denV Gene of Bacteriophage T4 Codes for Both Pyrimidine Dimer-DNA Glycosylase and Apyrimidinic Endonuclease Activities

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Recent studies have shown that purified preparations of phage T4 UV DNA-incising activity (T4 UV endonuclease or endonuclease V of phage T4) contain a pyrimidine dimer-DNA glycosylase activity that catalyzes hydrolysis of the 5' glycosyl bond of dimerized pyrimidines in UV-irradiated DNA. Such enzyme preparations have also been shown to catalyze the hydrolysis of phosphodiester bonds in UV-irradiated DNA at a neutral pH, presumably reflecting the action of an apurinic/apyrimidinic endonuclease at the apyrimidinic sites created by the pyrimidine dimer-DNA glycosylase. In this study we found that preparations of T4 UV DNA-incising activity contained apurinic/apyrimidinic endonuclease activity that nicked depurinated form I simian virus 40 DNA. Apurinic/apyrimidinic endonuclease activity was also found in extracts of *Escherichia coli* infected with T4 *denV*⁺ phage. Extracts of cells infected with T4 *denV* mutants contained significantly lower levels of apurinic/apyrimidinic endonuclease activity; these levels were no greater than the levels present in extracts of uninfected cells. Furthermore, the addition of DNA containing apurinic or apyrimidinic sites to reactions containing UV-irradiated DNA and T4 enzyme resulted in competition for pyrimidine dimer-DNA glycosylase activity against the UV-irradiated DNA. On the basis of these results, we concluded that apurinic/apyrimidinic endonuclease activity is encoded by the *denV* gene of phage T4, the same gene that codes for pyrimidine dimer-DNA glycosylase activity.

Bacteriophage T4 codes for functions that protect it from the lethal effects of UV radiation (23). Mutants defective in the *denV* gene (41) (formerly called the *v* gene [15]) are abnormally sensitive to UV radiation (15). Friedberg and King (10) demonstrated that when UV-irradiated DNA was incubated with extracts of T4 *denV*⁺-infected *Escherichia coli* and was sedimented in alkaline sucrose gradients, the average size of the DNA was reduced significantly compared with UV-irradiated DNA incubated with extracts of T4 *denV*⁻-infected cells or with unirradiated DNA incubated with either type of extract. These results led to the conclusion that the *denV* gene of phage T4 codes for a function(s) required for the incision of UV-irradiated DNA during pyrimidine dimer (PD) excision. Since this work an enzyme activity with an *M_r* of approximately 18,000 has been purified in a number of laboratories from T4 *denV*⁺-infected cells (4, 11, 32, 38, 43, 44), and in one study this activity was purified to apparent homogeneity (11). This activity has been shown to catalyze

nickings of UV-irradiated DNA but not unirradiated DNA under neutral conditions (6, 12, 25), creating phosphodiester bond breaks 5' with respect to PD in DNA (12, 43). This activity has been referred to as T4 UV endonuclease (11, 24) and endonuclease V of phage T4 (32, 43, 44).

Recent studies have shown that, like the so-called "corendonucleases" of *Micrococcus luteus* (14, 16), highly purified preparations of T4 UV endonuclease contain a PD-DNA glycosylase activity that catalyzes hydrolysis of the 5' glycosyl bonds of thymine-containing PD in DNA (4, 13, 16, 28, 33; E. H. Radany, J. D. Love, and E. C. Friedberg, in E. Seeberg and K. Kleppe, ed., *Chromosome Damage and Repair*, in press) in addition to the activity responsible for the hydrolysis of phosphodiester bonds at PD sites. Excision of PD in vivo after infection of UV-irradiated host cells by phage T4 *denV*⁺ is also mediated by PD-DNA glycosylase activity (E. H. Radany and E. C. Friedberg, submitted for publication). Therefore, it is reasonable to postulate that the endonuclease activity is not

an activity that directly catalyzes hydrolysis of phosphodiester bonds in the immediate vicinity of PD, but rather is an activity that incises the apyrimidinic sites created by the action of the PD-DNA glycosylase. Thus, we were interested in determining whether there is an apurinic/apyrimidinic (AP) endonuclease activity encoded by the *denV* gene that can act in concert with the PD-DNA glycosylase activity. If not, can the host- or phage-encoded AP endonuclease activity (or activities) responsible for incision of phosphodiester bonds at the apyrimidinic sites generated by the PD-DNA glycosylase be identified? In this study we examined these questions and concluded that both PD-DNA glycosylase and AP endonuclease activities are functions of the *denV* gene product of phage T4.

(Preliminary presentations of some of the results have been made [H. J. Edenberg, J. Supramol. Struct. 5(Suppl.):170, 1981; S. L. H. McMillan, E. H. Radany, and E. C. Friedberg, Fed. Proc. 40:1763, 1981].)

MATERIALS AND METHODS

Bacterial and phage strains. *E. coli* AB 1157 (*xth*⁺ *endI*⁺) and W3110 are maintained in the laboratory of E.C.F. Strains BW 9101 (*xth*⁻) and BW 9062 (*xth*⁻ *endI*⁻) were generously provided by B. Weiss, Johns Hopkins University, Baltimore, Md. Phages T4D (*denV*⁺) and T4v₁ (*denV*⁻) are maintained in the laboratory of E.C.F. The T4 temperature-sensitive mutants F431 and F794 were originally isolated by Sato and Sekiguchi (31) and were obtained from Pat Seawell, Stanford University, Stanford, Calif. Phages T4uvsx and T4uvsy (UV-sensitive mutant phages with no defect in PD excision [5]) were a gift from S. S. Wallace, New York Medical College, Valhalla, N.Y., and phages T4 rII H23 (*denB*), *nd28* (*denA*), and T4 Sa Δ9 (*denB*) *nd28* (*denA*) (defective in phage T4 endonucleases A and B [41]) were obtained from Elizabeth Kutter, Evergreen State College, Olympia, Wash.

Bacteria were routinely cultured in modified YT medium (5 g of yeast extract per liter, 10 g of tryptone [Difco Laboratories, Detroit, Mich.] per liter) containing 10 g of NaCl per liter. All phages were plaque purified, and the UV sensitivity or resistance of each was confirmed by the plaque assay described below before use. PM2 phage and its host *Pseudomonas* BAL 31 were obtained from S. Linn, University of California, Berkeley.

Cells and virus. CV-1 cells were obtained from M. De Pamphilis (Harvard University, Cambridge, Mass.) and were grown at 37°C in 150-mm plastic culture dishes (Lux Plastics) in minimal essential medium (autoclavable; type F-17; GIBCO Laboratories, Grand Island, N.Y.) supplemented with 2.0 mM glutamine and 10% fetal bovine serum (Flow Laboratories, Inc., Rockville, Md.). Stocks of simian virus 40 (SV40) were grown at a low multiplicity of infection (0.01 to 0.04 PFU/cell) from a stock originally obtained from P. Tegtmeyer (State University of New York, Stony-

brook). Confluent cells were infected with SV40 at an input multiplicity of approximately 2 PFU/cell and were kept in medium containing 2% fetal bovine serum.

Preparation of DNA substrates: native and apurinic form I PM2 DNA. Exponentially growing strain BAL 31 cells in 0.8% nutrient broth–2.6% NaCl–0.07% KCl at 28°C ($\sim 1.5 \times 10^8$ cells per ml) were infected with PM2 phage at a multiplicity of infection of approximately 10. After 7 to 10 min, deoxyadenosine (final concentration, 0.1 mg/ml) and [*methyl*-¹⁴C]thymidine (50 to 60 mCi/mmol; final concentration, 0.5 to 1.0 μ Ci/ml; New England Nuclear Corp., Boston, Mass., or Amersham Corp., Arlington Heights, Ill.) were added. Incubation was continued at 28°C for an additional 4 h. After cell lysis was complete, cell debris was sedimented by centrifugation at 7,000 rpm for 60 min, and polyethylene glycol 6000 (J. T. Baker Chemical Co., Phillipsburg, N.J.) and NaCl were added to the supernatant to final concentrations of 10% and 0.5 M, respectively. Phage were precipitated out of the suspension by keeping the lysate at 4°C overnight. The precipitate was harvested by centrifugation at 7,000 rpm for 60 min in a Sorvall GS3 rotor at 4°C. Then the precipitate was suspended in 1.0 M NaCl–20 mM Tris-hydrochloride (pH 7.5) and layered onto a discontinuous gradient containing 7.5 ml of 2.8 M cesium chloride (Harshaw Chemical Co.), 7.5 ml of 2.0 M cesium chloride, and 7.5 ml of 20% sucrose in 10.0 mM Tris-hydrochloride (pH 8.0)–20 mM EDTA. The gradient was centrifuged in a Beckman SW27 rotor for 2.5 h at 25,000 rpm and 20°C. Purified phage was recovered from the 2.0 M cesium chloride–2.8 M cesium chloride interface of the discontinuous gradient and dialyzed extensively against 10.0 mM Tris-hydrochloride (pH 7.5)–1.0 mM EDTA. Sodium dodecyl sulfate was added to a final concentration of 0.2%, and the preparation was extracted twice with equal volumes of buffered phenol (pH 6.5), twice with chloroform-isoamyl alcohol (20:1, vol/vol), and twice with ether. The DNA was then dialyzed against 10.0 mM Tris-hydrochloride (pH 7.5)–1.0 mM EDTA at 4°C. More than 95% of the DNA was in the form I configuration, as determined by gel electrophoresis (see below).

Depurination of form I PM2 DNA was performed as follows. DNA (75 to 120 μ g/ml) was dialyzed against 10.0 mM sodium acetate (pH 5.5)–100 mM NaCl for 4 h. The DNA was then incubated at 60°C for 60 min before being transferred to an ice bath. The solution was neutralized by adding 1/20 volume of 1.0 M Tris-hydrochloride (pH 7.5) and 1/100 volume of 100 mM EDTA. This protocol resulted in the production of an average of one alkali-labile (apurinic) site per PM2 DNA molecule, as determined by sedimentation of the DNA in alkaline sucrose gradients (data not shown).

SV40 and salmon sperm DNA substrates. ¹⁴C-labeled covalently closed circular SV40 form I DNA was extracted by the method of Hirt (17) from infected cells labeled between 22 and 70 h postinfection with 0.1 μ Ci of [¹⁴C]thymidine per ml. The DNA was extracted three times with chloroform-isoamyl alcohol and purified by ethidium bromide-cesium chloride centrifugation under the conditions described in the legend to Fig. 1. The ethidium bromide was extracted

with isoamyl alcohol, and the DNA was passed through Dowex AG50W-X8 (Bio-Rad Laboratories, Richmond, Calif.) and dialyzed against 10.0 mM Tris-hydrochloride (pH 8.0)–1.0 mM EDTA–10.0 mM NaCl (TEN buffer). Samples were irradiated with UV light from a germicidal lamp (General Electric Co., Schenectady, N.Y.) for 2 s at 5.4 W/m² (measured with an International Light IL770 germicidal radiometer) or were partially depurinated by the method of Shaper and Grossman (35), chilled on ice, and neutralized to pH 8.0 with Tris base. Salmon testis DNA (Sigma Chemical Co., St. Louis, Mo.) was purified by repeated chloroform-isoamyl alcohol (24:1, vol/vol) extractions, ethanol precipitated, and suspended in TEN buffer. Samples were depurinated in parallel with the SV40 DNA.

***E. coli* DNA.** [³H]thymine-labeled *E. coli* DNA was prepared by the method of Thomas et al. (36). This DNA had a specific activity of 1.5×10^4 cpm/μg, and UV irradiation of this DNA was performed by using a low-pressure germicidal lamp.

Depyrimidinated phage PBS2 DNA. DNA from bacteriophage PBS2 was prepared as described by Friedberg et al. (8), using a [¹⁴C]uracil radiolabel. Depyrimidinated sites were created by limited incubation of the DNA (70 μg/ml) with 500 U of uracil-DNA glycosylase from *Bacillus subtilis* (3) per ml in TEN buffer at 37°C for 20 min. This incubation released 15% of the labeled uracil from the DNA, thus creating a substrate in which the concentration of apyrimidinic sites was 1/20 the concentration of total nucleotide. The depyrimidination reaction was terminated by vigorous extraction of the DNA with chloroform and then with ether. The ether was removed by evaporation under a gentle airstream.

³H-labeled poly(dT)₂₀₀•poly(dA)₈₀₀₋₁₀₀₀. ³H-labeled polydeoxythymidylic acid₂₀₀ [poly(dT)₂₀₀] was synthesized with terminal deoxynucleotidyl transferase as described by Bollum et al. (1), using [³H]dTTP (52.8 Ci/mmol; ICN) and unlabeled dTTP at a molar ratio of 1:20. The polymer product had a specific radioactivity of 1.3×10^3 cpm/pmol. ³H-labeled poly(dT)₂₀₀ was annealed to polydeoxyadenylic acid₈₀₀₋₁₀₀₀ [poly(dA)₈₀₀₋₁₀₀₀; Collaborative Research, Inc., Waltham, Mass.] at a nucleotide ratio of 1:1 by heating the two compounds in TEN buffer at 60°C for 5 min and then slowly cooling the mixture. The copolymer was UV irradiated with a fluence of 600 J/m² at 254 nm. This fluence converted 6% of the [³H]thymine to thymine-thymine dimers, as measured by the procedure of Reynolds et al. (29).

Preparation of extracts of T4-infected cells. All extracts were prepared by using *E. coli* BW 9062 as the host, except for infections with phages T4*uvsx* and T4*uvsy*, for which *E. coli* W3110 (*sup*⁻) was the host. Extracts were prepared by using the following two methods.

(i) **Method 1.** Phage were added to 100-ml portions of exponentially growing bacteria in medium containing 5 g of yeast extract per liter, 10.0 g of tryptone (Difco) per liter, and 10.0 g of NaCl per liter at a multiplicity of infection of 5 to 10. Incubation was at 37°C for 15 min before chloramphenicol was added to a final concentration of 150 μg/ml. Cultures were cooled rapidly in a dry ice-ethanol bath and were then

centrifuged at 5,000 rpm for 20 min in a Sorvall GSA rotor at 4°C. Cell pellets were washed in 10% sucrose–1.0 mM EDTA–50 mM Tris-hydrochloride (pH 8.0) and were resuspended in 5.0 ml of the same buffer. Lysozyme (Worthington Diagnostics, Freehold, N.J.) was added to a final concentration of 0.5 mg/ml, and the mixture was incubated at 0°C for 60 min and then at 37°C for 6 min. After centrifugation in a Beckman 50Ti rotor for 90 min at 45,000 rpm and 3°C, the supernatant was collected and used as a crude extract.

(ii) **Method 2.** Portions of exponentially growing bacteria (800 ml) were infected, harvested, and washed as described above. The cells were suspended in 5.0 ml of 5.0 M NaCl–50.0 mM Tris-hydrochloride (pH 7.5) and subjected to pulsed sonication for a total of 2 min with a model 350 Sonifier (Branson Instruments Co., Stamford, Conn.). The lysates were clarified by high-speed centrifugation as described above. Solid polyethylene glycol 6000 and dextran T500 (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) were added successively to 5.0-ml portions of the supernatants at 4°C to final concentrations of 6 and 4%, respectively, and the preparations were stirred in the cold for 4 to 5 h. Each solution was centrifuged at 7,000 rpm for 30 min at 4°C in a Sorvall type SS34 rotor. The upper (polyethylene glycol) phase was recovered and dialyzed extensively against 6% polyethylene glycol–10.0 mM Tris-hydrochloride (pH 7.5)–1.0 mM EDTA at 4°C.

Preparation of T4 UV DNA-incising activity. T4 UV DNA-incising activity (T4 UV endonuclease or endonuclease V of phage T4) was prepared from frozen T4 *amN82*-infected *E. coli* cells (New England Bio Labs, Beverly, Mass.) by the method of Friedberg et al. (9). The phosphocellulose fraction was used in all experiments. The enzyme preparation used for experiments with SV40 DNA contained 110 filter-binding units/μl. (One filter-binding unit was defined as the amount of enzyme required to bind 1 fmol of SV40 DNA containing an average of 16 pyrimidine dimers per molecule to nitrocellulose filters when the method of Seawell et al. [32] was used.) The enzyme preparation used for the competition experiments with UV-irradiated poly(dT)•poly(dA) was purified through phosphocellulose as a PD-DNA glycosylase activity by using the photoreversal-dependent release of free thymine as an assay, as described below. The phosphocellulose fraction contained 8.5 U of DNA glycosylase activity per μg of protein. (One unit of T4 PD-DNA glycosylase activity hydrolyzes 1.0 pmol of glycosyl bonds in thymine-containing PD in UV-irradiated *E. coli* DNA per min at 37°C.)

Assay of PD-DNA glycosylase activity. PD-DNA glycosylase activity was assayed by a modification of the procedure originally described by Radany and Friedberg (28). This procedure measured the release of free thymine from radiolabeled DNA with thymine-containing PD after monomerization of the dimers by photoreversal. The release of free thymine was measured by thin-layer chromatography as previously described (28) or by the soluble radioactivity remaining after co-precipitation of reaction mixtures by sequential additions of equal volumes of 0.2 M zinc acetate and 0.2 M sodium carbonate (T. Bonura and E. C. Friedberg, unpublished data). This procedure

effectively precipitated phosphorylated species as small as mononucleotides (E. W. Radany, Jr., personal communication). The two methods yielded quantitatively identical results.

Measurement of PD-DNA glycosylase activity in extracts of T4-infected and uninfected cells. Each 50- μ l incubation mixture contained 7.0 μ g of *E. coli* DNA (approximately 10^5 cpm; either unirradiated or irradiated with 5 kJ/m²), 10.0 mM EDTA, 100 mM NaCl, 10.0 mM Tris-hydrochloride (pH 8.0), and 400 μ g of extract protein per ml. Routinely, these preparations were incubated at 37°C for 30 min, except when extracts of temperature-sensitive mutants were used. For these, incubation mixtures without DNA were established in duplicate. One tube of each extract was preincubated at 30°C for 2 min, and the other was preincubated at 43°C for 2 min. UV-irradiated DNA was then added, and all further incubations were at 30°C for 16 min. Reactions were terminated by adding sodium dodecyl sulfate and proteinase K (Boehringer Mannheim Corp., New York, N.Y.) to final concentrations of 0.5% and 100 μ g/ml, respectively. The tubes were incubated at 60°C for 80 to 85 min and were extracted with equal volumes of phenol, chloroform-isoamyl alcohol (20:1, vol/vol), and ether. The volume of each sample was adjusted to 50 μ l with 10.0 mM EDTA-100 mM NaCl-10.0 mM Tris-hydrochloride (pH 8.0). PD-DNA glycosylase activity was measured by the photoreversal-dependent release of free thymine, using thin-layer chromatography as described previously (28).

Assay of AP endonuclease activity in extracts of T4-infected and uninfected *E. coli*. Preparations contained 2 volumes of apurinic PM2 DNA prepared as described above, 1 volume of a mixture consisting of 10.0 mM Tris-hydrochloride (pH 7.5), 1.0 mM EDTA, and 0.4 mM dithiothreitol, and 1 volume of extract diluted to varying protein concentrations in 10.0 mM Tris-hydrochloride (pH 7.5)-1.0 mM EDTA. These preparations were incubated at 37°C for varying times for the standard reaction, except when extracts of temperature-sensitive mutants were used. The conditions for specific experiments are described below. Control incubation mixtures containing saturating amounts of *E. coli* endonuclease IV (21) were included in some experiments. This enzyme (a gift from T. Lindahl, Imperial Cancer Research Fund, London, England) was used to determine the maximum extent of nicking attainable at AP sites in DNA.

Reactions were terminated by transferring 20 μ l of the reaction mixture to an ice bath, followed by immediate addition of 20 μ l of ice-cold chloroform-isoamyl alcohol (20:1, vol/vol) with vigorous mixing. Samples were centrifuged in an Eppendorf model 5412 microcentrifuge for 2 min. The upper (aqueous) phase was recovered, and 5.0 μ l of electrophoresis marker dye (0.1% bromophenol blue, 0.1% xylene cyanol, 50% glycerol) was added. Electrophoretic separation of form I DNA and form II DNA was performed in neutral 0.8% agarose gels by using a model H1 horizontal gel electrophoresis system (Bethesda Research Laboratories, Bethesda, Md.). The electrophoresis buffer contained 50 mM Tris-hydrochloride (pH 8.0), 20 mM sodium acetate, 18 mM NaCl, and 2.0 mM EDTA. Electrophoresis was performed overnight at

room temperature; a total current of 30.0 mA was used.

After electrophoresis, DNA bands were visualized by staining the gels for 30 to 60 min in a solution containing 1.0 μ g of ethidium bromide per ml and exposing them to UV light. The gels were sliced into fractions incorporating individual bands; each fraction was solubilized by autoclaving for 5 min in 1.0 ml of 0.1 N HCl, and the radioactivity in each fraction was measured by liquid scintillation spectrometry.

Measurement of alkali-labile sites and phosphodiester bond hydrolysis in UV-irradiated SV40 DNA. Preparations (40 to 110 μ l) contained 0.1 M NaCl, 0.01 M EDTA, 0.01 M Tris-hydrochloride (pH 8.0), 1.6% ethylene glycol, 0.1 mg of bovine serum albumin per ml, varying amounts of untreated, UV-irradiated, or depurinated form I SV40 DNA, in some cases competing native or depurinated salmon testis DNA, and T4 UV DNA-incising activity (see below). These preparations were incubated at 37°C for 15 min. The reactions were stopped by transferring the tubes to an ice water bath, and samples were withdrawn immediately. These samples were either adjusted to 0.2 M NaOH and heated for 15 min at 50°C before analysis on alkaline sucrose gradients (for measurement of alkali-labile sites), added to premixed cesium chloride-ethidium bromide gradients, or adjusted to 0.2% sodium dodecyl sulfate-10% sucrose for analysis by 1.4% agarose gel electrophoresis (to measure phosphodiester bond hydrolysis). Agarose gels were run in 40 mM Tris base-20 mM sodium acetate-1.0 mM EDTA adjusted to pH 7.9 by adding acetic acid. Slices containing form I DNA, slices containing form II DNA, and the regions between these slices were transferred to glass scintillation vials, solubilized, and counted for radioactivity, as described above.

Measurement of phage survival after UV irradiation. T4 phage at a concentration of approximately 10^7 PFU/ml were suspended in 30 ml of phosphate-buffered saline in a 10.0-cm petri dish and exposed to UV irradiation at an incident fluence of 0.5 to 0.8 J/m² per s. After each successive dose increment, a 50- μ l sample was withdrawn and transferred to 5.0 ml of phosphate-buffered saline. The resulting suspension was diluted serially, and the titer of each dilution was determined on exponentially growing bacteria by standard plaque assay methods.

Protein determinations. Protein was measured by the method of Bradford (2).

RESULTS

T4 UV endonuclease contains AP endonuclease activity. In agreement with the results of numerous previous studies (6, 9, 11, 12, 24, 28, 32, 33, 43, 44), preparations of purified T4 UV endonuclease preferentially incised UV-irradiated DNA but not unirradiated DNA (Table 1). In addition, we observed that the purified T4 enzyme incised form I SV40 DNA containing apurinic sites produced by heat and acid treatment (Fig. 1 and Table 1), demonstrating the presence of an AP endonuclease activity.

TABLE 1. Incubation of T4 UV endonuclease with native, UV-irradiated, and apurinic SV40 DNAs^a

DNA	Amt (μg)	Amt of enzyme (filter-binding units)	No. of nicks per SV40 DNA molecule as measured by:		
			Alkaline sedi- mentation	Neutral sedi- mentation	Neutral gel elec- trophoresis
Untreated	10, 15	0	0.0	0.0	0.0
	10	110	0.01	0.02	
	15	1,100	0.02	0.02	0.03
UV-irradiated	10, 15	0	0.1	0.0	0.01
	10	110	2.14	1.97	
	15	1,100	2.00	1.91	2.01
Depurinated	10	0	0.33	0.01	0.03
	5	110	0.37	0.35	
	10	1,110	0.37	0.34	0.37

^a ¹⁴C-labeled SV40 form I DNA was either not treated, irradiated with 11 J of UV irradiation per m², or depurinated for 12 min as described in the text. The amounts of DNA and T4 UV endonuclease used are shown. After incubation for 15 min at 37°C, samples were withdrawn for analysis by either alkaline sedimentation (as described in the legend to Fig. 6), neutral sedimentation (as described in the legend to Fig. 1), or neutral gel electrophoresis (as described in the text). The average number of nicks per molecule was calculated from the fraction of molecules remaining unnicked, assuming a random (Poisson) distribution. The values shown are corrected for the small fraction of unirradiated, untreated DNA which was nicked before the start of the experiment (0.02 to 0.05 nicks per molecule in different experiments).

Exonuclease III of *E. coli* is not required for phage T4 DNA repair in vivo. A fortuitous co-purification of AP endonuclease(s) with T4 PD-DNA glycosylase is possible, as has been observed with purified *M. luteus* correndonucleases (16). Thus, studies with T4 UV DNA incising activity in vitro may not reflect the enzymology of PD excision by T4 *denV*⁺ phage in infected cells. The quantitatively major AP endonuclease activity of uninfected *E. coli* cells is associated with exonuclease III (37, 39). *xth*⁻ mutants (40), which are defective in this enzyme activity (39, 42), possess only 10% of the total AP endonuclease activity present in extracts of *xth*⁺ cells (22). To determine whether the AP endonuclease function of exonuclease III is required for the excision of PD from phage T4 in vivo, we compared the UV sensitivities of T4 *denV*⁺ phage in *xth*⁺ and *xth*⁻ hosts. Figure 2 shows that the UV sensitivity of the phage was indistinguishable in a wild-type strain and in four different *xth*⁻ strains, including mutants containing complete deletions of the *xth* gene. These results indicated that *E. coli* exonuclease III is not essential for the excision of PD from T4 DNA in vivo and suggested that some other specific AP endonuclease(s) is required instead.

Extracts of T4 *denV*⁺-infected *E. coli* cells contain more AP endonuclease activity than extracts of T4 *denV*⁻-infected cells. A strain of *E. coli* defective in both exonuclease III and endonuclease I activities was infected with either phage T4 *denV*⁺ or T4 *denV*⁻. The cultures were harvested, and extracts freed of most

of the nucleic acids were prepared as described above and adjusted to identical protein concentrations. The extracts were assayed for PD-DNA glycosylase activity, and, as expected, this activity was detected in the extracts of the T4 *denV*⁺-infected cells but not in the extracts of the T4 *denV*⁻-infected cells (data not shown). These extracts were incubated with form I PM2 DNA containing an average (by Poisson distribution) of one apurinic site per molecule under conditions of limiting enzyme activity (Fig. 3). The kinetics of degradation of form I apurinic PM2 DNA were significantly faster with extracts of T4 *denV*⁺-infected cultures than with equal amounts (as protein) of extracts of T4 *denV*⁻-infected cells. The level of AP endonuclease activity in extracts of T4 *denV*⁻-infected cells was no greater than the level in extracts of uninfected *E. coli* cells (Fig. 4). In all of our experiments the substrate concentrations used were well in excess of the apparent *K_m* of each extract for apurinic DNA (data not shown); thus, the differences in AP endonuclease activity shown in Fig. 3 and 4 could not be attributed to differences in affinity for substrate. Incubation of either extract with native PM2 DNA under the conditions described above showed no detectable loss of the form I species (data not shown).

Mutants of phage T4 temperature sensitive with respect to UV endonuclease activity have been isolated by Sato and Sekiguchi (31). Extracts of *E. coli xth*⁻ *endI*⁻ cells were prepared by the sonication-phase partitioning procedure,

FIG. 1. T4 UV endonuclease catalyzed hydrolysis of phosphodiester bonds in depurinated DNA. ^{14}C -labeled SV40 DNA was depurinated for 12 min at pH 5.2 and 70°C . Samples were incubated for 15 min at 37°C with (solid line) and without (dashed line) T4 UV endonuclease. Each incubation mixture (110 μl) contained 10.0 μg of ^{14}C -labeled SV40 DNA and 1,190 binding units of the T4 enzyme preparation in a solution containing 0.1 M NaCl, 0.01 M EDTA, 0.01 M Tris-hydrochloride (pH 8.0), 1.6% ethylene glycol, and 0.1 mg of bovine serum albumin per ml. Immediately after incubation 40 μl of the reaction mixture was diluted into a premixed cesium chloride solution (5.7 g of CsCl, 6.0 ml of TEN buffer) containing 380 μg of ethidium bromide per ml, centrifuged to equilibrium (42 h, 38,000 rpm, 20°C , Beckman 50Ti rotor), and fractionated. Carrier DNA was added to each fraction before acid precipitation with 1.0 M HCl and collection of the DNA on glass fiber filters. Radioactivity was determined in a scintillation solution containing 5.0 g of 2,5-diphenyloxazole per liter and 0.125 g of dimethyl-1,4-bis-(5-phenyloxazolyl)benzene per liter in toluene with a Beckman model LS-8000 liquid scintillation spectrometer. Density increased from right to left. The peak at the left contained form I (un nicked) DNA molecules. Depurination alone did not introduce nicks (dashed line); nicking after incubation with the enzyme demonstrated an AP endonuclease activity (solid line).

after infection with wild-type phage or with one of two temperature-sensitive mutant phages. Table 2 shows that the PD-DNA glycosylase activities in the extracts of the cells infected with the mutants were clearly temperature sensitive. Figure 4 shows the kinetics of degradation of apurinic PM2 DNA by these extracts, as well as by extracts of uninfected *E. coli* cells. At both the permissive and restrictive temperatures, the extracts of cells infected with wild-type phage catalyzed more rapid degradation of the DNA than did the extracts of cells infected with the temperature-sensitive mutants. A subsaturating concentration of an extract of *denV*⁺-infected cells was also assayed, and this experiment confirmed that the AP endonuclease activity in this extract was not temperature sensitive (data not shown). Extracts of cells infected with the two temperature-sensitive mutants showed levels of AP endonuclease activity indistinguishable from the levels in extracts of uninfected cells at either

30 or 43°C , although all three cell extracts showed greater lability at the higher temperature. Even at 20°C no increase in the level of AP endonuclease activity was observed in the extracts of cells infected with the temperature-sensitive mutants compared with extracts of uninfected cells (data not shown).

Results identical to those obtained with extracts of T4 *denV*⁺-infected cells were obtained with extracts of *E. coli* cells infected with T4 phages containing mutations in genes *x*, *y*, *denA*, and *denB* (data not shown). Thus, the observations with extracts of cells infected with *denV* mutants could not be attributed to a nonspecific result of random mutations affecting UV sensitivity or endonuclease production by phage T4; rather, the results presented above suggested that the *denV* gene of phage T4 codes for both PD-DNA glycosylase and AP endonuclease activities. The two temperature-sensitive mutants tested had a thermolabile PD-DNA glycosylase activity, but within the limits of sensitivity of our experiments, there was no detectable phage-

FIG. 2. Survival of UV-irradiated phage T4D in various bacterial indicator strains. Plaque-purified phage were irradiated, and survival values were measured as described in the text. The following host cells were used: AB1157 (*xth*⁺) (●), BW2021 (*xth*[−]) (○), BW2001 (*xth*[−]) (Δ), NH5016 (*xth*[−]) (▲), and BW9101 (*xth*[−]) (□).

FIG. 3. Kinetics of degradation of form I depurinated PM2 DNA by extracts of *E. coli* infected with T4 *denV*⁺ (□) or T4 *denV*⁻ (○) bacteriophage. The preparation of substrate and extracts (lysozyme procedure) is described in the text. Extracts were diluted in 10.0 mM Tris-hydrochloride (pH 7.5)–1.0 mM EDTA to a protein concentration of 10.0 µg/ml (exclusive of lysozyme). Each incubation mixture (0.14 ml) contained 8.4 µg of depurinated PM2 DNA, 30.0 mM Tris-hydrochloride (pH 7.5), 1.0 mM EDTA, 5.0 mM sodium acetate, 50.0 mM NaCl, 0.1 mM dithiothreitol, and 1.4 µg of extract. These mixtures were incubated at 37°C. At the indicated times 20-µl sam-

plified AP endonuclease activity at any temperature examined.

DNA containing apurinic or apyrimidinic sites competes for the PD-DNA glycosylase function of the T4 UV DNA-incising activity. If the PD-DNA glycosylase and AP endonuclease activities of the T4 enzyme are both functions of a single polypeptide, we would expect that the DNA glycosylase activity against UV-irradiated DNA containing PD would be competed for by DNA containing apurinic or apyrimidinic sites. Two sets of competition experiments were performed, in which either apu-

ples were removed from each incubation mixture and added to 20-µl amounts of ice-cold chloroform-isoamyl alcohol (20:1, vol/vol). A control sample containing no extract was incubated for 60 min at 37°C. Samples were mixed vigorously and centrifuged at low speed, and the upper (aqueous) phases were recovered for analysis of the DNA by gel electrophoresis as described in the text. The ordinate represents the percentage of form I PM2 DNA molecules remaining after incubation with extract, normalized to the percentage of form I molecules present in the incubation mixture without added extract. Non-depurinated PM2 DNA incubated with either extract was not degraded under the incubation conditions used.

FIG. 4. Kinetics of degradation of form I depurinated PM2 DNA by extracts from uninfected *E. coli* cells (● and ○) or from cells infected with phage T4 *denV*⁺ (■ and □), phage T4 *denV*⁻ ts431 (▲ and △), or phage T4 *denV*⁻ ts794 (▼ and ▽). Extracts were prepared by the sonication-phase partitioning method and were diluted in 10.0 mM Tris-hydrochloride (pH 7.5)–1.0 mM EDTA to 60 µg of protein per ml. Duplicate incubation mixtures (60 µl) each contained 10.0 mM Tris-hydrochloride (pH 7.5), 1.0 mM EDTA, 0.2 mM dithiothreitol, and 1.8 µg of extract. One set of incubation mixtures was preincubated at 30°C for 2 min (open symbols), and the other was preincubated at 43°C for 2 min (closed symbols). Depurinated PM2 DNA (4.5 µg in 60 µl of TEN buffer) was then added to each reaction mixture, and the preparations were incubated at 30°C for the times indicated. The reactions were terminated, and DNA was analyzed as described in the legend to Fig. 3.

rinic or apyrimidinic DNA sites were used as competitors. Table 3 shows the results of competition experiments in which depyrimidinated

TABLE 2. PD-DNA glycosylase activity in T4 temperature-sensitive mutants^a

Source of extract	Photoreversal-dependent radioactivity as free thymine (% of radioactivity at origin of chromatogram after preincubation at:		Activity at 43°C (% of activity at 30°C)
	30°C	43°C	
T4 <i>denV</i> ⁺ -infected <i>E. coli</i>	2.69	1.91	71.0
T4 <i>ts431</i> -infected <i>E. coli</i>	1.15	0.18	15.7
T4 <i>ts794</i> -infected <i>E. coli</i>	0.57	0.07	12.3
Uninfected <i>E. coli</i>	0		

^a Extracts of uninfected cells and cells infected with phage were prepared by the sonication-phase partitioning procedure and were assayed for PD-DNA glycosylase activity as described in the text. Preparations contained 400 µg of protein per ml (final concentration).

DNA was used. In these experiments competition for T4 PD-DNA glycosylase activity against UV-irradiated poly(dT)·poly(dA) was measured by assaying the enzyme for its ability to hydrolyze specifically glycosyl bonds in thymine-containing PD in DNA. Affected dimers (and no other sites in the DNA) are the sources of free thymine after monomerization by UV radiation (direct photoreversal) (28; Radany et al., in press). We chose as the substrate a copolymer of very high specific radioactivity so that measurable amounts of photolabile thymine would be generated in reactions that were a convenient volume and contained UV-irradiated DNA with a concentration of PD ($\sim 10^{-8}$ M) somewhat greater than the measured K_m of the T4 PD-DNA glycosylase for PD in DNA ($\sim 10^{-9}$ M; E. H. Radany and E. C. Friedberg, unpublished data). Under such conditions of relatively high PD concentration, competition should be relatively insensitive to weak interactions between enzyme and competing DNA. Table 3 shows that the protein molecule containing PD-DNA glycosylase activity interacted with apyrimidinic sites with an affinity comparable to its interaction with PD. More specifically, our data indicated that the K_m of the enzyme for DNA con-

TABLE 3. Competition for T4 PD-DNA glycosylase activity against UV-irradiated ³H-labeled poly(dT)·poly(dA) by apyrimidinic DNA^a

Expt	Competing DNA	Concn (M) in reaction mixture of:		PD-DNA glycosylase activity (fmol of [³ H]thymine-containing PD hydrolyzed per min at 37°C)
		Specific competing site (AP or PD)	Total nucleotide in competing DNA	
1	None	0	0	51
	Native <i>E. coli</i> DNA	0	6.0×10^{-6}	55
	Apyrimidinic PBS2 DNA	3.0×10^{-8}	6.0×10^{-6}	23
	Apyrimidinic PBS2 DNA	10.0×10^{-8}	6.0×10^{-6}	17
	Apyrimidinic PBS2 DNA	30.0×10^{-8}	6.0×10^{-6}	4
2	None	0	0	97
	Native <i>E. coli</i> DNA	0	1.2×10^{-5}	97
	UV-irradiated <i>E. coli</i> DNA	1.0×10^{-8}	1.2×10^{-5}	57
	UV-irradiated <i>E. coli</i> DNA	3.0×10^{-8}	1.2×10^{-5}	35
	UV-irradiated <i>E. coli</i> DNA	10.0×10^{-8}	1.2×10^{-5}	6

^a Reaction mixtures (200 µl) contained 200 pmol of UV-irradiated ³H-labeled poly(dT)·poly(dA) (3 pmol as thymine dimers) in a solution consisting of 100 mM NaCl, 10.0 mM Tris-chloride (pH 8.0), 10 mM (sodium salt), EDTA, 100 µg of bovine serum albumin per ml, and either depyrimidinated ¹⁴C-labeled PBS2 DNA (experiment 1) or UV-irradiated ¹⁴C-labeled *E. coli* DNA (7% thymine-containing PD) (experiment 2) to give the indicated concentrations of competing AP sites and PD. Native *E. coli* DNA was added to give the total competing nucleotide concentrations indicated. T4 UV DNA-incising activity (2.5 µU [experiment 1] or 5 µU as PD-DNA glycosylase activity units [experiment 2]) was added to reaction mixtures prewarmed to 37°C. Incubations at this temperature were for 20 min and were terminated by adding 0.1% sodium dodecyl sulfate. Native DNA was then added to the uncompleted reactions to give the same absorbance at 254 nm as the competed reactions. The reactions were subjected to 6 kJ of photoreversing fluences per m². The released thymine was measured as soluble radioactivity by liquid scintillation spectrometry after co-precipitation of the substrate and competing DNAs with 50 µl each of 0.2 M Zn(C₂H₃O₂)₂ and 0.2 M Na₂CO₃. Blank reactions were run without enzyme for determinations of the background-soluble radioactivity, and corrections for ¹⁴C spillover during liquid scintillation spectrometry were made when necessary. Thymine release under these incubation conditions was linear with respect to both enzyme concentration and incubation time.

taining apyrimidinic sites was only about twice as great as the K_m for DNA containing PD, since PD-DNA glycosylase activity was reduced by approximately 50% when the concentration of competing apyrimidinic sites was twice the concentration of substrate PD sites. Such a strong interaction with DNA containing apyrimidinic sites was not likely to result from simple product inhibition of a PD-DNA glycosylase activity that was physically unassociated with an AP endonuclease activity. As predicted, under these experimental conditions, native DNA was a very weak competitor.

The results of a second set of competition experiments are shown in Table 4 and Fig. 5. In these experiments we investigated competition for both PD-DNA glycosylase activity (measured by the generation of alkali-labile apyrimidinic sites in UV-irradiated DNA) and AP endonuclease activity (measured by hydrolysis of phosphodiester bonds under neutral conditions). When UV-irradiated SV40 DNA was incubated with T4 enzyme in the presence of DNA containing apurinic sites, both the number of alkali-labile sites and the number of strand breaks created in the DNA were reduced (Table 4). Table 4 also shows that native DNA did not compete significantly for activity that generated alkali-labile sites in UV-irradiated DNA (presumably PD-DNA glycosylase). However, such DNA did compete effectively for activity re-

quired for hydrolysis of phosphodiester bonds in UV-irradiated DNA (presumably AP endonuclease). The ability of native DNA to compete against UV-irradiated DNA containing apyrimidinic sites but not against UV-irradiated DNA with intact glycosyl bonds (Table 4) suggested that the glycosylic and endonucleolytic events stem from independent enzyme-substrate interactions. Support for this suggestion came from further experiments performed under conditions of limiting enzyme concentration, in which we found that incubation of UV-irradiated SV40 form I DNA with subsaturating levels of T4 enzyme resulted in a larger number of alkali-labile sites than phosphodiester bonds breaks (Fig. 6).

DISCUSSION

Bacteriophage T4 is apparently unique among all known bacterial viruses in that it codes for a gene product that is involved in the repair of UV damage to DNA. This product is encoded by the *denV* (DNA endonuclease V) gene (41) of phage T4. Mutants defective in this gene have the phenotype of abnormal UV sensitivity (15) and are defective in the excision of thymine-containing PD from their own DNA after infection of *E. coli* with UV-irradiated phage (27).

The *denV* gene product has been extensively purified and characterized in a number of laboratories (6, 9, 11, 12, 24, 32, 43, 44). These studies

TABLE 4. Competition for T4 UV endonuclease activity against UV-irradiated DNA by apurinic DNA^a

Expt	Competing DNA	Amt of enzyme (filter-binding units)	No. of nicks per SV40 DNA molecule as measured by:		
			Alkaline sedimentation	Neutral sedimentation	Neutral gel electrophoresis
1	None	22	1.74	1.16	
	Apurinic (9.6 μ g)	22	0.82	0.20	
	Apurinic (48.0 μ g)	22	0.28	0.07	
2	None	22	1.06		0.39
	Apurinic (30 μ g)	22	0.52		0.00
	Native (30 μ g)	22	1.04		0.03
	Apurinic (15 μ g) + native (15 μ g)	22	0.70		0.00
3	None	55	1.36	1.05	
	Apurinic (9.6 μ g)	55	1.05	0.27	
	Apurinic (72 μ g)	55	0.46	0.07	
	Native (50 μ g)	55	1.18	0.57	
	Apurinic (24 μ g) + native (25 μ g)	55	0.81	0.17	

^a Within each experiment all reactions contained equal amounts of ¹⁴C-labeled SV40 form I DNA irradiated with 11 J of UV irradiation per m² (5 μ g in experiment 1, 4 μ g in experiment 2, 10 μ g in experiment 3). Variable amounts of nonradioactive salmon testis DNA, either untreated or depurinated as described in the text for 55 min (experiments 1 and 3) or for 36 min (experiment 2), were added in the amounts shown before treatment for 15 min at 37°C with the amount of T4 UV endonuclease shown. Samples were withdrawn for determinations of the number of nicks introduced, as described in Table 1, footnote a. In all three experiments the amount of enzyme used was insufficient to nick all of the SV40 DNA (data not shown).

FIG. 5. PD-DNA glycosylase activity of T4 UV endonuclease was inhibited by added depurinated DNA. Incubation mixtures (75 μ l) contained 5 μ g of 14 C-labeled SV40 DNA irradiated with 11 J of UV irradiation per m^2 . Three reactions contained 22 binding units of enzyme, one without competing DNA (—), one with 9.6 μ g of depurinated salmon DNA (---), and one with 48 μ g of depurinated salmon DNA (.....). The fourth reaction contained neither enzyme nor competing DNA (-.-). After 15 min at 37°C, the reactions were terminated by adding 35 μ l of 0.4 M NaOH to 35 μ l of reaction mixture. The mixtures were incubated at 50°C for 15 min to complete β -elimination at alkali-labile sites and then centrifuged through alkaline sucrose gradients (5 to 20% [wt/vol] sucrose in 0.2 M NaOH–0.8 M NaCl–1.0 mM EDTA) at 49,000 rpm and 20°C for 90 min in a Beckman SW50.1 rotor. This treatment insured that all glycosylic cleavages (which created alkali-labile sites) were converted to scissions in the phosphodiester backbone, independent of any requirement for AP endonuclease. Thus, this procedure measured the total number of glycosylic cleavages made. Gradients were fractionated, and radioactivity was determined as described in the legend to Fig. 1. Sedimentation was from right to left; the peak at left represents form I (unnickd) DNA.

have indicated that this gene product is a protein having an M_r of 18,000 and no subunit structure. It catalyzes the production of single-strand breaks (nicks) in UV-irradiated DNA containing PD but not in unirradiated DNA or DNA containing a variety of other types of base damage. The nicks are located 5' with respect to the dimers and consist of 3' OH and 5' P termini. The enzyme-catalyzed nicking of UV-irradiated DNA occurs at a neutral pH and thus is not dependent on alkali-catalyzed β -elimination reactions at sites of base loss. The nicking of UV-irradiated DNA is not dependent on any known cofactors and occurs in the presence of EDTA. Based on these results, it was generally assumed that the *denV* gene product is an endonuclease that directly catalyzes the hydrolysis of phosphodiester bonds in the immediate vicinity of (but always 5' to) PD; hence, the terms T4 UV endonuclease (11, 24) and endonuclease V of phage T4 (32, 43, 44) have been used.

In the 1970s studies with *E. coli* (18) and *B. subtilis* (8) led to the discovery of a novel type of enzyme activity that catalyzes the release of free uracil from DNA containing that base by hydrolysis of the N-glycosylic bond linking the base to the deoxyribose-phosphate residue in polynucleotide chains. Lindahl et al. (20) designated this class of enzymes as DNA glycosylases, and since then a variety of other highly specific DNA glycosylases have been identified in extracts of procaryotic and eucaryotic cells (19). Recent studies by Grossman et al. (14) and Haseltine et al. (16) led to the demonstration that the so-called correndonucleases I and II of *M. luteus* contain a PD-DNA glycosylase activity that exclusively catalyzes the hydrolysis of the 5' glycosyl bond of dimerized pyrimidines in duplex UV-irradiated DNA.

FIG. 6. Treatment of UV-irradiated SV40 DNA with subsaturating levels of enzyme produced more alkali-labile sites than DNA strand breaks. [14 C]thymidine-labeled SV40 form I DNA was irradiated with 11 J of UV per m^2 (solid line) or was not irradiated (dashed line). Samples (8 μ g of DNA) were incubated for 15 min at 37°C with T4 enzyme (11 filter-binding units) in a total reaction mixture of 40 μ l. (A) One portion of each reaction mixture was adjusted to 0.2 M NaOH, incubated at 50°C, and sedimented in an alkaline sucrose gradient as described in the legend to Fig. 5. The average number of nicks per molecule was 1.57. (B) The other portion of each incubation mixture was diluted into premixed cesium chloride gradients and sedimented as described in the legend to Fig. 1. The average number of nicks per molecule was 0.63. The excess of nicks detected under alkaline conditions compared with those detected under neutral conditions showed that not all glycosylic cleavages which resulted in alkali-sensitive sites were followed by endonucleolytic scissions.

Radany and Friedberg (28) and Radany et al. (in press) studied the T4 UV endonuclease and demonstrated the presence of PD-DNA glycosylase activity by the photoreversal-dependent release of free thymine from UV-irradiated DNA preincubated with T4 enzyme. This phenomenon was not observed when UV-irradiated DNA was incubated with extracts of cells infected with phage T4 v_1 , a mutant defective in the *denV* gene (28). Thus, the *denV* gene codes for a PD-DNA glycosylase activity, a conclusion supported by independent studies in a number of other laboratories (4, 13, 33; Edenberg, J. Supramol. Struct. 5[Suppl.]:170, 1981). Coupled with the previously mentioned demonstration of phosphodiester hydrolase activity against UV-irradiated DNA, this information leads to the obvious hypothesis that the associated endonuclease is not a direct-acting endonuclease that recognizes PD in UV-irradiated DNA as a substrate, but is instead an AP endonuclease that recognizes apyrimidinic sites created by the action of the PD-DNA glycosylase. This hypothesis is supported by the direct demonstration in our study of endonuclease activity against DNA containing apurinic sites, confirming the observations of Seawell et al. (33).

The primary issue which we addressed in this study was the origin of this AP endonuclease activity. Extracts of uninfected *E. coli* cells contain a number of AP endonuclease activities (E. C. Friedberg, T. Bonura, E. H. Radany, and J. D. Love, in P. D. Boyer, ed., *The Enzymes*, vol. 14, in press), any one of which could be specifically or nonspecifically associated with the T4 PD-DNA glycosylase activity to constitute T4 UV DNA-incising activity. Alternatively, the AP endonuclease could be a phage T4 product encoded either by the *denV* gene or by some other gene. The results described below provide evidence that both the PD-DNA glycosylase and AP endonuclease activities are products of the *denV* gene of phage T4.

Extracts of T4 *denV*⁺-infected cells contain considerably more AP endonuclease activity than extracts of cells infected with either a constitutive mutant or one of two temperature-sensitive T4 mutants defective in the *denV* gene. Furthermore, the levels of residual AP endonuclease in the extracts of cells infected with T4 *denV* mutants are not greater than those in extracts of uninfected *E. coli* *xth*⁻ cells. Thus, there is no evidence from these experiments that T4 genes other than the *denV* gene code for AP endonuclease activity.

When UV-irradiated SV40 DNA and UV-irradiated ³H-labeled poly(dT)·poly(dA) were incubated with T4 enzyme in the presence of DNAs containing apurinic and apyrimidinic

sites, respectively, there was extensive competition by these DNAs for PD-DNA glycosylase activity. This competition was specific, since it was barely observed with native DNA. Furthermore, apyrimidinic sites competed for PD-DNA glycosylase activity nearly as strongly as PD themselves. These results are consistent with an enzyme-substrate interaction between the protein containing PD-DNA glycosylase activity and the apyrimidinic sites in DNA and indicate a physical association between PD-DNA glycosylase and AP endonuclease activities.

The conclusion that both PD-DNA glycosylase and AP endonuclease activities are functional domains of a single protein is consistent with our previous observation that an apparently homogeneous preparation of T4 UV endonuclease incises UV-irradiated DNA (11) and with our more recent observation that extensive purification of the T4 PD-DNA glycosylase activity is not associated with a detectable loss of phosphodiesterase activity (T. Bonura and E. C. Friedberg, unpublished data). A more systematic demonstration of the co-purification of these two activities is presented in the accompanying paper by Warner et al. (38). This conclusion is also consistent with the observation that phage T4 is not abnormally sensitive to UV radiation after infection of strains of *E. coli* defective in the major AP endonuclease (exonuclease III). It is of interest in this regard that both temperature-sensitive phage mutants examined in this study are apparently totally defective in AP endonuclease activity in vitro, whereas they retain significant levels of PD-DNA glycosylase activity at permissive temperatures. The temperature-sensitive T4 mutant *ts* F431 is not abnormally sensitive to UV radiation in either *xth*⁺ or *xth*⁻ hosts (data not shown), suggesting that some other AP endonuclease(s) can subsume this role in the excision repair of PD. The isolation of mutants of *E. coli* in which temperature-sensitive mutants do demonstrate increased UV sensitivity at the permissive temperature may help identify such AP endonucleases.

Figure 6 shows that during incubation of UV-irradiated DNA with T4 UV DNA-incising activity, alkali-labile sites are generated more rapidly than phosphodiester bond incisions. These results suggest that the PD-DNA glycosylase can dissociate from the alkali-labile (apyrimidinic) sites that it produces before AP endonuclease action at these sites. Indeed, the observation (Table 4) that AP endonuclease activity is competed for more strongly by native DNA than the PD-DNA glycosylase activity is, indicates that the PD-DNA glycosylase does dissociate from the intermediate alkali-labile sites

before AP endonuclease action in vitro. This conclusion has surprising implications for the mechanism of excision repair of PD in vivo. Uninfected *E. coli* cells contain a number of AP endonucleases that are not associated with known DNA glycosylases (19; Friedberg et al., in press). Therefore, one might predict that if the T4 enzyme functions by a two-step mechanism that involves separate enzyme-substrate encounters, these host AP endonucleases would be sufficient for incision of the apyrimidinic sites created by the PD-DNA glycosylase. We propose that the AP endonuclease encoded by the *denV* gene of phage T4 may have unique properties distinct from those of any of the host AP endonucleases. An obvious distinction could be that the T4 AP endonuclease has a particular affinity for the apyrimidinic sites specifically created by the PD-DNA glycosylase. This question is being investigated with purified enzyme in vitro.

The demonstration that the T4 UV DNA-incising activity contains a PD-DNA glycosylase provides a rational explanation for the previously described substrate specificity of this enzyme. Unlike the DNA-incising activity encoded by the *uvrA*, *uvrB*, and *uvrC* genes of *E. coli* (34), which apparently recognizes a wide range of DNA damage, including that caused by UV irradiation, mitomycin C, psoralen plus near-UV irradiation, and 4-nitroquinoline-1-oxide (26), the T4 enzyme is apparently specific for PD in DNA. Studies in other biological systems have indicated that both yeast (30) and human (45) cell lines that are defective in PD excision are sensitive not only to UV irradiation, but also to many other forms of base damage (7; R. H. Haynes and B. A. Kunz, in J. Strathern, J. Broach, and E. W. Jones, ed., *The Molecular Biology of the Yeast Saccharomyces*, in press). Thus, the T4 enzyme, and perhaps also the *M. luteus* enzyme, are probably not examples of a prototypic incising activity for damaged DNA.

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