UV Irradiation of *Escherichia coli* Modulates Mutagenesis at a Site-Specific Ethenocytosine Residue on M13 DNA. Evidence for an Inducible *rec*A-Independent Effect†

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Received November 18, 1992; Revised Manuscript Received February 8, 1993

ABSTRACT: Mutagenic action of chemical and physical mutagens is mediated through DNA damage and subsequent misreplication at sites of unrepaired damage. Most DNA damage is noninstructive in the sense that the causative chemical modification either destroys the template information or renders it inaccessible to the DNA polymerase. Noninstructive adducts possess high genotoxicity because they stop DNA replication. Replication past noninstructive adducts is thought to depend on induced functions in addition to the regular replication machinery. In *Escherichia coli*, noninstructive DNA damage leads to induction of the SOS regulon, which in turn is thought to provide the inducible functions required for replicative bypass of the lesion. Because of the absence of accessible template instruction, base incorporation opposite noninstructive lesions is inherently error-prone and results in mutagenesis. Ethenocytosine (εC), an exocyclic DNA lesion induced by carcinogens such as vinyl chloride and urethane, is a highly mutagenic, noninstructive lesion on the basis of its template characteristics in vivo and in vitro. However, mutagenesis at εC does not require SOS functions, as evidenced by efficient mutagenesis in *rec*A-deleted *E. coli*. Even though efficient mutagenesis in *rec*A-deleted cells shows a lack of SOS dependence, the question remains whether SOS induction can modulate mutagenesis opposite εC. To examine the possible contribution of SOS functions to mutagenesis at εC, we constructed an M13 duplex circular DNA molecule containing an εC residue at a unique site. The construct was transfected into nonirradiated or UV-irradiated *E. coli*. The frequency as well as specificity of the mutations induced under a number of conditions was determined by using a multiplex DNA sequencing technology. Without prior UV irradiation, approximately one-third of the progeny is mutant, the majority of mutations being C→T transitions. Prior UV irradiation of wild-type host cells results in a significant increase in mutagenesis with most of the increase accounted for by an increase in C→A transversions. Surprisingly, essentially identical effects were observed in irradiated *rec*A-deleted cells as well as in *umu*C-deficient cells, suggesting that the observed UV modulation of mutagenesis is independent of the SOS pathway. These observations suggest the existence of a *rec*A-independent UV-inducible mutagenic mechanism in *E. coli*.

On the basis of proposed mutagenic mechanisms, DNA lesions are classified into two broad categories: mispairing and noninstructive. Mutagenic mechanisms of mispairing lesions such as O^6-methylguanine or DNA uracil deriving from deamination of DNA cytosine are reasonably well understood: such lesions possess template information that can be decoded by DNA polymerase, but the information is wrong. The specificity of mutations is determined by the specific miscoding conferred on the base by the chemical modification. It is widely believed that mispairing lesions do not block replication and that mutagenesis opposite such lesions does not depend on induced functions (Walker, 1984, 1987).

In contrast, noninstructive lesions are either devoid of template activity, as in the case of abasic sites, or have their template activity blocked by steric hindrance, as in the case of bulky chemical adducts. Such adducts block DNA replication and are lethal unless they are either removed by DNA repair or "bypassed" by the replication apparatus. For nearly two decades, the *Escherichia coli* SOS phenomenon has been central to our understanding of mutagenesis by noninstructive DNA adducts (Defais et al., 1971; Radman, 1975; Witkin, 1976; Walker, 1984, 1987). According to the SOS hypothesis, arrest of DNA synthesis by such adducts induces the SOS regulon, and the induced SOS functions in turn facilitate translesion DNA synthesis (lesion bypass) by an unknown mechanism. Since base incorporation opposite a noninstructive site must be by definition error-prone, the ultimate result of SOS induction is proposed to be enhanced survival at the cost of increased mutagenesis. Since replication past noninstructive adducts requires induced (SOS) functions, mutagenesis at such lesions is SOS-dependent. Recent literature has proposed two separate steps in SOS–mutagenesis: the misincorporation step and the bypass step (Bridges & Woodgate, 1985).

An interesting feature of forced replication of noninstructive adducts is that base incorporation opposite such adducts is not random as one would expect on empirical grounds, i.e., incorporation probability is not equal to 0.25 for each of the four bases. Instead, adenine is preferentially incorporated opposite such lesions, followed by other bases (Kunkel, 1984; Rabkin & Strauss, 1984). This order of preference appears to be largely independent of the DNA polymerase as well as the chemical modification (as long as it is noninstructive) and has come to be regarded as an inherent property of DNA replication. Mutational specificity deriving from the so-called "adenine rule" for base misincorporation is considered to be a property of a noninstructive adduct.

Even though the above generalizations regarding mispairing and noninstructive mutagenic adducts are widely accepted,

† This work was supported by a grant from the NIH.

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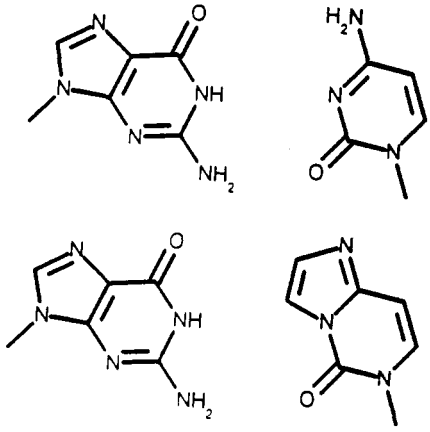

FIGURE 1: Structure of εC. (Top) A normal Watson–Crick G:C pair. (Bottom) A G:εC pair to show the effect of the etheno bridge on the Watson–Crick base-pairing positions of the cytosine.

recent experimental evidence raises questions concerning the validity and utility of this classification. In particular, recent evidence suggests that SOS dependence and template characteristics may be independent attributes of a mutagenic lesion; thus, it is possible to have SOS-dependent as well as SOS-independent mispairing lesions (Sambamurti et al., 1988; Sahasrabudhe et al., 1990, 1991). Similarly, SOS dependence is not a defining attribute of a noninstructive lesion: some require SOS functions for efficient mutagenesis, and some do not. This last point is particularly well made by the recently characterized mutagenic properties of ethenocytosine[1] (εC; Jacobsen et al., 1989; Jacobsen & Humayun, 1990; Simha et al., 1991; Palejwala et al., 1991, 1993), an exocyclic lesion induced by several carcinogens such as vinyl chloride and urethane (Leonard, 1984; Leithauser et al., 1990). Accumulated evidence has shown that εC, a noninstructive adduct by the criteria of chemical structure (Figure 1), in vitro template activity, and in vivo mutational signature, is highly mutagenic in both wild-type and *rec*A-deficient *E. coli*. εC appears to be not so much a unique mutagenic lesion as the first clearly recognized example of a noninstructive adduct that is SOS-independent for mutagenesis.

The experimental system used to characterize εC mutagenesis involved the transfection of M13 DNA modified with εC in either a regiospecific (Jacobsen et al., 1989) or site-specific (Palejwala et al., 1991) manner. Transfection of such DNA into wild-type or *rec*A-deficient *E. coli* was shown to result in efficient mutagenesis without a significant effect on phage survival. While these experiments indicate that SOS functions are not *required* for either bypass or efficient mutagenesis opposite εC, they leave unexplored the possibility that even if not required SOS functions can still *modulate* mutagenesis at εC. The experiments described in this article were originally undertaken to examine the effect of SOS induction on εC mutagenesis. This issue can be addressed by transfecting DNA bearing εC into SOS-induced cells, followed by the determination of mutational frequency and specificity. A rigorous examination of the effect of SOS induction on mutational specificity will, however, require sequence analysis of large numbers of mutant progeny phage isolated under a number of experimental conditions. The development of a multiplex sequence assay for quantitative analysis of mutational hot spots (Palejwala et al., 1993) has made such an examination practical. Here, we describe the application of the multiplex technology to uncover the effect of prior UV irradiation of *E. coli* on mutagenesis at a site-specific εC residue carried on M13 DNA.

[1] Abbreviations: εC, 3,N^4-ethenocytosine; RF-DNA, replicative-form DNA, ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; bp, base pair(s); nt, nucleotide(s); UVM, UV modulation of mutagenesis.

MATERIALS AND METHODS

Bacteriophage Strains. M13 AB28 is essentially M13mp2 except that the *lacZ*(α) gene segment in AB28 has the polylinker sequence from M13mp8 (Sambamurti et al., 1988). M13f2 is a derivative of M13 AB28 obtained by replacing a 26-nt segment within the polylinker region with a different synthetic 25-nt sequence. This replacement results in a −1 frameshift in the *lacZ*(α) gene. M13f2t, M13f2a, and M13f2Δ1 are essentially the same as M13f2 except for point mutations occurring at the center of the 25-nt "replacement" sequence in M13f2. DNA sequences of these strains are shown in Figure 1 of the preceding article in this issue (Palejwala et al., 1993).

DNA Preparations. Form II′$_{εC}$ is a circular duplex M13 DNA construct bearing a single site-specific εC residue at the center of a 17-nt-long single-stranded region prepared as described by Palejwala et al. (1991; see Figure 1 of the preceding article in this issue for the DNA sequence). Form II′$_C$ is a control DNA construct with normal cytosine in place of the site-specific εC found in form II′$_{εC}$. These DNAs were constructed as described in detail elsewhere (Palejwala et al., 1991; see Figure 1 of the preceding article for the relevant sequence). M13 viral ssDNAs used in multiplex assays were prepared as described in the preceding article in this issue (Palejwala et al., 1993).

Bacterial Strains. The following *E. coli* strains were gifts from K. Sambamurti (Sambamurti et al., 1988; Palejwala et al., 1991): KH2 Sup^0, Δ*lac–pro*, *trpE*9777; F′ LacI^{q}ZΔM15, Pro$^+$, KH2R Sup^0, Δ*lac–pro*, *trpE*9777, Δ(*srl*R–*rec*A)306::Tn10(Tet^R); F′ LacI^{q}ZΔM15, Pro$^+$; and KH2C Sup^0, Δ*lac–pro*, *trpE*9777, *umu*C122::Tn5(Kan^R); F′ LacI^{q}ZΔM15, Pro$^+$. The Δ(*srl*R–*rec*A)306 deletion in the KH2R strain is a total deletion of the *rec*A gene (Csonka & Clark, 1979). The KH2C strain was constructed by P1 transduction of the *umu*C122::Tn5(Kan^R) locus (Elledge & Walker, 1983) from *E. coli* GW2100 (gift of Dr. G. Walker) into *E. coli* KH2.

All strains were maintained on appropriately supplemented M9 minimal agar plates. Before use in transfection, the KH2R (*rec*A$^-$) and KH2C (*umu*C$^-$) strains were grown on minimal agar plates with appropriate nutritional supplements and tetracycline (12.5 μg/mL; KH2R) or kanamycin (50 μg/mL; KH2C). The UV sensitivity of all strains was initially examined by exposing a streak of each strain to UV (Sambrook et al., 1989). In comparison to wild-type cells, the *rec*A-deficient strain was highly UV sensitive by the streak test, whereas the *umu*C-deficient strain (KH2C) was not significantly more sensitive. To confirm the UV sensitivity phenotype, cells from each strain were suspended in buffer and UV-irradiated as described below, serially diluted, and plated on appropriate M9 agar plates. Figure 2 shows that, as compared to wild-type cells, KH2R cells have pronounced sensitivity to UV irradiation. As expected, KH2C cells are more sensitive than wild-type cells (Kato & Shinoura, 1977; Bagg et al., 1981; Elledge & Walker, 1983), but by no means as sensitive as KH2R cells (Walker, 1987).

UV Irradiation of Bacterial Cells. Each strain was grown overnight in LB medium at 37 °C with vigorous aeration. Fresh LB medium (250 mL in a 1-L flask) was inoculated with 1 mL of the overnight culture, and the cells were allowed to grow at 37 °C with vigorous aeration to an optical density

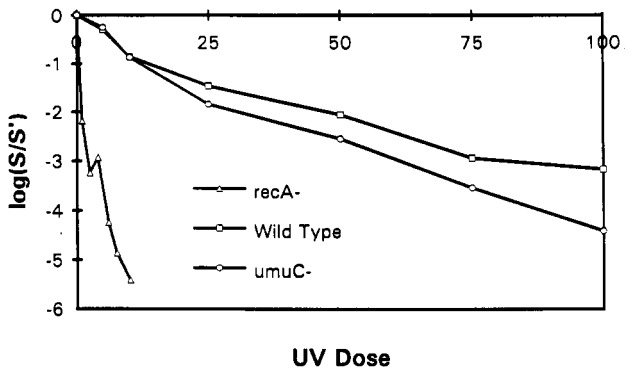

FIGURE 2: Effect of UV irradiation on survival of the three *E. coli* strains used in this study. UV dose is in J/m^2. Irradiated and control cells were plated as described in Materials and Methods to determine survival (S, irradiated cells; S', mock-irradiated cells): □, KH2 (wild type); ○, KH2C (*umuC*$^-$); △, KH2R (*recA*$^-$). Note that the *recA*$^-$ strain shows pronounced UV sensitivity, whereas the *umuC*$^-$ strain shows, as expected, much lower sensitivity as compared to the *recA*$^-$ strain.

of 0.3–0.4 (600 nm), corresponding to a cell density of $\sim 10^8$ cells/mL. Cells were pelleted by centrifuging the culture (250 mL) at about 1000*g* for 10 min at 4 °C. The pellet was resuspended in 50 mL of cold MN buffer (10 mM $MgSO_4$ and 100 mM NaCl) to achieve a cell density of about 5×10^8 cells/mL. UV irradiation was carried out using a 15-W General Electric G15T8 germicidal lamp calibrated after a 15-min warm-up period to a fluence rate of 100 μW/cm^2 using a Spectroline DM-25N UV meter (Spectronics Corporation, Westbury, NY). The resuspended cells (6 mL, 5×10^8 cells/mL) were dispensed into plastic Petri dishes held on ice and exposed to a UV dose of 0–25 J/m^2 for *E. coli* KH2R or 0–100 J/m^2 for *E. coli* KH2 and KH2C, as described. During each irradiation, the Petri dish was gently rocked back and forth on a bed of ice to ensure uniform UV exposure of cells and to keep the temperature low. UV irradiation as well as subsequent operations was carried out in the dark to minimize the photoreactivation of UV damage. After irradiation, cells were pelleted by centrifugation at 1000*g* for 10 min at 4 °C and resuspended in 3 mL ($\sim 10^9$ cells/mL) of transformation and storage solution (TSS; LB medium containing 10% (w/v) poly(ethylene glycol) 3350 (J. T. Baker), 5% (v/v) dimethyl sulfoxide (Sigma), and 20 mM $MgCl_2$; Chung et al., 1989). Mock-irradiated cells were put through identical manipulations except for omission of the UV irradiation.

Weigle Reactivation of UV-Irradiated M13 ssDNA. The procedures described by Bennett et al. (1988) were followed. M13 AB28 ssDNA, prepared as described previously (Palejwala et al., 1991), was irradiated (50 J/m^2) at a concentration of 40 μg/mL in water as described above. This treatment reduced survival (transfection efficiency) of ssDNA from an average of 10^5 pfu/μg to about 10^3 pfu/μg. Mock-treated DNA was put through identical procedures except for the UV irradiation. Actively growing cells were concentrated to a density of 5×10^8 cells/mL and UV-irradiated as described above. After irradiation, the cell suspension was centrifuged at 1000*g* for 10 min at 4 °C, and the pellet was resuspended in one-half volume of TSS so as to obtain a cell density of $\sim 10^9$ cells/mL. Mock-UV-irradiated cells were subjected to identical manipulations except for omission of the UV irradiation. The TSS-suspended cells were allowed to sit on ice for 10 min to induce full competence before transfection with UV-irradiated or mock-irradiated ssDNA. Transfection (50–200 ng of DNA per mL of competent cells) and subsequent plating of infectious centers were carried out as described (Palejwala et al., 1991). Weigle reactivation was expressed as the ratio of survival of irradiated ssDNA in UV-irradiated cells versus that in unirradiated cells.

Transfection, Determination of Survival, and Multiplex DNA Sequence Analysis. All transfections were carried by a modification of the dimethyl sulfoxide method of Chung et al. (1989) as described by Palejwala et al. (1991). Competent cells were prepared from UV-irradiated or mock-irradiated cells as described above. Transfection efficiency, determined by plating various dilutions of the competent cell–DNA mix as described (Palejwala et al., 1991), was approximately 10 000 pfu per 10 ng of form II'$_{\epsilon C}$ (or form II'$_C$) DNA. Transfection efficiency was not significantly affected by the strain or UV dose (within the dose range for each strain) as compared to the efficiency obtained for control M13 AB28 ssDNA. Progeny pooled DNA was prepared by mass liquid culture of approximately 5000 infectious centers and subjected to multiplex sequence analysis as described (Palejwala et al., 1993). Each analysis was accompanied by a set of standard DNA mixes to monitor the multiplex assay.

RESULTS

Experimental System. To characterize the effect of prior UV irradiation on mutagenesis opposite an ϵC residue, we used a site-specific experimental system based on phage M13 DNA. Form II'$_{\epsilon C}$ DNA is a construct in which a single ϵC residue has been placed at the center of a 17-nt-long single-stranded region in an otherwise duplex circular DNA molecule derived from phage M13. Form II'$_C$ is a control DNA construct in which the single ϵC residue has been replaced with a normal cytosine residue. The construction and characterization of these molecules have been described elsewhere in detail (Simha et al., 1991; Palejwala et al., 1991; see Figure 1 of the preceding article in this issue for the relevant DNA sequence). The experimental approach consists of transfecting UV-irradiated or nonirradiated *E. coli* cells with ϵC-bearing or control DNA, followed by plating the infectious centers to determine survival effects. The frequency and specificity of mutations induced by ϵC are determined by a multiplex DNA sequence analysis procedure described in detail by Palejwala et al. (1993). Briefly, the method consists of mass liquid culture of a part of the transfection mix to obtain a pool of progeny phage ssDNA. A 5'-end-labeled primer annealed to the progeny DNA pool is allowed to elongate in the presence of a DNA polymerase, a single selected dideoxynucleoside triphosphate (ddNTP), and one or two selected deoxynucleoside triphosphates (dNTP). The primer (a 19-mer) is poised at the site of the mutation such that the first base required for primer elongation is different for the wild-type and mutant fractions contained in progeny DNA pool. Primer elongation under these conditions will therefore yield products of different lengths for wild-type and mutant DNA templates contained within the DNA pool. Mutation frequency and specificity are deduced from a quantitative analysis of the appropriate limit–elongation products. The particular assays used here are designed to quantitate the three mutations known to be induced by ϵC, the predominant one being a C→T transition (~70%) followed by a C→A transversion and a single-base deletion (Jacobsen et al., 1989; Jacobsen & Humayun, 1990; Palejwala et al., 1993). Each multiplex assay is monitored by simultaneously determining the mutational frequency and specificity of a set of standard DNA mixes containing known proportions of authentic wild-type and mutant DNAs.

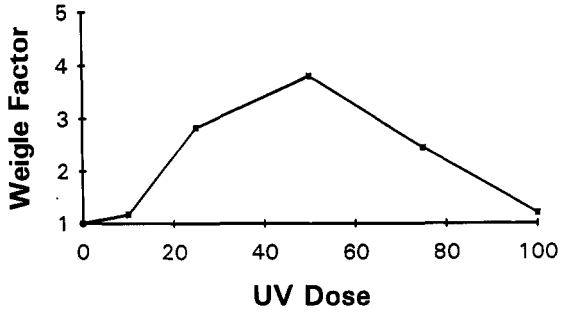

FIGURE 3: Weigle reactivation of UV-irradiated (50 J/m^2) M13 AB28 ssDNA in nonirradiated or UV-irradiated (10–100 J/m^2) *E. coli* KH2 (wild type) cells. UV dose is shown in J/m^2. For experimental procedures, see Materials and Methods. The Weigle factor is calculated as follows: Weigle factor = (survival of UV-irradiated M13 DNA in UV-irradiated cells)/(survival of UV-irradiated M13 DNA in nonirradiated cells). Plotted values are the averages of three independent experiments.

Weigle Reactivation of UV-Irradiated M13 AB28 ssDNA. Since the original intent of this work has been to examine the effect of SOS induction—achieved by prior UV irradiation of host cells—on an ϵC residue carried on transfected M13 DNA, it was necessary to demonstrate that SOS induction actually occurred under the experimental conditions used. To demonstrate SOS induction, we transfected UV-irradiated (50 J/m^2) M13 AB28 ssDNA into cells irradiated with UV wavelengths (0–100 J/m^2) and determined the transfection efficiency. Induction of SOS functions should enhance bypass of UV photoadducts, resulting in enhanced survival (Weigle reactivation) of transfected UV-treated ssDNA. The ratio of survival in irradiated cells and nonirradiated cells (the Weigle factor) is plotted as a function of UV dose to cells in Figure 3. The data show that UV irradiation of KH2 (wild-type) cells enhances survival of UV-treated ssDNA under the transfection conditions used. The maximal effect (a 4-fold enhancement) is observed at 50 J/m^2. Weigle reactivation was not observed in KH2R (*rec*A$^-$) or KH2C (*umu*C$^-$) cells (data not shown). It may be noted that UV-damaged DNA rather than ϵC-bearing DNA was used to demonstrate Weigle reactivation because ϵC does not significantly reduce survival of M13 DNA.

Effect of UV Irradiation of Wild-Type E. coli Cells on the Frequency and Specificity of Mutations Induced by ϵC. Wild-type *E. coli* cells were irradiated at a fluence of 0–100 J/m^2 and transfected with form II$'_{\epsilon C}$ or form II$'_C$DNA. Figure 4 shows examples of the results of multiplex sequence analyses of progeny phage obtained from each transfection, and Table I presents a quantitative summary of the data obtained from several experiments. In the mutation frequency assay used (Figure 4, upper panel), primer annealed to the wild-type template fraction (N = C; see sequence in legend for Figure 4) will be elongated to a dead-end 20-mer. In the mutant fraction (N = A, T, or Δ1), the primer will be elongated to a size longer than 20 nt. The results in Figure 4 (upper panel) can be summarized as follows: (a) When form II$'_C$ DNA (lanes labeled C) is transfected, the only elongation product observed is a 20-mer, implying that all progeny is wild type; in contrast, transfection of form II$'_{\epsilon C}$ DNA (lanes labeled ϵC) results in elongation products corresponding to wild-type (20-mer) as well as mutant fractions (>20-mer). (b) In the lanes labeled ϵC, the mutant bands (those with a length >20-mer) increase in intensity at the expense of the wild-type (20-mer) band as a function of UV dose. This result implies that prior UV irradiation of host cells increases mutagenesis at ϵC.

Figure 4 (lower panel) shows the results of a mutational specificity analysis of the same progeny pool DNA samples as seen in Figure 4 (upper panel). In the multiplex specificity assay, because of the absence of dGTP (or ddGTP), no primer elongation occurs on wild-type DNA. Both transitions (C→T) and 1-nt deletions (Δ1) give a 20-mer referred to as the transition band here. Transversion mutations (C→A) yield a 21-mer. As expected, all progeny DNA obtained by transfection of form II$'_C$ DNA is wild type (lanes labeled C; no elongation). DNA from transfection of form II$'_{\epsilon C}$ (lanes labeled ϵC) shows transition (20-mer) as well as transversion (21-mer) bands. When nonirradiated host cells (0 J/m^2) are transfected with form II$'_{\epsilon C}$, there are relatively few transversions as reflected by the much lower intensity of the 21-mer band compared to the 20-mer band. Prior UV irradiation of host cells causes a specific and dramatic increase in the 21-mer (transversion) band such that the intensities of the 20-mer and 21-mer bands are approximately the same. These results imply that UV irradiation of wild-type host cells specifically increases the proportion of C→A transversions among the progeny.

Table I summarizes data averaged from two independent sets of transfections, with each set of progeny DNAs subjected to three independent multiplex frequency and specificity analyses. The results establish the following points: (1) Prior UV irradiation of wild-type *E. coli* cells increases the already high mutation frequency at ϵC, nearly doubling it from the initial ~30% figure. (2) The increase appears to arise mostly from an increase in C→A transversions. (3) The effect appears to reach an early plateau at around 10 J/m^2 of UV fluence. In contrast, as previously shown in Figure 3, Weigle reactivation in the same competent cells appears to be maximal at 50 J/m^2.

Effect of UV Irradiation of recA- or umuC-Deficient E. coli Cells on the Frequency and Specificity of Mutations Induced by ϵC. Figure 5 shows examples of the results of multiplex analyses of progeny phage obtained by transfection of form II$'_{\epsilon C}$ or form II$'_C$ DNA into *rec*A-deleted *E. coli* cells, and Table II summarizes the data from several experiments. Figure 5 (upper panel) shows that transfection of control (form II$'_C$) DNA (lanes labeled C) yields only wild-type progeny (20-mer band), whereas transfection of form II$'_{\epsilon C}$ DNA yields both wild-type (20-mer) and mutant progeny (>20-mer). When compared to nonirradiated host cells (0 J/m^2), UV irradiation in the range of 5–25 J/m^2 results in a relative increase in the intensity of the mutant progeny (also see Table II). Figure 5 (lower panel) shows a specificity analysis of the same progeny DNAs as shown in Figure 5 (upper panel). Transfection of control DNA yields only wild-type phage progeny (lanes labeled C; no elongation), whereas transfection of ϵC-bearing DNA shows transition (20-mer) and transversion (21-mer) bands in addition to the wild-type (19-mer) band. Without UV irradiation of cells (0 J/m^2), transitions (20-mer) clearly constitute the predominating mutation. Prior UV irradiation results an increase in the proportion of transversions such that the relative proportion of transition and transversion mutations is approximately the same at a UV dose of 5–10 J/m^2. Table II summarizes the data from two independent transfection experiments, with progeny DNA from each experiment subjected to three multiplex frequency and three specificity assays. These data permit the following conclusions: (1) The frequency and specificity of mutations induced by ϵC in nonirradiated *rec*A-deleted cells are approximately the same as those found in nonirradiated *rec*A-proficient cells. (2) Prior UV irradiation of *rec*A-deleted *E.*

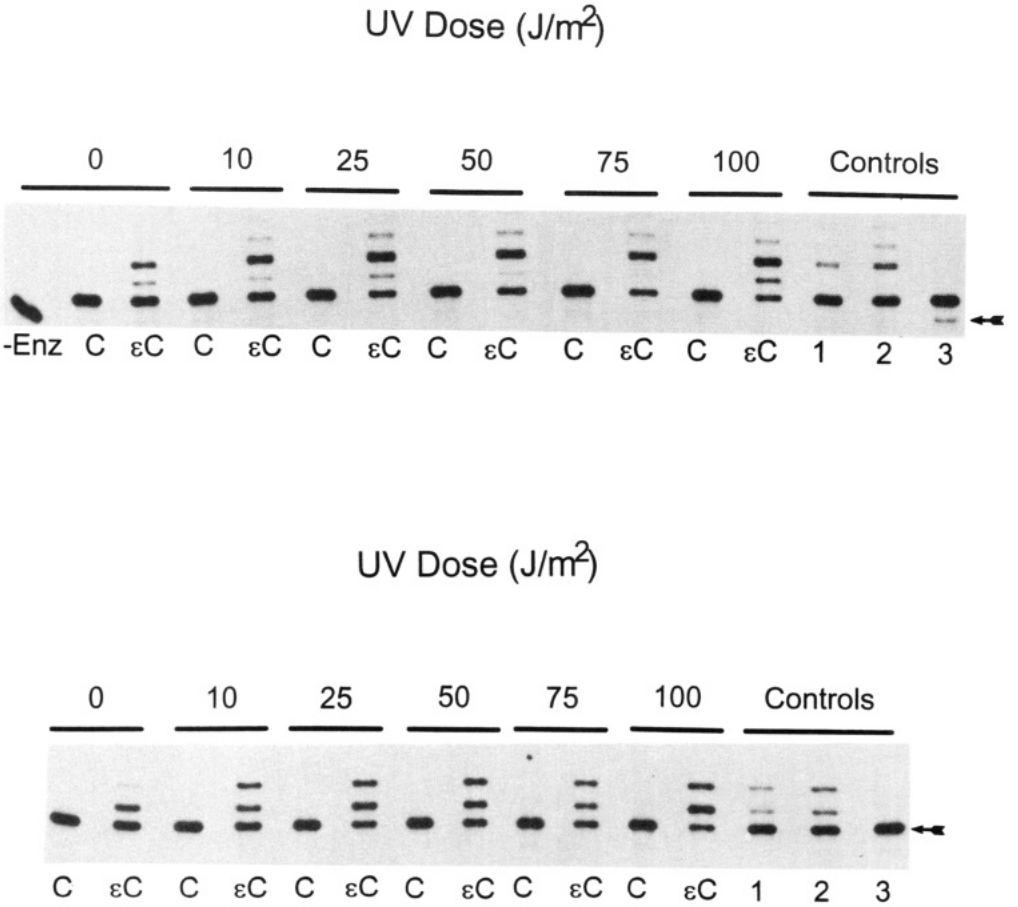

FIGURE 4: Multiplex mutation frequency (upper panel) and specificity (lower panel) analysis of pooled progeny phage DNA obtained by transfection of form II′$_{\epsilon C}$ DNA (lanes labeled εC) or the control form II′$_C$ DNA (lanes labeled C) into UV-irradiated *E. coli* KH2 (wild type) cells. Progeny DNA was isolated from each transfection, and the multiplex assays were conducted as described in detail by Palejwala et al., (1993). In these assays, a 5′-^{32}P-end-labeled 19-mer primer annealed to the DNA was elongated by T7 DNA polymerase (Sequenase Version 2.0; U. S. Biochemicals) in the presence of the nucleotide precursors indicated. The DNA sequence of the template in the vicinity of the primer 3′-OH terminus is 5′-AATTGTGGAGTTNTTATCGTGTGCAGCCAAGC, with N being the site of the mutation. The primer sequence is complementary to the underlined part of the template. Upper panel (mutational frequency analysis): The arrow at right shows the position of unelongated primer (a 19-mer). The first lane from left (–Enz) is a control in which the elongation mix was incubated without DNA polymerase and should therefore have only the unelongated primer. In the presence of ddGTP, dATP, and dTTP, wild-type template allows elongation of the 19-mer to a 20-mer. C→T transitions and C→A transversions give rise to a 22-mer, whereas the Δ1 mutation gives rise to a 21-mer. All progeny obtained by transfection with control DNA is wild type, as evidenced by elongation to a 20-mer, but not beyond (lanes labeled C). In contrast, transfection with form II′$_{\epsilon C}$ DNA results in wild-type (20-mer band) as well as mutant (21-mer and 22-mer bands) progeny (lanes labeled εC). It is apparent that in the εC lanes the relative intensity of the mutant bands (21-mer and up) increases as a function of UV irradiation at the expense of the wild-type band (20-mer), an observation that is expressed quantitatively in Table I. Mutation frequency is derived from the normalized densitometric signal present in the mutant bands as follows: the sum of the densitometric signal in bands corresponding to the 21-mer and up is divided by the sum of all bands from the 20-mer and up. Note that the relatively weak bands corresponding to a length of >22-mer found in εC lanes are believed to represent slippage–elongation products on transition templates as shown below.

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------G-T-T-T-T-T----------------
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These products accumulate because of the lack of 3′→5′ exonuclease activity in the mutant T7 polymerase used and the lack of all four dNTPs in the reaction mix. For the purposes of this assay, these bands are included in the mutant fraction. The frequency of Δ1 mutations is derived from the same gels as follows: the densitometric signal corresponding to the 21-mer band is divided by the sum of the signal in all bands from the 20-mer and up. Control lanes 1–3 represent analyses of standard DNA mixes in which the mutant fraction is, respectively, 20% (i.e., 80:20 ratio of wild-type and mutant DNAs), 30% (70:30), and 0% (all wild type). Controls such as these are included to monitor the multiplex assays. Lower panel (mutational specificity analysis): In the presence of ddATP and dTTP, C→T transitions and Δ1 mutations both yield a 20-mer, whereas C→A transversions give rise to a 21-mer (lanes labeled εC). (No elongation occurs on wild-type DNA because of the absence of dGTP; see lanes labeled C. Primer position is indicated by the arrow at right.) In the absence of UV irradiation, there are relatively few C→A transversions, as indicated by the relatively weak signal in the 21-mer band in lane εC at 0 J/m^2. UV irradiation results in a specific increase in C→A events, as indicated by the increase in the relative intensities of the 21-mer band. The changes observed are quantitatively expressed in Table I. The frequency of C→T mutations is extracted from the 20-mer signal as follows. The 20-mer signal is normalized by division with the sum of the signals in the 20-mer and 21-mer bands to obtain the combined frequency of C→T transitions and Δ1 mutations. The frequency of Δ1 mutations (determined as a part of the frequency assay above) is subtracted from this value to obtain the frequency of C→T mutations. The frequency of C→A transversions is determined from the normalized signal in the 21-mer band (signal in the 21-mer band divided by the combined signal in the 20-mer and 21-mer bands). Control lanes 1–3 are specificity analyses of the same standard DNA mixes as shown in the panel above. No elongation is seen on wild-type DNA (control lane 3) because there is no dGTP (or ddGTP). The mutant fractions in control lanes 1 and 2 consist of an equimolar mix of C→T transitions and C→A transversions. Therefore, the transition (20-mer) and transversion (21-mer) bands should have the similar intensities. It is worth noting that each experimental lane (lanes 1–12, lower panel) yields data equivalent, in principle, to those obtained by sequencing ~5000 individual phage clones.

Table I: Effect of Prior UV Irradiation of Wild-Type *E. coli* on the Frequency and Specificity of Mutations Induced by an εC Residue Borne on M13 DNA

UV to cells (J/m^2)	mutation frequency (%) (±SD)[a]	mutation specificity (%) (±SD)[b]		
		C→T	C→A	Δ1
0	35 (±2)	20 (±2)	3 (±2)	8 (±1)
10	54 (±5)	17 (±2)	27 (±5)	5 (±1)
25	62 (±4)	28 (±1)	25 (±2)	5 (±3)
50	62 (±3)	25 (±1)	30 (±3)	3 (±1)
75	62 (±5)	20 (±1)	32 (±6)	4 (±1)
100	73 (±1)	27 (±2)	32 (±6)	10 (±7)

[a] Averages derived by analyzing progeny DNA pools obtained in two separate transfection experiments, with each pool subjected to three multiplex frequency assays as described in the text and in Palejwala et al. (1993) (SD, standard deviation; numbers are rounded to nearest integer). Note that the mutation frequency determined by the frequency assay is slightly more (about 5% more) than that obtained by summing up the frequencies of the three specific mutations shown. This difference is probably due to technical reasons. [b] Averages derived by analyzing progeny DNA pools obtained in two separate transfection experiments, with each pool subjected to three multiplex specificity assays as described in the text and in Palejwala et al. (1993) (SD, standard deviation; numbers are rounded to nearest integer).

coli cells results in a significant increase in mutation frequency. Most of the increase in mutation frequency can be accounted for by an increase in C→A transversions. Therefore, prior UV irradiation of *rec*A-proficient as well as *rec*A-deleted *E. coli* cells results in a significant modulation of mutagenesis provoked by εC.

Figure 6 shows the mutational frequency (upper panel) and specificity (lower panel) analyses of progeny phage obtained by the transfecting control or εC-bearing DNAs into the *umu*C-deficient strain KH2C. The upper panel shows that mutation frequency increases with UV dose, and the lower panel shows that UV irradiation specifically enhances C→A transversions. Table III summarizes data from several experiments and shows that the UV modulation of mutagenesis opposite εC occurs in *umu*C-deficient *E. coli* and that the major features of this effect are similar in wild-type, *rec*A-deficient, and *umu*C-deficient strains.

DISCUSSION

Mutagenesis by εC in UV-Irradiated E. coli. The experiments described in this article were originally undertaken to address the question of whether SOS induction could modulate mutagenesis opposite an εC residue. To induce SOS functions, we used the classical approach of UV irradiation of cells. To confirm that SOS induction occurred in the same competent cells that were used for transfection of εC-bearing M13 DNA, we demonstrated Weigle reactivation of UV-damaged M13 ssDNA. Under these conditions, significant modulation of mutagenesis was observed, suggesting that UV irradiation did induce a mutagenic mechanism. The major UV effect was an enhancement of C→A transversions.

The observed UV effect can be accommodated by the SOS hypothesis if one assumes additional lesion-specific and sequence-specific effects operating at the time of lesion bypass. Here, one takes the view that due to a structural feature of εC, such as the enhanced base-stacking promoted by the etheno ring, translesion DNA synthesis does not require SOS functions. If SOS functions are available, they can participate in translesion synthesis, and their participation can influence base misincorporation opposite the lesion.

What cannot be readily accommodated by the SOS hypothesis, however, is the unanticipated finding that essentially the same UV modulation is also observed in *rec*A- and *umu*C-deficient strains. According to our current understanding, the SOS regulon is normally in a state of repression because the LexA repressor binds to the promoter region of each member gene so as to prevent transcription. SOS induction leads to the activation of the recA protein to a form (recA*) that actively promotes proteolytic cleavage of the LexA repressor (Walker, 1984; Echols and Goodman, 1991). The recA protein is required not only for derepressing the SOS regulon but also for activation of umuD to umuD′. In addition, recA is hypothesized to participate in base misinsertion opposite the lesion as well as in lesion bypass. Since the *rec*A⁻ strain used here has suffered a total deletion of the *rec*A gene, it is unlikely that the observed UV modulation of mutagenesis is mediated by known SOS functions.

These results can be explained by two classes of hypothetical mechanisms: (a) UV irradiation *induces* a hitherto unrecognized second mutagenic mechanism (referred to as UVM for UV modulation) distinct from the SOS pathway. This hypothesis implies that in wild-type strains UV irradiation may result in the availability of both mechanisms (i.e., SOS and UVM) and that effects previously attributed to SOS functions may be the combined effects of the two pathways. (b) UV irradiation *suppresses* a damage repair or mismatch correction system, leading to an apparent increase in mutagenesis. Befure further discussion of the above two hypotheses, it is necessary to briefly review what is known about the in vivo mutagenic properties of εC.

Mutagenic Properties of εC. Because the etheno ring directly blocks the Watson–Crick face of the base (Figure 1), εC is expected to have the template characteristics of a noninstructive lesion. Accordingly, in an in vitro DNA replication system, εC displays properties expected for such a lesion: *E. coli* DNA polymerase I does not incorporate any base opposite a site-specific εC residue on an oligonucleotide template at low dNTP (micromolar) concentrations. At high dNTP (millimolar) concentrations, base incorporation is observed, and the relative incorporation of the four bases follows the adenine rule (Simha et al., 1991). Furthermore, the in vivo mutagenic specificity observed by transfecting regiospecifically or site-specifically modified M13 DNA shows that εC has the specificity of noninstructive adducts (Jacobsen et al., 1989; Palejwala et al., 1993). However, εC differs from other noninstructive adducts such as abasic sites (Lawrence et al., 1990) in the following regards: (a) εC does not reduce survival in either wild-type or *rec*A-deficient cells; and (b) the specificity and frequency of mutations are essentially the same in wild-type, *rec*A⁻, and *umu*C⁻ cells, suggesting that mutagenesis opposite εC does not require classical SOS functions.

Simha et al. (1991) demonstrated that the incorporation of dGMP opposite εC by DNA polymerase I is inefficient. Furthermore, once dGMP is incorporated opposite εC, continued elongation (bypass) is inefficient in vitro compared to the result when dTMP or dAMP is incorporated opposite εC. These findings lead to the naive expectation that dGMP incorporation opposite εC in vivo may also be similarly inefficient. However, transfection of M13 DNA bearing a single site-specific εC lesion shows that up to 70% of the progeny resulting from in vivo translesion synthesis is wild type. This result cannot be ascribed to known DNA damage repair mechanisms because of the experimental strategy employed. The DNA construct used in these experiments placed the adduct at the center of a 17-nt-long single-stranded DNA in an otherwise duplex circular molecule (see Figure 1 of the preceding article in this issue for the relevant sequence).

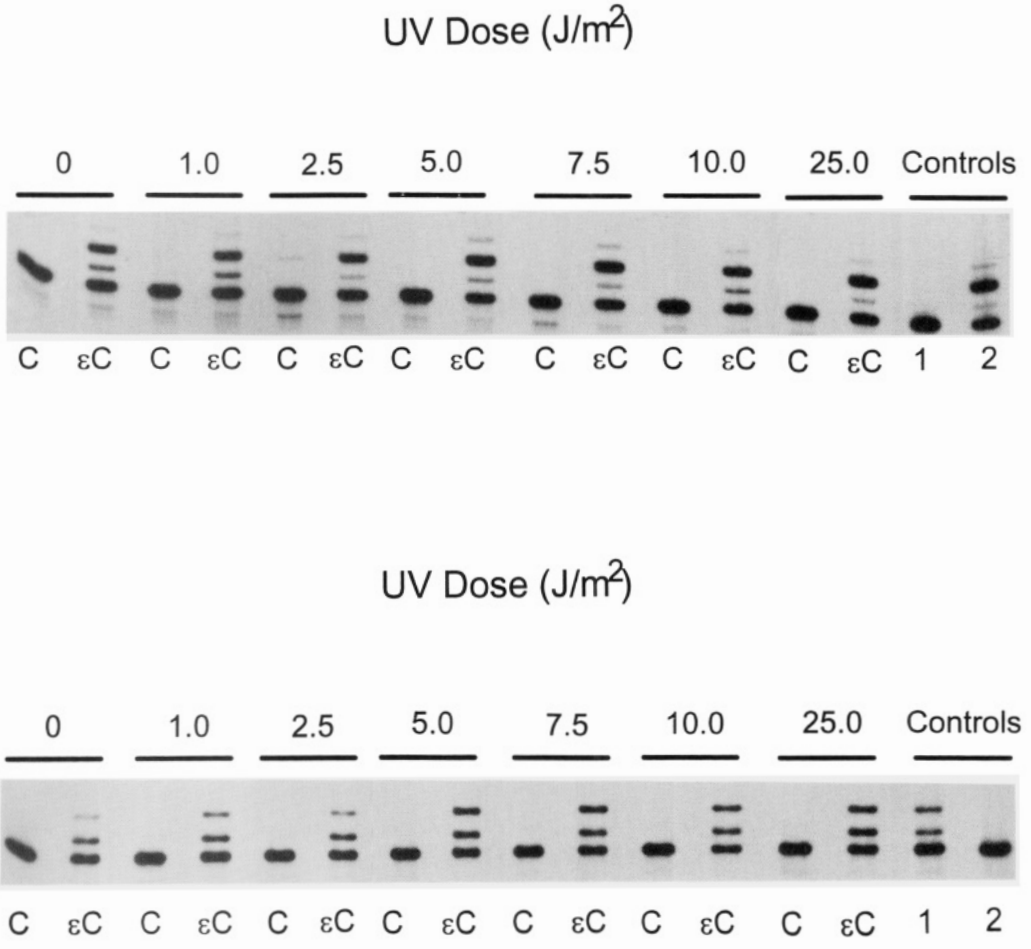

FIGURE 5: Multiplex mutation frequency (upper panel) and specificity (lower panel) analysis of pooled progeny phage DNA obtained by transfection of form II′$_{\epsilon C}$ DNA (lanes labeled εC) or the control form II′$_{C}$ DNA (lanes labeled C) into *E. coli* KH2R (*rec*A-deficient) cells subjected to various doses of UV. Assay procedures are as described in the legend for Figure 4 and in Palejwala et al. (1993). Despite the lower UV dose range 1–25 J/m^2 (as compared to 10–100 J/m^2 used for wild-type cells), it can be seen that mutational frequency increases as a function of irradiation (upper panel; also see Table II). There is a specific increase in C→A transversions (lower panel) as a result of UV irradiation, as seen by the increase in the relative intensity of the 21-mer band as a function of UV irradiation. In the upper panel, control lane 1 is an analysis of wild-type DNA; all elongation proceeds to a 20-mer, but not beyond. Control lane 2 is a frequency analysis of a 50:50 mix of wild-type and mutant DNAs. In the lower panel, control lane 1 is an analysis of a 70:15:15 mix of wild-type, C→T, and C→A DNAs, respectively. The resulting 20-mer and 21-mer bands are expected to have equal intensities. Control lane 2 is wild-type DNA on which no primer elongation can occur.

Table II: Effect of Prior UV Irradiation of *rec*A-Deficient *E. coli* on the Frequency and Specificity of Mutations Induced by an εC Residue Borne on M13 DNA

UV to cells (J/m^2)	mutation frequency (%) (±SD)[a]	mutation specificity (%) (±SD)[b]		
		C→T	C→A	Δ1
0	33 (±3)	20 (±1)	6 (±3)	8 (±2)
1	37 (±3)	20 (±4)	12 (±2)	5 (±3)
2.5	42 (±6)	25 (±2)	11 (±2)	6 (±3)
5	51 (±3)	23 (±3)	22 (±5)	4 (±1)
7.5	42 (±10)	15 (±5)	23 (±7)	2 (±1)
10	50 (±3)	17 (±1)	26 (±2)	6 (±2)
25	52 (±2)	22 (±3)	26 (±2)	3 (±1)

[a] See footnote *a* for Table I. [b] See footnote *b* for Table I.

This arrangement in effect forbids base or nucleotide excision repair *before* translesion synthesis because any strand scission (assuming that efficient excision can occur in a single-stranded region) will be futile: absence of the complementary sequence will prevent restoration of the excised base(s), and moreover, linearization of M13 DNA is lethal. It is unlikely that a significant part of the transfected DNA suffers excision and linearization, because (a) εC does not reduce survival, and (b) almost all progeny phage recovered contain the DNA sequence flanking the εC residue, implying that progeny derived almost entirely from parental DNA molecules in which replicative bypass of εC occurred. Excision repair *after* translesion synthesis is expected to be similarly futile because the mutation has already been fixed. (It is formally possible, as pointed out by a referee, that during translesion synthesis the nucleotide incorporated opposite a lesion is a rare conformational variant of a normal base such as a syn conformer. In this situation, subsequent removal of the original adduct by excision repair will result in gap-filling synthesis past a base in the syn conformation and may result in (further) mutagenesis. Because of the syn–anti transition and because the parental M13 minus strand may be repeatedly utilized as a template for progeny phage production, this scenario predicts infectious centers with more than one type of progeny phage. Our unpublished results do not support the existence of mixed plaques at significant levels.)

Therefore, the finding that up to 70% of progeny phage from nonirradiated cells have a normal cytosine at the site of εC leaves open the following possibilities: (1) The in vivo replication machinery is substantially different in its base incorporation specificity as compared to the simple in vitro model system of Simha et al. (1991). Mutation fixation opposite εC in vivo presumably occurs during gap-filling in the form II′ molecule (see Figure 1). Since polymerase I as well as polymerase III can carry out gap-filling DNA synthesis,

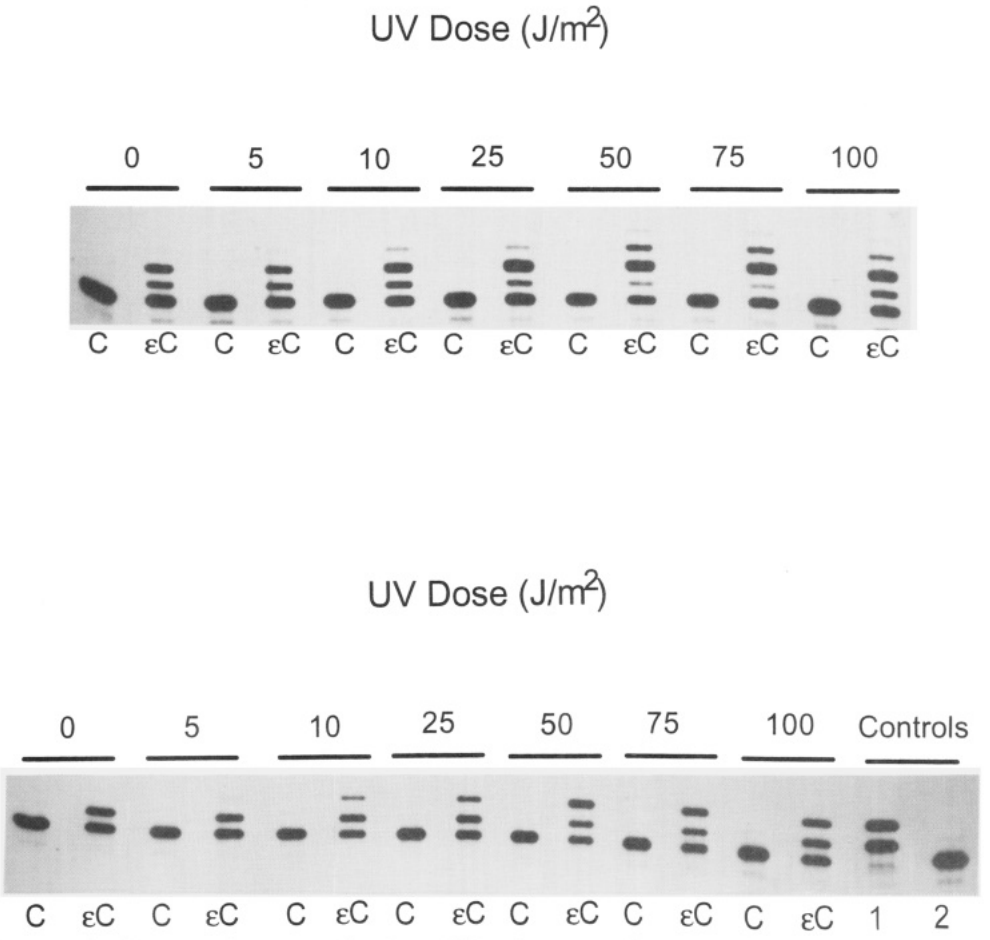

FIGURE 6: Multiplex mutation frequency (upper panel) and specificity (lower panel) analysis of pooled progeny phage DNA obtained by transfection of form II′$_{\epsilon C}$ DNA (lanes labeled εC) or the control form II′$_{C}$ DNA (lanes labeled C) into UV-irradiated *E. coli* KH2C (*umu*C-deficient) cells. Assay procedures are as described in the legend for Figure 4 and in Palejwala et al. (1993). It can be seen that mutational frequency increases as a function of irradiation (upper panel; also see Table III). There is a specific increase in C→A transversions (lower panel) as a result of UV irradiation, as seen by the increase in the relative intensity of the 21-mer band as a function of UV irradiation (also see Table III). In the lower panel, control lane 1 is an analysis of a 50:50 mix of C→T and C→A mutant DNAs. The resulting 20-mer and 21-mer bands are expected to have equal intensities. Control lane 2 is an analysis of wild-type DNA on which no primer elongation is expected.

Table III: Effect of Prior UV Irradiation of *umu*C-Deficient *E. coli* on the Frequency and Specificity of Mutations Induced by an εC Residue Borne on M13 DNA

UV to cells (J/m^2)	mutation frequency (%) (±SD)[a]	mutation specificity (%) (±SD)[b]		
		C→T	C→A	Δ1
0	33 (±4)	24 (±5)	2 (±1)	8 (±3)
5	32 (±2)	21 (±2)	3 (±2)	8 (±5)
10	49 (±3)	30 (±2)	9 (±3)	8 (±5)
25	51 (±2)	25 (±5)	18 (±1)	5 (±2)
50	61 (±8)	22 (±2)	31 (±12)	4 (±1)
75	54 (±7)	20 (±1)	28 (±8)	2 (±1)
100	59 (±1)	20 (±4)	27 (±3)	8 (±3)

[a] See footnote *a* for Table I. [b] See footnote *b* for Table I.

the difference between the in vitro results (using polymerase I) and the in vivo results may be simply due to the polymerase utilized in gap-filling. (2) A nonexcisive damage repair mechanism (analogous to photoreactivation or O^6-methylguanine repair) capable of replacing εC with normal cytosine exists and is capable of acting with a relatively high efficiency on ssDNA. (3) A post-replicative mismatch correction mechanism that selectively removes bases incorporated opposite εC exists and is responsible for selecting wild-type progeny. In this last, somewhat complex scenario, A and T are preferentially incorporated opposite εC, but are continuously removed by the hypothetical post-replicative mismatch correction system. Even though G incorporation is inefficient relative to that of A or T, the G:εC pair is not a favored substrate for the mismatch correction system. The efficient removal of A from an A:εC pair and of T from a T:εC pairs recycles the template for further rounds of bypass replication and ultimately leads to an enrichment for wild-type progeny. The template recycling predicted by this model does not necessarily reduce survival as measured here. This is so because any temporal delay in the phage development cycle will not be observable when transfected cells are immediately plated and incubated overnight to determine the number of infectious centers. Finally, even though mismatch correction systems are believed to act on mismatches involving normal DNA bases, there is now evidence that certain mismatch correction systems can in fact remove "normal" bases incorporated opposite damaged DNA bases (Michaels et al., 1992).

Suppression of DNA Repair or Mismatch Correction as a Possible Mechanism for UVM. As discussed above, repair or mismatch correction systems can select for wild-type progeny. Thus, a hypothetical nonexcisive damage repair system may remove εC from about 70% of the transfected form II′$_{\epsilon C}$ DNA molecules and thereby account for the unexpectedly large fraction of wild-type progeny in nonirradiated cells. UV irradiation may modulate mutagenesis by suppressing this repair mechanism so as to increase the proportion of lesion-bearing molecules available for translesion synthesis. Alternatively, UV suppression of the hypothetical N:εC mismatch correction system discussed above will have

a similar effect because there will be no enrichment for wild-type progeny. Both of the above UV suppression hypotheses can account for the UV enhancement of mutation frequency, but cannot readily account for a specific increase in C→A transversions without additional assumptions.

Other Mechanisms for UVM. While available data do not permit a detailed discussion of the mutation-promoting activity observed here, three interesting possibilities deserve mention. (a) UV irradiation causes a switch in the DNA polymerase utilized in translesion synthesis on a transfected genome. For example, repair of chromosomal DNA damage inflicted by UV may in effect sequester *E. coli* DNA polymerase I, thereby allowing another polymerase to carry out gap-filling in the transfected form II′$_{\epsilon C}$ DNA. (b) UV irradiation triggers an imbalance in intracellular nucleotide pools. UV irradiation is known to effect the levels of intracellular dNTPs and, in particular, to enhance the levels of dTTP (Suzuki et al., 1983; Das & Loeb, 1984; Kunz & Kohalmi, 1991). Increased dTTP levels may lead to more frequent incorporation of dTMP opposite ϵC, thereby enhancing C→A transversions. (c) UV irradiation induces a gene (or genes) that is not a member of the SOS regulon. The resulting gene product directly or indirectly alters base misincorporation opposite a mutagenic lesion. UV irradiation, like other agents that cause DNA damage or other cellular stress, may induce not only the SOS regulon but also other genes to various degrees (Neidhardt & VanBogelen, 1987). Among the non-SOS *E. coli* genes known to be induced by UV are the heat-shock proteins GroEL and DnaK (Krueger & Walker, 1984). Interestingly, *E. coli gro*EL and *gro*ES mutants are defective in the *umu*D′C-dependent mutagenic pathway (Donnelly & Walker, 1989; Liu & Tessman, 1990), but it is not clear whether the reported *umu*D′C dependence is at the level of base misinsertion versus mutation recovery (lesion bypass). The data presented here are consistent with the possibility that UV induces a *rec*A-independent mutation-promoting activity. Whether this activity requires, or is augmented by, the *gro*ES and *gro*EL gene products is one of the many interesting questions that remain to be investigated.

ACKNOWLEDGMENT

We thank Drs. K. Sambamurti and G. Walker for strains and anonymous referees for several helpful suggestions.

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