis and pool imbalance (PALEJWALA *et al.* 1993b). (b) A simple repair-depletion model is depicted. Here one proposes that a constitutively expressed nonexcisive mechanism is available in uninduced cells. This activity repairs most of the ϵC to C before replication, accounting for low mutagenesis in uninduced cells. UVM results from depletion of this activity as a result of massive chromosomal DNA damage inflicted by UV or alkylating agents. Note that this model cannot account for the observed UVM reactivation without additional assumptions. (c) A complex repair-depletion model requiring template recycling is depicted. Here, translesion synthesis in uninduced cells gives rise to ϵC:A, ϵC:T, ϵC:G "pairs" (only the ϵC:G and ϵC:T pairs are shown for the sake of simplicity). However, the ϵC:A and ϵC:T pairs (but not the ϵC:G pair) are substrates for a hypothetical DNA N-glycosylase that removes the base inserted opposite the ϵC lesion. Subsequent repair endonuclease and exonuclease cleavage steps create a gap opposite ϵC, and thus recycle the template for additional rounds of translesion synthesis. Template recycling will therefore select for ϵC:G pairs, accounting for low mutagenesis in uninduced cells. UVM results from depletion of the repair glycosylase by massive chromosomal DNA damage. As a result, template recycling is reduced, and the observed mutagenesis directly reflects the base misinsertion pattern of the DNA polymerase.

where a *dinD::lacZ* fusion gene (a marker for SOS induction) is not derepressed at significant levels (V. A. PALEJWALA, G. WANG, H. S. MURPHY, and M. Z. HUMAYUN, unpublished data). These observations define UVM as an inducible mutagenic phenomenon that may represent a previously unrecognized cellular response to genotoxic stress.

That DNA damage may be involved in UVM is suggested by the observation that the UVM effect is enhanced in Δ*ada* cells known to be defective in alkylation damage repair. It is interesting that methyl iodide can efficiently induce the adaptive response but not the SOS response (TAKAHASHI and KAWAZOE 1987). Methyl iodide is thought to induce the adaptive response by directly methylating the Ada protein rather than by damaging DNA. Therefore, its inability to induce UVM is consistent with the idea that DNA damage may be involved in UVM induction. It may be noted that methyl iodide appears to increase M13 survival by about twofold without a concomitant increase in mutation fixation at the ϵC residue borne on the transfected M13 ssDNA. The significance of the reactivation effect without mutagenesis is unclear at this time.

Current views on mutation fixation at noninstructive DNA lesions have been largely shaped by the SOS hypothesis that originally postulated that DNA replication arrest induced by unrepaired bulky lesions leads to the coordinated expression of a number of cellular functions that are normally expressed at low levels (reviewed in RADMAN 1975; WITKIN 1976). Among the induced functions are hypothetical factors that can modify the replication apparatus such as to allow it to overcome the replication arrest at the cost of mutagenesis ("SOS mutagenesis"). The so-called "error-prone" replication was subsequently recognized as requiring two discrete steps (BRIDGES and WOODGATE 1985), namely, the base misinsertion step and the bypass (base extension) step. The term bypass refers to the resumption of replication at the newly inserted 3′ nucleotide that by definition cannot form a correct base pair with the noninstructive template site.

The mutagenic specificity of certain noninstructive lesions such as abasic sites (KUNKEL 1984; RABKIN and STRAUSS 1984; LOEB and PRESTON 1986; LAWRENCE *et al.* 1990; ECHOLS and GOODMAN 1991; GOODMAN *et al.* 1993, 1994) has been interpreted to mean that SOS proteins are involved in the "A-rule" misinsertion and bypass replication at such sites. Accordingly, it has been observed that cells incapable of SOS induction such as

recA or *lexA*(Ind$^-$) mutant cells display hypersensitivity as well as a reduced mutagenic response to agents that inflict certain types of DNA damage. The lower mutagenic response accompanying increased lethality has been interpreted to mean that SOS functions are required for *both* the misinsertion and bypass steps. However, as argued below, reduced mutagenesis in SOS-deficient cells can result from a failure of misinsertion *or* a failure of bypass.

The major features of the regulation of the SOS regulon are quite well described (reviewed in WALKER 1984, 1987; ECHOLS and GOODMAN 1991): SOS gene expression is repressed by the binding of the LexA repressor to the SOS gene promoters. Unrepaired noninstructive DNA damage leads to replication arrest, exposing single-stranded DNA to which the RecA protein binds and undergoes a dATP-dependent conformational transition to the RecA* ("coprotease") form. The RecA* protein derepresses the SOS regulon through proteolytic destruction of the LexA protein and activates the UmuD protein (encoded by the SOS gene *umuD*) to the proteolytically cleaved active form UmuD'. In addition to the above two SOS roles, the RecA* protein bound to the damaged template DNA at the site of the replication arrest has been proposed to facilitate error-prone replication as a part of a hypothetical "mutasome" consisting of RecA*, UmuD', and UmuC proteins (WOODGATE *et al.* 1989; ECHOLS and GOODMAN 1991; RAJAGOPALAN *et al.* 1992; FRANK *et al.* 1993). Despite the wealth of information available on the SOS regulon, the *recA, umuD* and *umuC* genes, and the availability of the purified gene products in appropriate activated form, it has not been possible to describe the mechanism of error-prone replication. While this frustrating lack of insight may be a reflection of the complexity of the phenomenon, the possibility exists that induced error-prone replication may require inducible activities in addition to those provided by the SOS regulon. In an interesting recent study, SOMMER *et al.* (1993) have shown that *among SOS genes,* the induction of the *umuD/C* operon is both necessary and sufficient for UV mutagenesis. It is important to note, however, that SOMMER *et al.* (1993) measured SOS mutagenesis in UV-irradiated cells. As considered below, it may be difficult to separate the base insertion and extension steps for certain DNA lesions. Because UV irradiation also induces the UVM phenomenon (PALEJWALA *et al.* 1994), the involvement of inducible activities in addition to those encoded by SOS genes cannot be ruled out. The results reported by SOMMER *et al.* (1993) are, therefore, not inconsistent with the possibility that SOS proteins may function predominantly at the base extension (bypass) step, rather than at the base insertion step.

The UVM phenomenon may have previously gone unrecognized because the base misinsertion step cannot be separated from the bypass step for the more commonly used model noninstructive lesions such as AP sites and certain bulky adducts (class 1 noninstructive lesions). Other noninstructive lesions such as ϵC may not require SOS functions for the bypass step (class 2 noninstructive lesions). We suggest that the SOS independence of class 2 noncoding lesions allows recovery of mutations in Δ*recA* cells, thus enabling one to experimentally isolate the mutation fixation step from the bypass step. We have previously speculated that improved base-stacking ability possibly conferred by the coplanar etheno ring in ϵC may eliminate or reduce dependence on SOS functions for the bypass step (PALEJWALA *et al.* 1993a).

It is intriguing that pretreatment of cells with alkylating agents results in a significant enhancement of survival of transfected M13 ssDNA bearing a site-specific ϵC residue (Table 2) and that this reactivation effect does not appear to require the *recA* or *ada* genes. This observation is reminiscent of Weigle reactivation, the name given to the paradoxical increase in the survival of UV-irradiated phage λ in UV-irradiated *vs.* unirradiated *E. coli* (WEIGLE 1953). Whether UVM reactivation makes a contribution to Weigle reactivation is among the interesting questions regarding UVM that remain to be addressed.

For the sake of simplicity, we have so far focused on base misinsertion opposite ϵC as the mechanism for UVM. However, our experiments measure the end result of mutation and do not directly address questions on the pathway for the observed inducible mutagenesis. In fact, two major classes of mechanisms can account for the UVM effect: induction mechanisms and depletion mechanisms. In Figure 5a, we depict an induction mechanism in which an unspecified alteration of DNA replication ("UVM replication") has the effect of altering base misinsertion opposite a noncoding lesion (X). In this particular case, it is proposed that base insertion opposite ϵC in uninduced cells follows the order G>A>T, whereas in UVM-induced cells, it is T/A>G. Figure 5b shows a simple depletion mechanism based on the sequestration of a hypothetical nonexcisive repair (direct repair) enzyme. Here, it is assumed that in uninduced cells, a prereplicative repair mechanism efficiently repairs the ϵC residue (see Figure 5 legend). UVM results from a transient depletion of this activity. Figure 5c shows a more complex depletion model based on the loss of a hypothetical postreplication mismatch correction activity (see Figure 5 legend). While each of the hypotheses summarized in Figure 5 has specific strengths, it is clear that much more needs to be learned about UVM before one can support or eliminate any single hypothesis.

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