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Uracil-DNA Glycosylase Causes 5-Bromodeoxyuridine Photosensitization in *Escherichia coli* K-12

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An *Escherichia coli* uracil-DNA glycosylase-defective mutant (*ung-1 thyA*) was more resistant than its wild-type counterpart (*ung*$^+$ *thyA*) to the killing effect of UV light when cultured in medium containing 5-bromouracil or 5-bromo-2′-deoxyuridine (BrdUrd). The phenotype of resistance to BrdUrd photosensitization and the uracil-DNA glycosylase deficiency appeared to be 100% cotransduced by P1 phage. During growth with BrdUrd, both strains exhibited similar growth rates and 5-bromouracil incorporation into DNA. The resistant phenotype of the *ung-1* mutant was observed primarily during the stationary phase. In cells carrying 5-bromouracil-substituted DNA, mutations causing resistance to rifampin and valine were induced by UV irradiation at a higher frequency in the wild type than in the *ung-1* mutant. This Ung-dependent UV mutagenesis required UmuC function. These results suggest that the action of the uracil-DNA glycosylase on UV-irradiated 5-bromouracil-substituted DNA produces lethal and mutagenic lesions. The BrdUrd photosensitization-resistant phenotype allowed us to develop a new, efficient method for enriching and screening *ung* mutants.

5-Bromouracil (BrUra) is a thymine analog and is readily incorporated into DNA. Cells carrying such BrUra-substituted DNA (BrUra-DNA) are much more sensitive than cells carrying unsubstituted DNA to visible or UV light (for reviews, see references 11 and 12). Although the molecular mechanism for the photosensitization by 5-bromo-2′-deoxyuridine (BrdUrd) has not been completely elucidated, this phenomenon has been widely used for enriching for specific mutations (26).

As a possible mechanism for BrdUrd sensitization to UV killing, Krasin and Hutchinson (16) have proposed that a single photochemical event in a BrUra residue in the DNA produces a double-strand break that is as lethal as gamma ray-induced double-strand breaks. This is consistent with a hypothesis that an unrepaired double-strand break is a lethal lesion in *Escherichia coli* (15).

UV irradiation markedly enhances debromination of BrUra in the DNA, so that the major photochemical products in the BrUra-DNA are uracil residues and single-strand breaks (11, 12). The molecular mechanism of uracil excision repair from uracil-containing DNA involves the liberation of uracil bases by uracil-DNA glycosylase and the subsequent apyrimidinic endonuclease cleavage of phosphodiester bonds at apyrimidinic sites left in the DNA (for reviews, see references 19 and 20).

In this study we examined possible participation of uracil-DNA glycosylase in the repair of lethal damage produced in either nonirradiated or UV-irradiated BrUra-DNA in vivo by use of wild-type and *ung* mutant strains. We present two lines of evidence: (i) the uracil-DNA glycosylase deficiency did not alter the cytotoxic effect of BrdUrd under nonirradiated conditions, and (ii) the uracil-DNA glycosylase action itself produced lethal and mutagenic damage in UV-irradiated BrUra-DNA. The latter activity (ii) is a biologically important new mechanism for the BrdUrd photosensitization. By utilizing this phenotype and mechanism, we developed a new selection procedure for enriching *ung* mutants of *E. coli* K-12.

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MATERIALS AND METHODS

Chemicals. BrUra, BrdUrd, and rifampin were purchased from Sigma Chemical Co. (St. Louis, Mo.). Thymidine (dThd), L-valine (Val), and tetra-*n*-butyl-ammonium-hydroxide were purchased from Wako Pure Chemical Industries, Ltd. (Japan). Poly(dA-dT), dUTP, dATP, and *E. coli* DNA polymerase I were purchased from Boehringer GmbH (Mannheim, Federal Republic of Germany). [2-^{14}C]dThd and [5-^{3}H]dUTP were purchased from Amersham International (Amersham, United Kingdom), and [6-^{3}H]BrdUrd was from New England Nuclear Corp. (Boston, Mass.). *N*-Methyl-*N*′-nitro-*N*-nitrosoguanidine was from Nacalai Tesque, Inc. (Japan).

Bacteria and media. Table 1 lists the *E. coli* K-12 derivative strains used. M9S medium and L broth were previously described (31). The Ca^{2+}- and Mg^{2+}-free M9S medium (M9S buffer) was used for washing or dilution of cells. M9S growth medium was supplemented with Casamino Acids, dThd (200 μM), and, when necessary, other nutrients. For BrdUrd substitution in the medium, a total concentration of dThd plus BrdUrd was fixed to be 200 μM. The percent substitution of BrdUrd (or BrUra) for dThd (or thymine) in the M9S growth medium (or DNA) was calculated as 100 [BrdUrd]/([dThd] + [BrdUrd]) where square brackets indicate concentrations. L broth was supplemented with thymine (100 μg/ml). All strains were grown at 37°C.

Genetic manipulations. Selection of thymine-requiring strains with trimethoprim and generalized transduction with P1 *vir* phage were carried out as described by Miller (24).

UV-light sensitivity. A fresh single colony of each strain was inoculated into M9S growth medium and grown overnight. Cultures were diluted (1/99) with M9S growth medium containing various amounts of dThd plus BrdUrd and grown

TABLE 1. *E. coli* K-12 strains used

Strain	Description	Source or reference
BD10	W3110 *ung-1*	T. Horiuchi (6)
BD1137	*thi-1 argH1 nadB4 purI66 pyrE41 lacY1 malA1 xyl-7 rha-6 ara-13 gal-7 rpsL9 tonA2* or *tonA22 supE44* $T2^r$ *rel-1*?	T. Horiuchi (6)
BD1153	BD1137 *ung-1 nadB*$^+$ *purI*$^+$ *pyrE*$^+$	T. Horiuchi (6)
BD1154	BD1137 *ung*$^+$ *nadB*$^+$ *purI*$^+$ *pyrE*$^+$	T. Horiuchi (6)
BDT1153	BD1153 *thyA*	Trimethoprim selection of BD1153, this paper
BDT1154	BD1154 *thyA*	Trimethoprim selection of BD1154, this paper
NK6042	Δ(*gpt-lac*)*5 nadB51*::Tn*10 relA1 spoT1 thi-1* λ$^-$	CGSC[a] strain, B. Bachmann
NKT6042	NK6042 *thyA*	Trimethoprim selection of NK6042, this paper
YY368	NKT6042 *nadB*$^+$ *ung-1*	BD10(P1) × NKT6042; nadB$^+$ Ung$^-$ transductant, this paper
YY369	NKT6042 *nadB*$^+$ *ung*$^+$	BD10(P1) × NKT6042; nadB$^+$ Ung$^+$ transductant, this paper
ZA12	WP_2 *umuC122*::Tn*5*	T. Ohta (8)
YY370	YY369 *umuC122*::Tn*5*	P1(ZA12) × YY369; Kn^r transductant, this paper
YY371	YY368 *umuC122*::Tn*5*	P1(ZA12) × YY368; Kn^r transductant, this paper
KA197	*thi-1 pheA97 relA1* λ$^-$ *spoT1*	CGSC strain, B. Bachmann
KAT197	KA197 *thyA*	Trimethoprim selection of KA197, this paper
YY372	NKT6042 *ung-10*	MNNG[b] treatment of NKT6042, this paper
YY373	NKT6042 *ung-20*	MNNG treatment of NKT6042, this paper
YY374	NKT6042 *ung-30*	MNNG treatment of NKT6042, this paper
KD2157	*ilv-145 metE46 his-4 trpC3 pro thi mtl-1 malA1 ara-9 galK2 lac-114 rpsL ton thyA*::Tn*5 thyR*	H. Nakayama (25)
AB1157	*thr-1 leu-6 proA2 his-4 thi-1 argE3 lacY1 galK2 ara-14 xyl-5 mtl-1 tsx-33 rpsL31 supE44*	P. Howard-Flanders (8)
ABT1157	AB1157 *thyA*::Tn*5*	KD2157(P1) × AB1157; Kan^r transductant, high-thymine-requiring derivative of AB1157, this paper
YY376	ABT1157 *nadB*::Tn*10 ung-10*	YY372(P1) × ABT1157; Tet^r Ung$^-$ transductant, this paper
YY378	ABT1157 *nadB*::Tn*10 ung-20*	YY373(P1) × AB1157; Tet^r Ung$^-$ transductant, this paper
YY380	ABT1157 *nadB*::Tn*10 ung-30*	YY374(P1) × AB1157; Tet^r Ung$^-$ transductant, this paper

[a] CGSC, *E. coli* Genetic Stock Center, Yale University, New Haven, Conn.
[b] MNNG, *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine.

again to the stationary phase (six to seven generations). After dilution with ice-cold M9S buffer, cell suspensions at a density of $\sim 5 \times 10^7$ cells per ml were irradiated with 254-nm UV light (or 315-nm UV light, used only in Fig. 1D) with stirring at 4°C. Cells were spread on L-broth agar to determine colony-forming abilities. The 254- and 315-nm UV sources were a 10 W Panasonic germicidal lamp and a 20 W Toshiba FL20 S. E. lamp (peak wavelength, 315 nm), respectively. The plastic dish lid was used as a filter (51.4% transmission at 315 nm and ≤0.3% transmission at ≤283 nm). The 254- and 315-nm UV fluence rates were 2.0 and 1.0 J/m^2 per s, respectively, as determined with a Topcon model UVR-254 dosimeter (Tokyo Kogaku Kikai K. K., Japan) and a Macum model UV.103 dosimeter with a B-310 nm filter (Macum Photometrics Ltd., Scotland), respectively.

BrUra content in DNA. (i) Uptake of BrdUrd and dThd. The relative uptakes of BrdUrd and dThd into DNA at each time during growth in BrdUrd-substituted M9S growth medium (BrdUrd medium) were measured by a slight modification of the Rydberg method (27). Briefly, freshly prepared overnight cultures were diluted (1/99) with 50% BrdUrd medium containing 50 nCi of [2-^{14}C]dThd (51.4 mCi/mmol) per ml and 1 μCi of [6-^{3}H]BrdUrd (26.6 Ci/mmol) per ml and incubated again. Samples of $\sim 10^8$ cells were withdrawn at the desired times and cooled in ice. RNA was hydrolyzed with NaOH (final concentration, 0.5 N) for 1 h at 37°C. The lysates were neutralized with HCl, and trichloroacetic acid (final concentration, 7%) was added. After 30 min on ice, acid-insoluble radioactivity was collected on a Whatman GF/C filter and measured with a scintillation counter. The radiochemical purity of the labeled BrdUrd was determined to be 98% by New England Nuclear Corp. Nonradioactive uracil or dUrd was not added to the medium.

(ii) HPLC analysis of acid hydrolysates of BrUra DNA. DNA extracted from bacteria as described by Berns and Thomas (1) was treated with RNase. High-molecular-weight DNA was separated from small molecules such as RNA degradation products by passing through a Sephadex G50 column (23). Contaminating RNA was further removed by alkaline hydrolysis (0.3 N KOH at 37°C for 18 h). Purified DNA was hydrolyzed in a sealed glass tube with HCOOH (98 to 100%) at 175°C for 30 min. The hydrolysate was dried and dissolved in 50 mM KH_2PO_4–15% (vol/vol) methanol–0.5 mM tetra-*n*-butyl-ammoniumhydroxide (solvent A). A Gilson model 302 high-pressure liquid chromatography (HPLC) apparatus was used for analysis of DNA bases. Each sample was injected into a 25-cm Microsorb C_{18} column and eluted with solvent A. The column effluent was monitored by A_{260}. The bases were identified by their specific retention times based on the authentic bases and quantified by their peak heights relative to those of the standard solutions. The retention times of the bases were as follows: cytosine (5.5 min), uracil (6.0 min), guanine (6.6 min), thymine (7.6 min), BrUra (8.7 min), and adenine (9.2 min).

UV-induced mutation frequency. Cell dilutions (3 ml of $\sim 10^8$ cells per ml) were irradiated with 254-nm UV light, and 0.1-ml samples were plated for measurement of survival as described above. The remaining cells were grown for 6 h (four to eight generations) in M9S growth medium for expression of mutations. They were then spun down, washed, and suspended with 0.3 ml of M9S buffer, and

0.1-ml samples of appropriate dilutions were plated on L broth agar containing 100 μg of rifampin per ml or on M9S medium agar containing Val (40 μg/ml) and other necessary nutrients. For viability, the same sample was plated on L-broth agar. After incubation for 48 h, visible colonies were counted. Mutation frequency was expressed as the number of Rif^r or Val^r colonies per viable cell.

Uracil-DNA glycosylase assay. Our previous uracil-DNA glycosylase assay method was employed (29–31). Briefly, crude cell extracts were prepared by ultrasonic disruption of cells in 50 mM Tris hydrochloride (pH 7.5)–1 mM dithiothreitol–1 mM EDTA–10% glycerol. The protein content of extracts was estimated by the method of Bradford (2) with a protein assay kit (Bio-Rad Laboratories, Richmond, Calif.) and bovine gamma globulin as a protein standard. The substrate poly(dA-[^{3}H]dU) was synthesized by polymerizing dATP and [^{3}H]dUTP (14.8 Ci/mmol) on poly(dA-dT) templates with *Escherichia coli* DNA polymerase I (30). Poly(dA-[^{3}H]dU) was not diluted with poly(dA-dT). The basic reaction mixture for uracil-DNA glycosylase included 10 mM Tris hydrochloride (pH 7.5), 1 mM dithiothreitol, 5 mM EDTA, poly(dA-[^{3}H]dU) (60 pmol of uracil residues, 80,000 dpm), and various amounts of extract protein in a final volume of 100 μl. The mixture was incubated at 37°C for 5 min, and the reaction was terminated on ice by adding 50 μl of 1-mg/ml ice-cold bovine serum albumin and 75 μl of 20% trichloroacetic acid. After 30 min on ice, samples were spun down. Then 200 μl of the acid-soluble supernatant was taken and neutralized with 200 μl of 0.6 N NaOH. Acid-soluble radioactivity was determined with scintillation counter with Triton X-100–toluence (1/2) containing 0.6% 2,5-diphenyloxazole and 0.01% 1,4-bis(5-phenyloxazolyl) benzene as the scintillation fluid. Under these conditions, 1 μg of ung^+ cell extract released ca. 30 pmol of uracil residues from the substrate into the acid-soluble fraction, whereas *ung-1* mutant extracts did not release more uracil residues than the background level determined without extracts. All of the radioactivity released by the ung^+ cell extract arose solely from free uracil bases, determined by HPLC as described before (29).

Screening procedure for uracil-DNA glycosylase-defective mutants. The parental strain NKT6042 was mutagenized by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine as described by Miller (24). The induced mutation frequencies detected by Val^r marker were 9.5×10^{-5} (experiment 1) and 1.2×10^{-4} (experiment 2), whereas the frequency for the untreated control was 2.9×10^{-7}. Mutagenized cells were grown overnight in M9S medium containing 200 μM dThd. For BrdUrd-plus-UV selection, 20 μl of the overnight culture was subcultured in 880 μl of M9S growth medium containing 100 μM dThd. Cells were aerated continuously for 90 min. Then 100 μl of 1 mM BrdUrd was added (final concentration, 100 μM), and the culture was shaken for 8 h (six generations). Cultures of $\sim 2 \times 10^9$ cells per ml were diluted 1:50 into M9S buffer and UV irradiated at 60 J/m^2. A sample of cell suspension was plated for determination of survival, and the remaining culture was grown overnight in M9S growth medium containing 200 μM dThd. After three (experiment 1) or four (experiment 2) cycles of this selection, cells were spread on L-broth agar to form colonies. From these plates, 5 (experiment 1) and 10 (experiment 2) colonies were picked randomly and examined for both UV sensitivity of cells grown in 50% BrdUrd medium and uracil-DNA glycosylase activity of cell extracts.

Spontaneous mutation frequency. A fluctuation test was carried out as described by Hoess and Herman (10).

RESULTS

Resistant phenotype of an *ung* mutant to BrdUrd photosensitization. To avoid variation of BrUra incorporation into DNA exhibited by low-thymine-requiring strains (*thyA drm* and *thyA dra*) (9, 27), we used high-thymine-requiring strains that all had a single *thyA* mutation.

The thymine-requiring derivatives of the isogenic set of strains, BDT1154 (ung^+ *thyA*) and BDT1153 (*ung-1 thyA*), were independently isolated from minimal plates containing trimethoprim. BDT1154 (ung^+) grown to the stationary phase in the BrUra-substituted medium showed increasing UV hypersensitivity with greater BrUra substitution in the medium (Fig. 1A). Under the same conditions, BDT1153 (*ung-1*) was more resistant to UV killing (Fig. 1B), suggesting that the uracil-DNA glycosylase deficiency renders cells resistant to the BrdUrd sensitization to UV killing.

thyA strains completely isogenic except for the *ung* gene region were constructed by P1 transduction. The *ung* gene is located at 56 min between the *nadB* and the *pheA* genes on the *E. coli* genetic map (6). A P1 lysate prepared on strain BD10 ($pheA^+$ *ung-1* $nadB^+$) was applied to strain NKT6042 ($pheA^+$ ung^+ *nadB51*::Tn*10* *thyA*). Of 96 $NadB^+$ transductants examined, 33 exhibited an Ung^- phenotype, indicating 34.4% cotransducibility. In another cross between BD10 (P1) and KAT197 (*pheA* ung^+ $nadB^+$ *thyA*), 12 among 54 $PheA^+$ transductants exhibited an Ung^- character (22.2% cotransduction frequency). These cotransduction frequencies between *ung* and *nadB* or between *ung* and *pheA* were similar to previous results (6). After growth in 50% BrdUrd medium, all of the above 45 Ung^- transductants were more resistant than 105 Ung^+ transductants to UV killing. These results indicate that the phenotype of resistance to BrdUrd photosensitization is very closely linked with the *ung* mutation.

A representative set of YY369 (ung^+) and YY368 (*ung-1*) was arbitrarily selected from these transductants for further studies described below. Both strains showed the same UV sensitivity after growth in unsubstituted medium (Fig. 1C). When grown in 50% BrdUrd medium, however, the *ung-1* mutant was more resistant than the wild-type strain to 254-nm UV light (Fig. 1C) and to 315-nm UV light (Fig. 1D). Approximately 10-fold higher doses of 315-nm UV light than 254-nm UV light were required to achieve the same killing of both strains, whether or not they contained BrUra-DNA (Fig. 1C and D).

Growth and UV-light sensitivity of bacteria during culture with BrdUrd. The wild-type strain and the *ung-1* mutant were grown in 0, 50, and 100% BrdUrd-substituted media. These strains exhibited the same number of viable cells all through the culture in either medium. They exhibited doubling times of 36 min in unsubstituted medium and 42 min in 50% BrdUrd medium during exponential growth. In 100% BrdUrd medium, the viable cell number of both strains did not increase and was held almost constant throughout the culture despite an 11-fold increase of A_{660}. After 7 h of culture, cells were microscopically filamentous. Therefore, it may be possible that cells stop DNA replication but continue protein synthesis during culture in 100% BrdUrd medium.

Change in UV sensitivity as a function of culture time with and without BrdUrd was examined (Table 2). Without BrdUrd, both the wild-type and *ung-1* strains showed a slightly higher UV sensitivity during exponential growth than during the stationary phase (Table 2). During exponential growth in 50% BrdUrd medium, the UV sensitivity of the

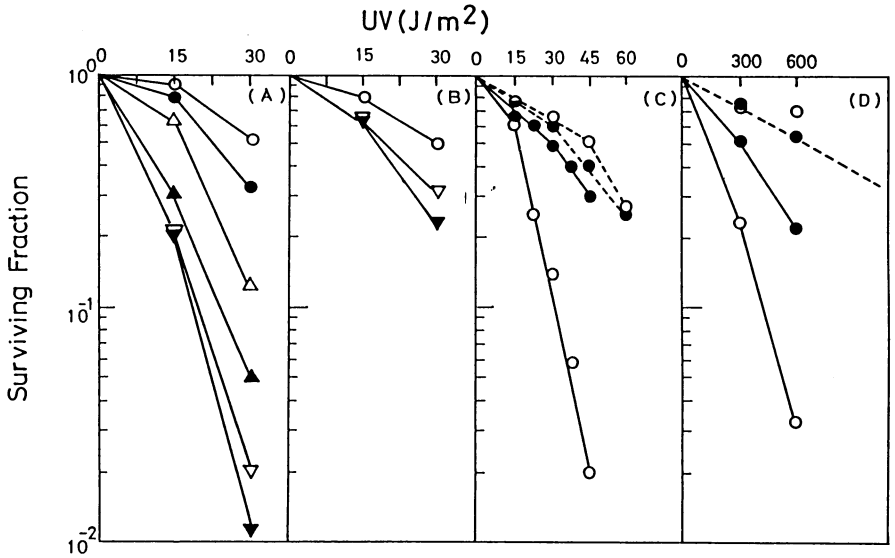

FIG. 1. BrdUrd sensitization of UV killing in wild-type and isogenic *ung-1* strains. Cells grown for 17 h in M9S growth medium containing various amounts of BrdUrd (or BrUra) were examined for 254-nm UV sensitivity. In panels A and B, growth medium contained 400 μM thymine and various amounts of BrUra. In panels C and D and all other experiments described below, the medium used contained a fixed 200 μM concentration of BrdUrd plus dThd. (A) BDT1154 (*ung*$^+$) and (B) BDT1153 (*ung-1*), grown in media with BrUra at the following concentrations: ○, 0%; ●, 10%; △, 20%; ▲, 40%; ▽, 50%; ▼, 75%. (C) YY369 (*ung*$^+$) (○) and YY368 (*ung-1*) (●), grown in 0% BrdUrd medium (– – –) or 50% BrdUrd medium (——). (D) YY369 (○) and YY368 (●), grown as in panel C but irradiated with 315-nm UV light; The D_{37} of cells carrying unsubstituted DNA was ~980 J/m^2 (see the text).

wild-type and *ung-1* mutant increased similarly with culture time. However, as soon as the cells approached the stationary phase after 6 h of culture, the *ung-1* mutant became more resistant than the wild-type strain to UV (Table 2). In 100% BrdUrd medium, the *ung-1* mutant was similar to the wild-type in UV sensitivity up to the first 1 h of the culture, whereas the wild type became 100-fold more UV sensitive than the *ung-1* mutant during the subsequent incubation

TABLE 2. UV-light sensitivity of YY369 (*ung*$^+$) and YY368 (*ung-1*) strains during growth in unsubstituted medium or BrdUrd medium[a]

Culture time (h)	Survival (%) in the following medium:					
	Unsubstituted		50% BrdUrd		100% BrdUrd	
	ung$^+$	*ung-1*	*ung*$^+$	*ung-1*	*ung*$^+$	*ung-1*
0	40	70	60	60	30	30
1	40	50	30	50	4	10
3	30	30	20	20	0.02	0.1
5	20	30	2	4	0.004	0.3
6	ND	ND	2	20	0.001	0.2
7	60	50	0.9	10	ND	ND
24	80	60	1	30	0.03	7

[a] Fresh overnight cultures were diluted (1/99) and subcultured in M9S growth medium containing 0 or 50% BrdUrd, or overnight cultures were once subcultured in unsubstituted M9S growth medium to the early log phase and then washed twice and recultured in 100% BrdUrd medium. Samples were withdrawn periodically during culture to monitor survival. Individual samples of each culture were exposed to a constant dose of UV light (40 J/m^2 for 0 and 50% BrdUrd medium; 30 J/m^2 for 100% BrdUrd medium). Survival is shown as a percentage of survival of unirradiated controls. The growth phase was determined by counting viable cell numbers at various times during culture (data not shown). The exponential phase was observed during growth from 1 to 5 h in unsubstituted or 50% BrdUrd media, and the stationary phase was observed from 6 to 24 h. In 100% BrdUrd medium, the viable cell number did not increase (see the text). ND, Not determined.

(Table 2). These results suggest strongly that the presence of uracil-DNA glycosylase activity causes considerable BrdUrd photosensitization during the growth-arrested phase.

BrUra content in DNA. BrUra substitution for thymine in DNA in the wild-type strain and the *ung-1* mutant during growth in 50% BrdUrd medium was determined by incorporation of [^{3}H]BrdUrd and [^{14}C]dThd. The extents of substitutions in the DNA of these strains increased similarly during the exponential phase (6% after 3 h of culture), reached a maximum of almost 30% at around 5 h, and sustained that level during the subsequent stationary phase.

The BrUra contents in the DNA of the strains grown in 50 and 100% BrdUrd media for 7 h were directly measured by HPLC. Molar guanine/cytosine and adenine/(thymine plus BrUra) ratios were 1.1 and 0.96, respectively. The wild-type cell DNAs had 24 and 38% BrUra substitutions after growth in the 50 and 100% BrdUrd media, respectively, and the *ung-1* mutant DNA exhibited much the same figures of 23 and 37% substitutions, respectively, under the same *thyA* background. In addition, a small, similar amount of uracil was detected in the acid hydrolysates of the wild-type and *ung-1* DNAs. Since it appeared that there was more uracil in samples without alkaline hydrolysis (data not shown), it seemed to come from small quantity of contaminating RNA but not from spontaneous debromination of BrUra-DNA in vivo.

UV-induced mutation frequency. Yields of Rif^r or Val^r mutants were measured in the wild type, the *ung-1* mutant, and their *umuC* derivatives by using cells grown in 0 and 50% BrdUrd media.

Without UV irradiation, both the Rif^r and Val^r mutation frequencies were slightly higher in strains carrying BrUra-DNA than in strains with unsubstituted DNA (Table 3), indicating that BrdUrd itself is a weak mutagen. Furthermore, both the Rif^r and Val^r mutation frequencies in the

TABLE 3. Frequency of UV-induced Rif^r and Val^r mutations in the *ung*$^+$ and *ung-1* isolates and their *umuC122*::Tn*5* derivatives carrying unsubstituted or BrUra-DNA

DNA	Relevant genotype	Survival[a] (%), 30 J/m^2	Mutation frequency (10^8)					
			Rif^r			Val^r		
			0 J/m^2	30 J/m^2	UV induced[b]	0 J/m^2	30 J/m^2	UV induced[b]
BrUra substituted	*ung*$^+$	7	10	1,729	1,719	12	1,060	1,048
	ung-1	45	32	286	254	90	484	394
	ung$^+$ *umuC122*	1	3	121	118	12	101	89
	ung-1 umuC122	30	47	136	89	38	94	56
Unsubstituted	*ung*$^+$	57	2	319	317	0.7	102	101
	ung-1	84	27	195	168	80	174	94
	ung$^+$ *umuC122*	40	0.6	3	2	1	5	4
	ung-1 umuC122	59	8	12	4	25	20	

[a] Survival (percentage of that of unirradiated control) after UV irradiation at 30 J/m^2 was determined as described in Materials and Methods.
[b] UV-induced mutation frequency was calculated as (mutation frequency observed at 30 J/m^2) − (mutation frequency observed without UV irradiation).

ung-1 mutants were always higher than those in their *ung*$^+$ counterparts when they carried unsubstituted DNA (e.g., 14-fold for Rif^r [27 versus 2] to 114-fold for Val^r [80 versus 0.7]) (Table 2). The hypermutability seems to be due to a weak mutator phenotype of the *ung* mutant, as described previously by Duncan and Weiss (7).

UV irradiation of the wild-type strain carrying unsubstituted DNA caused levels of UV-induced mutation frequencies similar to those of the *ung-1* mutant (twofold [317 versus 168] for Rif^r and the same [101 versus 94] for Val^r at 30 J/m^2) (Table 3). Most of such UV-induced mutations did not occur in respective isogenic *umuC* derivatives (Table 2), confirming the previous observations by Elledge and Walker (8).

On the contrary, UV-induced mutation frequencies of the wild-type strain carrying BrUra-DNA were higher than those of the *ung-1* mutant (sevenfold [1,719 versus 254] for Rif^r and threefold [1,048 versus 394] for Val^r at 30 J/m^2, a UV dose at which the wild-type strain was more sensitive to UV than the *ung-1* mutant) (Table 3). The *ung*$^+$ *umuC* and the *ung-1 umuC* strains exhibited similar mutation frequencies for both markers at the UV dose of 30 J/m^2, although the *ung*$^+$ *umuC* strain was more sensitive than the *ung-1 umuC* strain to UV (Table 3). These results indicate that uracil-DNA glycosylase causes the major part of UV-induced Rif^r and Val^r mutations in BrUra-DNA and that most of the Ung-dependent mutagenesis requires the UmuC function.

It is noteworthy that even the *ung*$^+$ *umuC* derivative with BrUra-DNA exhibited higher UV-induced mutation frequency than the same strain carrying unsubstituted DNA at 30 J/m^2 (59-fold for Rif^r [118 versus 2], 22-fold for Val^r [89 versus 4]) (Table 3). The same was true for the *ung-1 umuC* derivative (Table 3). Therefore, UV also induced mutations significantly in BrUra-DNA in the absence of the UmuC function.

Enrichment of *ung* mutants by repeated alternating BrdUrd exposure and UV irradiation. The phenotype of resistance to BrdUrd photosensitization exhibited by the *ung-1* mutant was used to devise a new screening procedure for *ung* mutants. The mutagenized cell population of the parental strain, NKT6042 (*ung*$^+$ *nadB51*::Tn*10 thyA*), was first grown in the 50% BrdUrd medium to the stationary phase and then irradiated with UV light, and the surviving cells were grown to the stationary phase in the unsubstituted medium. During three (experiment 1) or four (experiment 2) replications of this selection, the UV sensitivity of the cell population grown in BrdUrd medium decreased (Fig. 2A). After the final selection, 5 (experiment 1) and 10 (experiment 2) colonies were randomly selected. All 15 clones, when grown in 50% BrdUrd medium, were similarly more UV resistant than the parental strain (the five clones of experiment 1 shown in Fig. 2B). One (experiment 1, clone 4) and two (experiment 2, clones 5 and 7) clones were defective in glycosylase activity (<0.2% of the NKT6042 parental strain) (Table 4), indicating that about 20% of the clones surviving several selection cycles were *ung* mutants.

The BrdUrd sensitization-resistant phenotype of the three putative *ung* mutant strains, YY372, YY373, and YY374, was cotransduced with the *nadB51*::Tn*10* (38 to 50%) or the *pheA*$^+$ (11 to 22%) mutant by P1 *vir* phage as frequently as with the *ung-1* mutant as described above. Furthermore, all of the resistant transductants were also defective in uracil-DNA glycosylase activity (data not shown). This indicates that they are alleles of the *ung-1* mutation. They were designated as *ung-10*, *ung-20*, and *ung-30*, respectively. The mutant *ung-10* (experiment 1) was of independent origin from *ung-20* and *ung-30* mutants (experiment 2). These mutations were transduced by P1 phage into strain AB1157 carrying *thyA*::Tn*5* (Table 1) and were examined for spontaneous mutation frequency (Rif^r cells per 10^8 viable cells): the median frequencies were 0.6 (wild type, 43 subcultures), 8.6 (*ung-10*, 32 subcultures), 4.9 (*ung-20*, 21 subcultures), and 14.5 (*ung-30*, 11 subcultures). Thus the *ung* mutants exhibited 8 to 24 times higher mutation frequencies than did the wild-type strain, as expected with *ung* as a weak mutator (7).

The clones surviving the selection with BrdUrd plus UV, were examined for BrUra substitution level in DNA (Table 4). The three *ung* mutant clones exhibited 31 to 33% substitution, similar to that of the parental strain (35%). All of the other resistant clones tested had much lower BrUra substitution levels of 5 to 16%, which is presumably the source of their resistance to BrdUrd photosensitization.

DISCUSSION

We have studied the biological effects of uracil-DNA glycosylase on viability, UV sensitivity, and UV mutagenesis of *E. coli* K-12 thymine-requiring bacteria (*thyA*) that had incorporated BrUra into their DNA. HPLC analyses of DNA bases revealed the same extents of about 24 and 38% BrUra substitution for thymine in the wild-type and *ung-1* mutant DNAs by growth in 50 and 100% BrdUrd media,

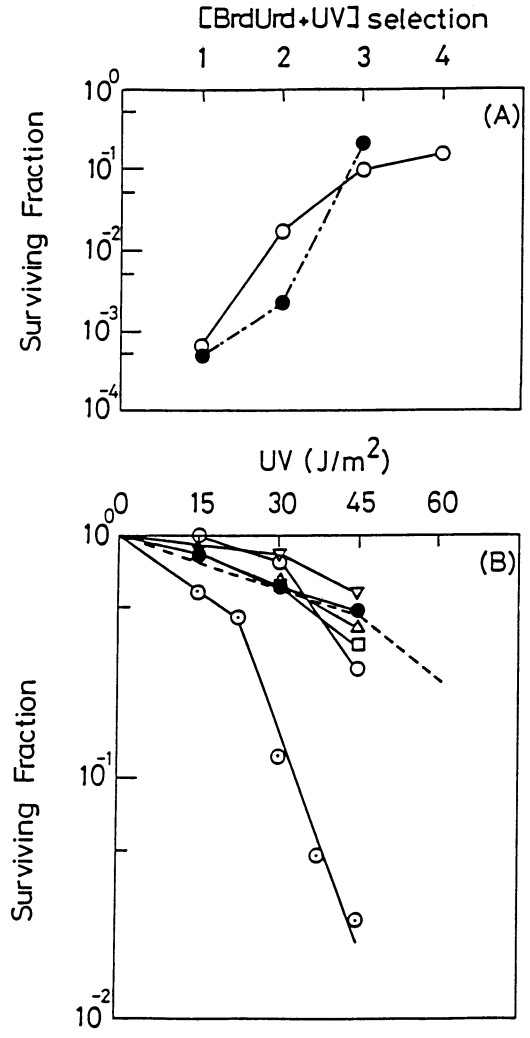

FIG. 2. BrdUrd-plus-UV selection. (A) UV (60 J/m^2) sensitivity of the cell population grown in 50% BrdUrd medium during a repeat of the BrdUrd-plus-UV selection. Symbols: ●, experiment 1; ○, experiment 2. (B) After three BrdUrd-plus-UV selection cycles (experiment 1), five clones picked up randomly were grown in 50% BrdUrd medium and examined for UV sensitivity. The UV sensitivity of NKT6042 grown in unsubstituted medium is also shown (– – –), and other clones grown in unsubstituted medium exhibited the same sensitivity levels as NKT6042. Symbols: ⊙, NKT6042 (parental strain); △, clone 2; ●, clone 4 (*ung-10*); □, clone 6; ▽, clone 8; ○, clone 10.

respectively. This finding is compatible with in vitro evidence that BrUra itself in DNA is not a substrate for *E. coli* uracil-DNA glycosylase (21). The BrdUrd medium retarded cellular growth, but the presence or absence of uracil-DNA glycosylase had essentially no effect on growth. On the other hand, UV irradiation of cells with BrUra-DNA demonstrated the remarkable characteristic that the absence of uracil-DNA glycosylase caused more UV resistance to killing (Fig. 1). Strain AB1157 with *thyA*::Tn*5* and *ung-152*::Tn*10* double mutations was also more resistant than the isogenic *ung*$^+$ strain to BrdUrd photosensitization (data not shown). Therefore, the resistant phenotype is commonly observed in *ung* mutants.

The BrdUrd sensitization factor (12), defined as the ratio of D_{37} (UV dose needed to reduce the surviving fraction to

TABLE 4. Percent BrUra substitution in DNA[a]

Expt	Strain or clone	Uracil-DNA glycosylase activity[b] (pmol of uracil/μg/min [%])	Deoxynucleoside incorporated into DNA (nmol/10^8 viable cells)		% BrUra in DNA
			BrdUrd	dThd	
	NKT6042 (parent)	4.6 (100)	303	575	35
1	Clones				
	2		94	734	11
	4 YY372 (*ung-10*)	<0.01 (<0.2)	330	671	33
	6		124	638	16
	8		119	737	14
	10		96	722	12
2	Clones				
	4		67	642	9
	5 YY373 (*ung-20*)	<0.01 (<0.2)	304	671	31
	6		66	652	9
	7 YY374 (*ung-30*)	<0.01 (<0.2)	322	643	33
	8		36	678	5
	10		75	712	9

[a] Percent BrUra substitution was determined in DNA of cells grown in 50% BrdUrd medium containing [^{14}C]dThd and [^{3}H]BrdUrd for 17 h as described in Materials and Methods.

[b] Clones 2, 6, 8, and 10 (experiment 1) and 4, 6, 8, and 10 (experiment 2) exhibited as much uracil-DNA glycosylase activity as the NKT6042 parental strain.

37% of the unirradiated control) for cells grown in media without BrdUrd to D_{37} for those in 50% BrdUrd were 2.8 (wild type) and 1.8 (*ung-1* mutant) for 254-nm UV, and 4.8 (wild type) and 2.3 (*ung-1* mutant) for 315-nm UV (Fig. 1C and D). Two factors may be involved in the BrdUrd photosensitization: (i) approximately a half of the sensitization in wild-type cells is due apparently to the presence of uracil-DNA glycosylase; and (ii) the remaining half, corresponding to the extent of sensitization in *ung-1* cells, may be due either to other enzyme(s), such as endonuclease V (4), acting on uracil-containing DNA or to different lesions such as double-strand breaks and alkali-labile damage generated in UV-irradiated BrUra-DNA (11, 12). The *ung-1* mutant phenotype of differential resistance to BrdUrd photosensitization was observed only during growth arrest either in the stationary phase or in 100% BrdUrd medium (Table 2). The wild-type and the *ung-1* mutant cells were almost equally sensitive to BrdUrd photosensitization during exponential growth (Table 2). In this case, single-strand breaks directly induced by UV irradiation in the parental BrUra strands near and at replication forks may produce double-strand breaks at forks, resulting in an effective block to replication and effective cell killing.

A plausible explanation for uracil-DNA glycosylase-dependent photosensitization [see (i) above] may be the enhanced degradation of UV-irradiated BrUra-DNA by the concerted action of uracil-DNA glycosylase and apyrimidinic endonuclease. Assuming that these enzymes may produce single-strand breaks rapidly at many sites of the major photoproducts, uracil residues, in the BrUra-DNA, the enhanced degradation of the DNA would occur before the repair by DNA polymerase and DNA ligase. In support of this, Warner and Duncan (28) reported that 30% uracil-containing T4 phage DNA replicated well as the uracil-free phage in *ung-1* cells, whereas the former phage failed to replicate and more than 50% of uracil-containing DNA was degraded very rapidly by uracil-DNA glycosylase in *ung*$^+$

cells. Therefore, breakdown of uracil-containing DNA is initiated by uracil-DNA glycosylase in *ung*$^+$ cells but not in *ung-1* cells.

With respect to mutation induction under the *umuC* regulation, frequencies of UV-induced Rif^r and Val^r mutations were higher in the wild type than in the *ung-1* mutant at the UV doses that caused more wild-type cell killing compared with that of the *ung-1* mutant (Table 3). Therefore, lethal damage produced in UV-irradiated BrUra-DNA by uracil-DNA glycosylase may also induce an excess of Rif^r and Val^r mutations in *ung*$^+$ cells over those in *ung-1* cells. Uracil-DNA glycosylase-dependent DNA breakage (see above) may induce deletion mutations directly or base-change mutations through *UmuC*-dependent error-prone repair (8, 14). The forward Rif^r and Val^r mutations have been shown to include both base-pair substitutions and deletions (13, 17, 18). The DNA sequences of the present Rif^r and Val^r mutations produced after BrdUrd photosensitization remain to be studied.

All colonies developed after three or four cycles of the BrdUrd-plus-UV selection were more UV resistant than the parental strain when grown in 50% BrdUrd medium, and about 20% of them were defective in uracil-DNA glycosylase. However, the remaining 80% had normal uracil-DNA glycosylase activity. They incorporated dThd into DNA normally but incorporated less BrdUrd, so that they exhibited 5 to 16% BrUra substitutions in DNA compared with 35% replacement in the parental strain (Table 4). They were high-thymine-requiring strains (data not shown), indicating that they had not acquired secondary *drm* and/or *dra* mutations (27). The gene defects in these strains remain to be elucidated.

Mutants deficient in uracil-DNA glycosylase activity were isolated from *Bacillus subtilis* (22), *E. coli* K-12 (6), and the smut fungus *Ustilago maydis* (30) by direct enzyme assay of crude extracts prepared from a large number of mutagenized colonies. This method is laborious and time consuming. T4 phage carrying uracil-containing DNA showed a much higher plating efficiency on *ung* mutant cells compared with that on wild-type cells (6, 28). Based on this observation, Duncan (5) devised a screening procedure specific for *ung* mutants, where transducing λ phage carrying uracil-containing DNA (with the Kan^r marker) was infected to a mutagenized parental strain and *ung* mutants were isolated from surviving Kan^r lysogens. Based on the same idea, Burgers and Klein isolated *Saccharomyces cerevisiae ung* mutants (3). The present BrdUrd-plus-UV selection method has several advantages: ease of manipulation, rapid isolation of the mutants, and lack of requirement for uracil-containing phage or plasmid DNA. The BrdUrd sensitization mechanism in higher eucaryotes may involve uracil-DNA glycosylase, as it does in bacteria. Thus it would be interesting to apply this screening procedure to isolate uracil-DNA glycosylase-defective mutants of higher eucaryotes.

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