Demonstration of Pyrimidine Dimer-DNA Glycosylase Activity In Vivo: Bacteriophage T4-Infected *Escherichia coli* as a Model System

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An approach to the detection of pyrimidine dimer-DNA glycosylase activity in living cells is presented. Mutants of *Escherichia coli* defective in *uvr* functions required for incision of UV-irradiated DNA were infected with phage T4 *denV*⁺ or *denV*⁻ (defective in the T4 pyrimidine dimer-DNA glycosylase activity). In the former case the *denV* gene product catalyzed the incision of UV-irradiated host DNA, facilitating the subsequent excision of thymine-containing pyrimidine dimers. Isolation of these dimers from the acid-soluble fraction of infected cells was achieved by a multistep thin-layer chromatographic system. Exposure of the dimers to irradiation that monomerizes pyrimidine dimers (direct photoreversal) resulted in the stoichiometric formation of free thymine. Thus, in vivo incision of UV-irradiated DNA dependent on a pyrimidine dimer-DNA glycosylase can be demonstrated.

The recent demonstrations of a two-step mechanism for the enzymatic incision of DNA containing pyrimidine dimers (PD) (2, 4, 7, 10, 13) represent an important advance in understanding the enzymology of DNA repair processes in living cells exposed to UV radiation. Incision of DNA mediated by the action of PD-DNA glycosylase and subsequent apyrimidinic endonuclease activities (Fig. 1) has been well established in vitro for the UV DNA-incising activity from *Micrococcus luteus* (4, 7) and for the *denV* gene product of coliphage T4 (10, 13). It is of interest to establish whether a similar enzymatic mechanism operates in any other organisms amenable to laboratory investigation. Thus far there have been no reports of PD-DNA glycosylase activities in extracts of any other cell type, and studies from this laboratory have failed to detect such an activity in extracts of the yeast *Saccharomyces cerevisiae*, human KB cells grown in culture, *M. radiodurans*, and *Escherichia coli*. (J. D. Love, E. H. Radany, S. McMillan, and E. C. Friedberg, unpublished data). Such negative results obviously do not eliminate the possible existence of these enzyme activities in these or other organisms, since the enzyme activities may be particularly labile in cell-free systems or may be present in amounts too small to be detected by the assays currently in use.

In principle, it is possible to overcome the limitations of in vitro enzymology and to demonstrate PD-DNA glycosylase activity in vivo by

recovering and analyzing the PD excised during the repair of DNA in irradiated cells (Fig. 1). In the present study, we have addressed this question by studying PD excision in *uvr*⁻ mutants of *E. coli* infected with phage T4. Our results show that PD-DNA glycosylase activity in vivo can be detected after infection with phage T4 *denV*⁺ and that this activity accounts quantitatively for all thymine-containing PD excised from the DNA of irradiated host cells. Neither PD-DNA glycosylase activity nor PD excision was detected in uninfected *uvr*⁻ cells or in such cells infected with a T4 phage defective in the *denV* gene. The approach used in these experiments should be generally useful for the analysis of PD excision repair in a variety of biological systems.

MATERIALS AND METHODS

Bacterial and phage strains. The *E. coli* K-12 strains SR314 (*uvrA6 uvrB5*) and SR143 (*uvrA6*) are both derivatives of strain W3110 and were obtained from Kendrick C. Smith, Stanford University. Both strains are thymine auxotrophs. Phage T4 M2 [*nd28 (denA) SaΔ9 (denB)*] was obtained from Elizabeth Kutter, Evergreen State College, Olympia, Wash., and phage T4v₁ (*denV*⁻) is maintained in our laboratory. Phage lysates (~10¹⁰ PFU/ml) were prepared by infection of *E. coli* W3110 followed by low-speed centrifugation of lysates and dialysis of the supernatants against 100 mM NaCl -1 mM MgCl₂ -1 mM CaCl₂. Lysates were stored over chloroform at 4°C.

Radioactive labeling of bacterial DNA. *E. coli* cells were grown at 37°C overnight in RT medium (1) supplemented with 0.5% glucose, 0.1% vitamin-free

FIG. 1. Diagrammatic representation of experimental protocol. *E. coli uvr⁻* cells are radiolabeled in their DNA with thymine. UV irradiation generates thymine-containing PD. Host DNA containing these lesions is incised after infection with phage T4 *denV⁺* by a two-step mechanism involving cleavage of the 5' glycosyl bond of a dimer and subsequent hydrolysis of a phosphodiester bond (here shown as the 3' bond) at the apyrimidinic site. Excision in the 5' → 3' direction generates acid-soluble oligonucleotides containing PD from which free thymine can be released by monomerization of the dimers by using photoreversal. The release of free thymine is thus a quantitative measure of PD-DNA glycosylase activity.

casein hydrolysate, and 2 μ g of thymine per ml. The cell cultures were diluted to a density of $\sim 2 \times 10^7$ cells/ml in fresh RT medium plus supplements containing 10 to 20 μ Ci of [3 H]thymidine per ml (Amersham Corp.; 53 Ci/mmol) and reincubated at 37°C to a density of 2×10^8 cells/ml. Radioactive thymidine was chased by the addition of 10 μ g of unlabeled thymidine per ml and further incubation for 15 min at 37°C. Cultures were chilled in an ice water bath, harvested by filtration through membrane filters (0.8 μ m, type AA; Millipore Corp.), and washed with 2 volumes of unsupplemented RT buffer. The washed cells were

resuspended in 0.5 volume of RT buffer at 25°C, thereby increasing their density to 4×10^8 cells/ml.

Irradiation and post-UV incubation. Washed cells were UV irradiated under a low-pressure germicidal lamp (GE no. G8T5) in a 9-cm-diameter petri dish with stirring. Irradiations were at 4°C at an incident dose rate of 70 J/m² per min, and corrections for effective dose were made by the method of Morowitz (8). Sham-irradiated cells were held at 4°C. Equal portions of cells were withdrawn immediately after irradiation and fixed in an equal volume of 200 mM potassium phosphate, pH 8.0–20% formaldehyde for measurement of

the initial thymine-containing PD content in DNA (9). Equal portions of irradiated or unirradiated cells were diluted into equal volumes of prewarmed (37°C) RT medium containing 2× supplements and, where indicated, were infected with phage T4 at a multiplicity of ~10. Phage-infected cells were incubated at 37°C for 10 to 20 min, and uninfected cells were incubated for ~45 min as specified in the legends to the tables and figures. Incubations were terminated by chilling cultures to 4°C. Cultures were centrifuged at 4°C for 5 min in a Sorvall SS-1 rotor at 9,000 rpm. The pelleted cells from each incubation were suspended in 2 ml of cold RT buffer and divided into four equal portions in 1.5-ml conical tubes. These suspensions were recentrifuged at 4°C for 2 min in an Eppendorf microfuge (model 5412). The supernatants were discarded, and the pellets were frozen in a dry ice-alcohol or liquid nitrogen bath. The four frozen pellets from each experimental sample were thawed in 100 µl of 10 mM EDTA, pH 8.0, and refrozen for storage.

Preparation of acid-soluble fractions of cell extracts. Two of the four frozen cell portions from each incubation were thawed to 4°C, and disruption of cells was completed by the addition of Brij 58 (Sigma Chemical Co.; 0.2% final concentration) and incubation at 4°C for 20 min. Cold trichloroacetic acid (10%, final concentration) was added to each sample with vigorous mixing. Acid-insoluble material was pelleted by centrifugation for 5 min in an Eppendorf microfuge. The pellets were dissolved in 200 µl of 1 N NaOH for measurement of acid-insoluble radioactivity. The acid-soluble supernatant fractions were immediately extracted seven times successively with 1.0 ml of ice-cold diethyl ether. This exhaustive extraction served to remove much of the [³H]thymidine as well as the Brij and trichloroacetic acid present in these fractions. The volume of the fractions was reduced considerably by the extraction, typically from 120 to ~35 µl. Residual ether was removed by evaporation under a gentle air stream. Five microliters of 100 mM Tris-hydrochloride, pH 8.0–10 mM EDTA was added to each acid-soluble fraction, and the final volume of each sample was brought to 50 µl with distilled water.

Photoreversal-dependent formation of free thymine in acid-soluble fractions. Small volumes (5 to 10 µl) of each acid-soluble fraction were spotted 2 cm from the bottom of silica gel thin-layer plates (20 by 20 cm; Brinkmann Instruments Inc.). Plates were developed twice in the same direction to a distance of 16 cm with water-saturated ethyl acetate-*n*-propanol (4:1, vol/vol). This preliminary chromatography served to remove all remaining [³H]thymidine from the origin regions of the thin-layer plates (Fig. 2). The origin regions of those chromatograms that were not designated to receive photoreversal irradiation were masked with cardboard, and each plate was exposed to a dose of 6 kJ of low-pressure germicidal (254 nm) radiation per m². Thin-layer plates were then developed a final time with the same solvent to a distance of 8 cm. Plates were fractionated into 0.5-cm segments and processed for measurement of radioactivity as described by Reynolds et al. (12).

Measurement of thymine-containing PD in acid-soluble fractions. The origin regions of the thin-layer plates containing samples not subject to photoreversal irradiation were excised, and essentially all of the radioactivity (present as nucleotides) was eluted into 0.5 ml of

1 N HCl. The HCl was removed by lyophilization, the residues were dissolved in 200 µl of 97% formic acid for hydrolysis, and thymine-containing PD were measured as described by Reynolds et al. (12). Elution of the origin regions directly into formic acid was not successful because the formic acid dissolved the binder in the silica gel matrix, leaving a residue after hydrolysis that interfered with the mobility of thymine-containing PD during thin-layer chromatography.

Measurement of thymine-containing PD in acid-insoluble DNA. The cells fixed in formaldehyde immediately after irradiation were washed twice in 10 mM Tris-hydrochloride, pH 8.0–10 mM EDTA and resuspended in 0.5 ml of this solution. The remaining two frozen cell portions from each incubation were also brought to a volume of 0.5 ml in this solution. All samples were deproteinized by incubation overnight at 37°C with 100 µg of proteinase K per ml (Boehringer Mannheim Corp.) plus 0.5% sodium dodecyl sulfate, followed by extraction twice with an equal volume of buffered phenol. DNA was precipitated with 10% (final concentration) trichloroacetic acid, and thymine-containing PD were measured by thin-layer chromatography of hydrolysates (12).

RESULTS

It has been previously demonstrated that infection of lethally irradiated *uvr*[−] mutants of *E. coli* with T4 phage results in a *denV* gene-dependent reactivation of cell viability (so-called *v*-gene reactivation) (3, 6). Presumably, the *denV* gene product mediates the incision step of the excision repair of host DNA containing PD. We therefore decided to investigate PD-DNA glycosylase activity *in vivo* by examining the thymine-containing PD excised from labeled host DNA after infection with phage T4. In all of these studies, the *E. coli* hosts used were defective in the *uvrA* and sometimes the *uvrB* function in order to avoid possible complications arising from expression of the host UV DNA incision enzyme activity (14).

It was recognized at the outset that in the present experiments incision of UV-irradiated DNA *in vivo* would be followed by excision of small oligonucleotides containing PD. Hence, putative hydrolyzed glycosyl bonds in PD would be isolated in the acid-soluble phase of cell extracts (Fig. 1). The detection of photoreversal-dependent free thymine released from these acid-soluble oligonucleotides would thus be complicated by a very high background of the radioactive precursor (thymine or thymidine) used initially to label the DNA of the cells. This problem was overcome by the use of the multi-step thin-layer chromatography system described in Materials and Methods. Acid-soluble fractions from ~2 × 10⁸ cells were maintained in volumes of 5 to 10 µl and spotted near the bottom of silica gel thin-layer plates (Fig. 2).

FIG. 2. Multistep thin-layer chromatographic assay for measurement of photoreversal-dependent free thymine in acid-soluble fractions. The acid-soluble fraction from an extract of 5×10^8 [^3H]thymine-labeled irradiated cells that had been infected with phage T4 *denV*⁺ was spotted on two adjacent lanes of a silica gel thin-layer plate and developed twice in the same direction to a distance of 16 cm as described in the text. These two developments resolved $\sim 10,000$ cpm of [^3H]thymidine from $\sim 44,000$ cpm of nucleotide species that remained at the origin in each lane (open and closed circles). The origin region of one lane was irradiated with a UV dose of 6 kJ/m² to photoreverse PD, and the plate was developed a third time to a distance of 8 cm. Note the appearance of free thymine without the detection of any further free thymidine (closed circles). Essentially no free thymine was detected in the third development of the lane that was not subject to photoreversal irradiation (open circles).

Residual labeled thymidine was removed by two preliminary solvent developments, and photoreversal of pyrimidine dimers was then carried out directly on the plates by irradiation of the origin regions with UV light. The thin-layer plates were then subjected to development with solvent for a third time. In this way, free thymine generated by the combined action of PD-DNA glycosylase *in vivo* and the subsequent splitting of thymine-containing dimers by photoreversal was readily detectable and quantifiable (Fig. 2). The use of multiple developments in the same direction allowed the analysis of control samples not exposed to photoreversal on a lane immediately adjacent to the corresponding sample exposed to photoreversal. Thus, an accurate correction for the background of photoreversal-independent radioactivity in the position of free thymine could be made (Fig. 2).

E. coli uvrA⁻ *uvrB*⁻ cells were radiolabeled in their DNA, UV irradiated, and infected with phage T4 M2. This phage is *denV*⁺ but is defective in T4 endonucleases II and IV and was used initially to minimize potential phage-induced degradation of the host genome. (However, degradation of host DNA to an acid-soluble form by T4 phage proficient in endonucleases II and IV was not observed under our experimental conditions [data not shown].) Infection was allowed to proceed at 37°C for 15 to 20 min, after which acid-soluble fractions were processed and analyzed as described in Materials and Methods (Table 1, Fig. 3). Formation of free thymine was detected only when the acid-soluble nucleotide fractions from phage T4-infected cells were exposed to high fluences of UV light (photoreversal irradiation). This procedure is known to monomerize preexisting PD (15), and it has been

TABLE 1. Release of free thymine from the acid-soluble fraction of T4-infected *E. coli uvr⁻* cells^a

Cell type	Fraction of acid-insoluble ³ H radioactivity in PD after incubation (×10 ³) ^b	Nucleotide ³ H radioactivity after incubation (cpm × 10 ⁻⁶) ^{c,d}		Calculated radioactivity in excised PD in acid-soluble fraction (cpm)		Photoreversal-dependent free thymine (cpm) ^{d,e}	
		Acid-insoluble fraction	Total (acid-soluble + acid-insoluble fractions)	³ H ^f	¹⁴ C ^g	³ H	¹⁴ C
Irradiated ^h							
T4-infected	3.26 ± 0.13	1.37	1.41	2,260 ± 310		677 (100)	
		1.41	1.45	2,320 ± 320		790 (114)	
Uninfected	4.54 ± 0.22	2.92	2.98	960 ± 910	700	<30	<5
		3.30	3.36	1,050 ± 1,030		<30	
Unirradiated,							
T4-infected	0	2.59	2.61	0	700	<30	<5

^a *E. coli* SR314 (*uvrA6 uvrB5*) (100 ml at 2×10^8 colony-forming units/ml) was labeled with [³H]thymidine, washed, and suspended at twice the original density in buffer as described in the text. A 30-ml portion of the cells was irradiated with an effective UV dose (254 nm) of 50 J/m²; the remaining cells were held without irradiation. Equal portions of irradiated (10 ml) and unirradiated (20 ml) cells were infected with phage T4 M2 (multiplicity = 10) for 20 min at 37°C. Irradiated cells (20 ml) were incubated without phage infection for 45 min at 37°C. The acid-soluble fractions from duplicate portions of cells were prepared as described in the text and analyzed for photoreversal-dependent formation of free thymine. The values listed on each line of the table refer to radioactivity in the various forms in the entire 50- μ l acid-soluble fraction from one cell pellet. Two cell pellets were analyzed for each experimental condition, using irradiated cells.

^b The thymine-containing PD contents of the acid-precipitable fractions are the means of four determinations $\pm$ standard error.

^c Nucleotide radioactivity excludes residual [³H]thymidine in the acid-soluble fractions. Radioactivity in acid-soluble nucleotide is defined as the radioactivity that remains at the origin of thin-layer chromatograms after two successive developments with solvent as described in the text. All acid-insoluble radioactivity is in nucleotide.

^d Duplicate determinations on equal portions of the same acid-soluble fraction agreed within 5%.

^e Calculated as the difference at the position of free thymine between radioactivity in chromatograms of the photoreversed and nonphotoreversed samples (see Fig. 2 and 3). The position of thymine was determined in these and all other experiments by cochromatography of authentic [¹⁴C]thymine in adjacent lanes. The numbers in parentheses indicate the calculated percentage of thymine-containing PD that are photolabile (i.e., contain a hydrolyzed glycosyl bond), assuming that 50% of the radioactivity associated with these dimers could be released as free thymine and that only 60% of the dimers were monomerized by photoreversal radiation (see text).

^f Calculated as the difference in acid-insoluble ³H radioactivity in PD before and after incubation. For the first line of the table this is $[(4.77 \times 10^{-3}) \times 1.41 \times 10^6 \text{ cpm}] - [(3.26 \times 10^{-3}) \times 1.37 \times 10^6 \text{ cpm}] = 2,260 \text{ cpm}$. The valid approximation is made here that the acid-insoluble nucleotide radioactivity in a cell sample before incubation equals the total nucleotide radioactivity in that sample after incubation; this approximation accounts for the small amount (2 to 3%) of acid-insoluble radioactivity made acid soluble during post-UV incubation. The confidence limits on calculated radioactivity in excised PD is the sum of the confidence limits for acid-insoluble ³H radioactivity in PD before and after incubation.

^g As a control to detect artifactual release of free thymine from dimer-containing oligonucleotides, [¹⁴C]thymine-labeled *E. coli* DNA (7,000 cpm/ μ g) was UV irradiated to give 7% thymine-containing PD. This DNA was degraded to acid-soluble products by incubation with pancreatic DNase. Incubations (0.5 ml) contained 300 μ g of DNA, 5 μ g of pancreatic DNase, 10 mM Tris-hydrochloride, pH 8.0, 10 mM MgCl₂, and 2 mM CaCl₂. Incubations were at 37°C for 1 h. Reactions were terminated by the addition of EDTA (10 mM, final concentration) followed by heating to 90°C for 20 min. A portion of this material containing 1.5 μ g of degraded DNA was added to some portions of lysed cells (as indicated) just after the addition of Brij 58 (see text for details). Recovery of this ¹⁴C-labeled DNA after preparation of the acid-soluble fractions was quantitative.

^h Fraction of acid-insoluble radioactivity in PD before incubation equals $(4.77 \pm 0.09) \times 10^{-3}$.

shown (2, 7, 10, 11) that the photoreversal-dependent formation of free thymine indicates that one of the glycosyl bonds associated with thymine-containing dimers was hydrolyzed before the irradiation. Note that essentially no thymine was observed in chromatograms of samples that were not subjected to photoreversal irradiation.

The specificity of this procedure as a measure

of PD-DNA glycosylase activity was indicated by the observation that essentially no photoreversal-dependent free thymine was observed when irradiated *uvr⁻* cells were not infected with phage T4 or when unirradiated cells were infected with phage T4 (Table 1, Fig. 3). As an additional control, UV-irradiated [¹⁴C]thymine-labeled DNA was digested to acid-soluble fragments with pancreatic DNase as described in

FIG. 3. Detection of photoreversal-dependent formation of free thymine in the acid-soluble fractions of UV-irradiated *E. coli* cells infected with phage T4 *denV*⁺ (bottom panel). Only the region of the thin-layer chromatogram relevant to the third development (see Fig. 2) is shown. The arrow marks the position of authentic [¹⁴C]thymine. The origin of the small peak of radioactivity in front of the thymine peak is not known, and it was not included in the calculation of free thymine. Typical results obtained with irradiated cells that were uninfected (middle panel) and with phage-infected unirradiated cells (top panel) are shown for comparison.

Table 1. When this acid-soluble material was added to the unfractionated extract immediately after lysis of ³H-labeled cells and carried through the rest of the experiments, no photoreversal-dependent release of free [¹⁴C]thymine was detected (Table 1). This result suggested that artifactual hydrolysis of glycosyl bonds in thymine-containing PD did not take place during the experimental manipulations.

The data presented in Table 1 also demonstrate the predicted stoichiometric relationship

between the amount of photoreversal-dependent free thymine and the calculated radioactivity present as thymine-containing PD in the acid-soluble fraction. If only one glycosyl bond of each thymine-containing dimer excised in vivo was hydrolyzed and all such dimers were reversed, exactly 50% of the radioactivity associated with these dimers should have been measured as free thymine (11) (Fig. 1). At the photoreversing fluence used in these experiments (6 kJ/m²), only 60% of the excised dimers in the acid-soluble fraction were actually reversed (Fig. 4) because the photoreversal reaction obeys first-order kinetics and would require infinite UV fluence to effect complete dimer monomerization. Thus, under our experimental conditions we would expect a maximum of ~30% of the thymine in PD to be released as free thymine after photoreversal. As indicated in Table 1, the amount of ³H radioactivity actually measured as free thymine was about 30% of the calculated ³H radioactivity associated with acid-soluble pyrimidine dimers. Thus, apparently all detected dimer excision from *E. coli* DNA after

FIG. 4. Photokinetics of free thymine release from UV-irradiated DNA. Acid-soluble fractions of *E. coli* SR314 infected with phage T4 M2 (*denV*⁺) were prepared and analyzed as described in the text and in the legend to Table 1, except that the photoreversal dose was varied as indicated. The acid-soluble fraction contained 6.48% (2,400 cpm; average of four determinations) of the total radioactivity in nucleotide (37,000 cpm) as thymine-containing PD. Based on these measurements, the maximum amount of radioactivity detectable as free thymine from complete photoreversal of dimers would be 1,200 cpm (half of 2,400 cpm, since only one glycosyl bond is hydrolyzed in each dimer; see Fig. 1). The best-fit first-order rate law extrapolates to 1,040 cpm maximum free thymine release (dotted line), a value in reasonable agreement with the 1,200 cpm calculated above. In this experiment, a photoreversal fluence of 6 kJ/m² corresponds to 63% of maximum thymine release. This value was reproducible to $\pm 4\%$ in multiple experiments.

infection with phage T4 *denV*⁺ was mediated by a PD-DNA glycosylase-dependent pathway of incision. Table 2 shows the results of a similar experiment in which a specific comparison was made between *uvr*⁻ *E. coli* infected with either T4 *denV*⁺ or T4 *denV*⁻ phage and demonstrates the strict dependence of the formation of photoreversal-dependent free thymine on the presence of a functional PD-DNA glycosylase gene.

DISCUSSION

We sought to establish whether it is possible to demonstrate a specific mechanism of incision of DNA containing PD in living cells. In the present study, we exploited the phenomenon of *denV* gene-dependent reactivation of *uvr*⁻ strains of *E. coli* and asked whether a putative DNA glycosylase/apurimidine endonuclease mechanism accounts quantitatively for the incision of thymine-containing PD excised from *E. coli* DNA in vivo. Our results show that no significant PD-DNA glycosylase activity was demonstrable during post-UV incubation of irradiated, uninfected *E. coli* strains defective in the *uvrA* or *uvrB* gene. After infection of irradiated *E. coli* with T4 *denV*⁺ phage, approximately one-third of all thymine-containing PD were excised from acid-precipitable *E. coli* DNA. When excised dimers present in the acid-soluble fraction were monomerized by direct photoreversal, quantitative determinations of the amount of free thymine generated and of the amount of excised thymine-containing PD showed that apparently all such dimers contained a single hydrolyzed glycosyl bond. Free thymine formation was not observed with extracts of unirradiated cells that were infected with phage T4. The apparent hydrolysis of gly-

cosyl bonds in DNA was strictly dependent on a functional *denV* gene; thus, it seems reasonable to conclude that incision of DNA in vivo (at least of that fraction of PD excised under our experimental conditions) was catalyzed by the T4 PD-DNA glycosylase.

Quantitatively similar results were obtained in experiments with *E. coli uvr*⁺ cells infected with phage T4 *denV*⁺ (data not shown). Such results were not unexpected, since it has been shown by others that, after infection of *E. coli uvr*⁺ cells with phage T4 or T5, an ATP-dependent UV DNA incising activity (presumably that coded by the *uvrA*, *uvrB*, and *uvrC* genes) is inactivated (18). Thus, such cells are effectively phenotypically *uvr*⁻. Surprisingly, in uninfected *E. coli uvr*⁺ cells, evidence for some PD-DNA glycosylase activity was also detected; the results of these studies will be reported in detail elsewhere (E. H. Radany and E. C. Friedberg, manuscript in preparation).

The data in Table 2 allow for a calculated estimate of the average total number of nucleotides excised from *E. coli* DNA per PD excision event (and hence indirectly the average repair synthesis patch size), assuming that all excised material was acid soluble. The values quoted as ³H acid-soluble radioactivity in nucleotide represent 1.81 and 1.58% of the total (acid-soluble plus acid-insoluble) radioactivity as nucleotides and oligonucleotides in extracts of *E. coli* infected with T4 *denV*⁺ and T4 *denV*⁻ phage, respectively. We attribute the small but highly reproducible difference between these two values to excision repair. Of this difference, about one-half (0.13%) was accounted for by the measured radioactivity in excised thymine-containing PD. At the UV fluence used in these experiments, the amounts of CT, TC, TT, and CC dimers

TABLE 2. Release of free thymine is dependent on a functional *denV* gene of phage T4^a

Phage	Nucleotide ³ H radioactivity		Photoreversal-dependent free thymine ([³ H]radioactivity; cpm)
	Acid soluble (cpm × 10 ⁻³)	Total (cpm × 10 ⁻⁵)	
T4 <i>denV</i> ⁺	6.4	3.40	142 ^b
	8.8	5.06	173 ^c
T4 <i>denV</i> ⁻	6.0	3.58	<20
	7.9	5.32	27
None	6.0	3.90	<20
	6.8	4.04	21

^a *E. coli* SR143 (*uvrA6*) (100 ml) was labeled and harvested as described in Table 1. The concentrated cells were irradiated with a UV fluence of 50 J/m² (254 nm), divided into three equal portions, and diluted into prewarmed 2× RT medium containing T4 phage (multiplicity = 10) as indicated. Incubation was for 10 min for phage-infected cells and for 40 min for uninfected cells (both at 37°C). The acid-soluble fractions of cells were prepared as described in the text. Ten-microliter portions of each fraction were analyzed for the photoreversal-dependent release of free thymine, and the remainder was processed for measurement of thymine-containing PD. The acid-soluble fractions from T4 *denV*⁺-infected cells contained (b) 490 and (c) 590 cpm in thymine-containing PD per 10-μl portion. No thymine-containing PD were detected in the acid-soluble nucleotide fractions from T4 *uvr*⁻-infected or uninfected cells. Acid-soluble and total nucleotide were determined as described in Table 1. Values listed are the radioactivity measured in the 10-μl portions of acid-soluble fractions.

formed are approximately equal (7). Thus, on the average each PD contains one labeled thymine; i.e., each labeled thymine measured in acid-soluble PD corresponds to two pyrimidine nucleotides excised when all (including CC) dimer species are taken into account. If we also assume that other nucleotides excised together with each PD reflect the average base composition of *E. coli* bulk DNA, then each labeled thymine in nondimer acid-soluble radioactivity reflects on average the presence of four nucleotides. Thus, the specific radioactivity of bulk DNA is one-half that of total excised PD when considered on a per-nucleotide basis. We can calculate, therefore, that six nucleotides (two dimer plus four nondimer) are lost from the DNA with each dimer excision event. Haseltine et al. (7) have shown that PD tend to occur more frequently in relatively thymine-rich sequences in *E. coli* DNA. Thus, it is likely that the average repair patch size is even smaller than that calculated, since the measured radioactivity in nondimer thymine would represent a larger fraction of the adjacent nucleotides made acid-soluble during PD excision than in bulk *E. coli* DNA.

It is interesting to note that the majority of studies on uninfected *E. coli*, in which incision is mediated by the ATP-dependent DNA incising activity coded by the *uvrA*, *uvrB*, and *uvrC* genes (14), have yielded repair patch sizes of 20 to 30 nucleotides, with a small proportion of longer patches estimated to be several hundreds of nucleotides in length (see reference 5). Further studies using independent techniques are needed to verify these apparent differences. If such differences do exist, it is tempting to speculate that the particular enzymatic mechanism by which DNA containing PD is incised (i.e., PD-DNA glycosylase/apurimidine endonuclease mechanism or direct-acting endonuclease mechanism) determines the extent of the subsequent excision-related degradation of the DNA and hence the repair patch size.

In conclusion, the experimental approach used in these studies is potentially applicable to any system of living cells from which radiolabeled excised oligonucleotides containing PD can be harvested. In particular, it could be valuable in investigating those systems where PD-DNA glycosylase activity cannot be demonstrated in vitro. The demonstration of photoreversal-dependent release of free thymine from oligonucleotides containing PD is evidence for the action of a PD-DNA glycosylase in that system. Where free thymine is not detected, a positive control might be provided by introduction of the T4 PD-DNA glycosylase (16, 17) or even the *denV* gene itself into the cells in question. A negative result might also be meaningfully established by incubating dimer-containing

oligonucleotides in vitro with a known PD-DNA glycosylase activity, thereby demonstrating the enzyme-dependent generation of photolabile free thymine. Efforts to establish the incubation conditions for enzyme-catalyzed hydrolysis of *N*-glycosylic bonds associated with thymine dimers in small oligonucleotides are in progress in our laboratory.

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