Mechanism of Action of a Mammalian DNA Repair Endonuclease†

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ABSTRACT: The mechanism of action of a DNA repair endonuclease isolated from calf thymus was determined. The calf thymus endonuclease possesses a substrate specificity nearly identical with that of *Escherichia coli* endonuclease III following DNA damage by high doses of UV light, osmium tetroxide, and other oxidizing agents. The calf thymus enzyme incises damaged DNA at sites of pyrimidines. A cytosine photoproduct was found to be the primary monobasic UV adduct. The calf thymus endonuclease and *E. coli* endonuclease III were found to possess similar, but not identical, DNA incision mechanisms. The mechanism of action of the calf thymus endonuclease was deduced by analysis of the 3′ and 5′ termini of the enzyme-generated DNA scission products with DNA sequencing methodologies and HPLC analysis of the material released by the enzyme following DNA damage. The calf thymus endonuclease removes UV light and osmium tetroxide damaged bases via an *N*-glycosylase activity followed by a 3′ apurinic/apyrimidinic (AP) endonuclease activity. The calf thymus endonuclease also possesses a novel 5′ AP endonuclease activity not possessed by endonuclease III. The product of this three-step mechanism is a nucleoside-free site flanked by 3′- and 5′-terminal phosphate groups. These results indicate the conservation of both substrate specificity and mechanism of action in the enzymatic removal of oxidative base damage between prokaryotes and eukaryotes. We propose the name redoxy endonucleases for this group of enzymes.

Base damage to cellular DNA occurs following exposure to ultraviolet light, ionizing radiation, certain environmental chemicals, and reactive oxygen species. If left uncorrected, DNA damage can result in cell death, mutation, and neoplastic transformation. Prokaryotes and eukaryotes have evolved systems for reversing DNA damage, and several classes of DNA repair enzymes have been identified (Friedberg & Bridges, 1983).

One group of prokaryotic enzymes removes modified bases from DNA in a two-step mechanism. These steps consist of an *N*-glycosylase activity that incises the bond between the damaged base and corresponding deoxyribose and an apurinic/apyrimidinic (AP) endonuclease activity that cleaves the phosphodiester bond on the 3′ side of the base-free sugar. The *Micrococcus luteus* and T4 endonucleases that incise UV-irradiated DNA at sites of cyclobutane dimers and *Escherichia coli* endonuclease III, an enzyme that recognizes a broad spectrum of base modifications, are both examples of this type of enzyme (Haseltine et al., 1980; Gordon & Haseltine, 1980; Demple & Linn, 1981).

Although no mammalian enzymes similar to the pyrimidine dimer specific endonucleases have been described, several enzymes that are broadly similar to *E. coli* endonuclease III have been identified. Such enzymes are relatively small in size, are active in the absence of divalent cations, and have been found in human (Brent, 1983; Breimer, 1983), bovine (Bachetti & Benne, 1975; Helland et al., 1986), and rodent cells (van-Lanker & Tomura, 1974; Nes, 1980). These enzymes recognize DNA damage induced by oxidizing agents, ionizing radiation, and high doses of UV light. No previous studies have provided a detailed characterization of these enzymes with respect to their broad substrate specificities and mechanisms of action.

We have determined the substrate specificity of a calf thymus endonuclease and have found it to be nearly identical with that of *E. coli* endonuclease III. The calf thymus endonuclease is similar to a calf thymus enzyme described by Bachetti and Benne (1975) and to activities present in human cells described by Breimer (1983) and Brent (1983). Both the calf thymus endonuclease and *E. coli* endonuclease III incise DNA at sites of damaged pyrimidines following exposure to high doses of UV light, osmium tetroxide, and other oxidizing agents. These findings suggest that the mechanisms of action of the calf thymus endonuclease and *E. coli* endonuclease may be similar. We investigated the mechanism of action of the calf thymus endonuclease by analysis of the 3′ and 5′ termini of the DNA scission products and by analysis of the modified bases released by the calf thymus and *E. coli* enzymes using DNA sequencing and HPLC methodologies. End-labeled DNA fragments of defined sequence and [^{3}H]-poly(deoxyribonucleotides) damaged with UV light or osmium tetroxide were utilized as enzyme substrates. The results reported here demonstrate that the calf thymus endonuclease and *E. coli* endonuclease III recognize and remove the same types of pyrimidine base damage via similar mechanisms.

Materials and Methods

Enzymes and Chemicals. Restriction enzymes and DNA polymerase I (Klenow fragment) were purchased from New England Biolabs and Bethesda Research Laboratories. *Micrococcus luteus* UV-specific endonuclease [endodeoxyribonuclease (pyrimidine dimer), EC 3.1.25.1] was prepared as described (Riazuddin & Grossman, 1977). T4 polynucleotide kinase and terminal deoxynucleotide transferase were purchased from Bethesda Research Laboratories. Calf alkaline

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phosphatase was from Boehring-Mannheim. Deoxyribodipyrimidine photolyase (EC 4.1.99.3) was provided by Dr. Aziz Sancar, University of North Carolina. *E. coli* endonuclease III was a gift of Dr. Richard Cunningham, SUNY, Albany, NY. [γ-^{32}P]ATP (sp act. 3000 Ci/mmol) and [α-^{32}P]dNTPs (sp act. 3000 Ci/mmol) were purchased from either New England Nuclear or Amersham.

Isolation of Calf Thymus Endonuclease. The purification of the calf thymus endonuclease used in this study will be described in detail elsewhere (Helland et al., 1986). Homogenized, sonicated tissue was subjected to high-speed centrifugation, and the 100000*g* supernatant was fractionated by chromatography on DEAE-cellulose, phosphocellulose, Sephacryl S-200, Sephadex G-75, and sulphopropyl-cellulose columns, respectively. This procedure resulted in a 400-fold purification of the enzyme that was free from nonspecific endonuclease activities (Helland et al., 1986). Gel filtration chromatography on Sephadex G-75 (superfine) indicated that the size of the calf thymus endonuclease was approximately 30000 daltons. The activity of the enzyme was monitored following each purification step by the ability to nick UV-irradiated, supercoiled DNA (Helland et al., 1986). This enzyme preparation possesses the same general properties as that reported by Bachetti and Benne (1975).

Preparation of Defined-Sequence DNA Damage Substrates. A series of 3′ restriction fragments were prepared from M13 mp8 RF (Messing & Viera, 1982) and pUC18 (Yanisch-Perron et al., 1985) for use as DNA damage substrates. Restriction fragments were 3′ end labeled (Chan et al., 1985) or 5′ end labeled (Doetsch et al., 1985) and isolated on preparative polyacrylamide gels as described (Doetsch et al., 1985). The DNA damage substrates derived from pUC18 were as follows: 211-bp[1] *Sal*I, *Pvu*II negative strand (3′ end labeled fragment B); 112-bp *Sal*I, *Pvu*II positive strand (3′ end labeled fragment C); 113-bp *Sal*I, *Pvu*II negative strand (5′ end labeled fragment E); 211-bp *Sal*II, *Pvu*II positive strand (5′ end labeled fragment F). The DNA damage substrates derived from M13 mp8 RF were 123-bp *Hin*dIII, *Bst*N1 positive strand (3′ end labeled fragment A), and 48-bp *Hin*dIII, *Bst*N1 negative strand (3′ end labeled fragment D). End-labeled DNA damage substrates were suspended in 10 mM Tris-HCl, pH 7.5, and 1 mM EDTA (TE buffer).

Preparation of [^{3}H]Poly(deoxyribonucleotides). Poly(dA-[^{3}H]dT) was synthesized as described (Cunningham & Weiss, 1985) in a reaction mixture (100 μL) containing 50 mM Tris-HCl, pH 7.5, 10 mM $Mg(OAc)_2$, 2 mM 2-mercaptoethanol, 0.01 mM $d(A-T)_n$, 0.55 mM dATP, 0.5 mM [*methyl*-^{3}H]dTTP (Amersham, 43 Ci/mmol), and 10 units of DNA polymerase I (Klenow fragment), incubated 8 h, 37 °C. NaOAc was added to 0.3 M, extracted 3 times with 2 volumes of phenol–chloroform–isoamyl alcohol (20:19:1 v/v/v), ethanol-precipitated, resuspended in 200 μL of TE buffer, applied to a Sephadex G-50 column (1 cm × 20 cm), and eluted with 10 mM Tris, pH 7.5, 1 mM EDTA, and 0.2 M NaCl (TEN buffer). Fractions containing poly(dA-[^{3}H]dT) were pooled, ethanol-precipitated, and resuspended in TE buffer.

Poly(dG-[^{3}H]dC) was synthesized in a reaction mixture (100 μL) containing 0.2 M potassium cacodylate, 20 mM Tris-HCl, pH 6.9, 2 mM dithiothreitol, 0.5 mM $CoCl_2$, 1.7 mM $d(pA)_3$ primer, 1.0 mM dGTP, 1.0 mM dCTP, 25 μM [5-^{3}H]dCTP (New England Nuclear, 26.3 Ci/mmol), 500 μg/mL BSA, and 38 units of terminal deoxynucleotide transferase, incubated 7 h, 37 °C (Maniatis et al., 1982). The reaction mixture was applied to a Sephadex G-50 column (1 cm × 20 cm) and eluted with TEN buffer. Fractions containing poly(dG-[^{3}H]dC) were pooled, ethanol-precipitated, and resuspended in TE buffer.

Calf Thymus Endonuclease Strand Scission Assay. The 3′ end labeled or 5′ end labeled DNA substrates were incubated with 1 unit of calf thymus endonuclease in 100 mM KCl, 10 mM Tris, pH 8.0, 10 mM 2-mercaptoethanol, and 10 mM EDTA (40 μL final volume), 30 min, 37 °C. One unit of calf thymus endonuclease is defined as the amount of enzyme necessary to produce one nick per genome with 0.1 μg of ^{3}HϕX174 in 30 min, 37 °C (Helland et al., 1986). Following incubation, DNA samples were extracted 3 times with phenol–chloroform–isoamyl alcohol (20:19:1 v/v/v), ethanol-precipitated, and subjected to gel electrophoresis.

E. coli Endonuclease III Strand Scission Assay. End-labeled DNA substrates were incubated with an excess (2000 units) of *E. coli* endonuclease III (Cunningham & Weiss, 1985) under the same conditions used for calf thymus endonuclease incubations. The same procedures were also followed for calf thymus endonuclease and *E. coli* endonuclease III digestions of osmium tetroxide damaged poly(dA-[^{3}H]dT) and UV-damaged poly(dG-[^{3}H]dC) with the exception that λ DNA (5 μg) was added as carrier and the DNA was ethanol-precipitated in the absence of 0.3 M NaOAc. The ethanol supernatants were the source of enzyme-released material in the HPLC experiments.

M. luteus Pyrimidine Dimer Specific Endonuclease Assay. UV-irradiated DNA substrates were treated with the *M. luteus* pyrimidine dimer specific endonuclease (37 °C, 30 min) for the detection of cyclobutane dimers as previously described (Haseltine et al., 1980). Following incubation, the DNA samples were processed as described for calf thymus endonuclease prior to electrophoresis.

Photoreactivation of Cyclobutane Dimers. UV-irradiated DNA substrates were incubated with 4000 units (saturating amounts) of *E. coli* photoreactivating enzyme (photolyase) to remove cyclobutane dimers as previously described (Sancar & Sancar, 1984). Samples were processed as above prior to electrophoresis.

5′ and 3′ Phosphatase Digestions. DNA scission products generated by enzyme or chemical treatments were ethanol-precipitated, resuspended in 50 μL of TE buffer, and digested with 0.05 unit of calf alkaline phosphatase, 6 h, 37 °C, as described (Gordon & Haseltine, 1981). DNA scission products were digested with the 3′ phosphatase associated with T4 polynucleotide kinase (Cameron & Uhlenbeck, 1977; Kleppe & Lillehaug, 1979) in reaction mixtures (20 μL) containing 25 mM MES, pH 6.0, 8 mM $MgCl_2$, 5 mM 2-mercaptoethanol, and 15 mM NH_4OAc, processed, and ethanol-precipitated as described for the other enzyme digestions.

Hot Alkali Treatment of UV-Damaged and Apurinic DNA. Damaged DNA substrates were treated with 1 M piperidine, 90 °C, 30 min, for cleavage of DNA at sites of UV-induced (6-4) photoproducts (Lippke et al., 1981) and at apurinic sites (Maxam & Gilbert, 1980).

UV Light and Osmium Tetroxide Damage of End-Labeled DNA Substrates and [^{3}H]Poly(deoxyribonucleotides). End-labeled DNA substrates and poly(deoxyribonucleotides) (5-μL droplets, on ice) were UV-irradiated (254 nm) with a dose of 10000 J/m^2 from two GE 15T8 germicidal lamps. DNA substrates were subjected to enzyme or chemical

[1] Abbreviations: bp, base pair; MES, 2-(*N*-morpholino)ethanesulfonic acid; (6-4) photoproducts, 6–4′-(pyrimidin-2′-one)–pyrimidine class of ultraviolet light induced photoproducts; Tris-HCl, tris(hydroxymethyl)-aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

treatments immediately following irradiation.

End-labeled DNA substrates and poly(deoxyribonucleotides) were exposed to osmium tetroxide (700 μg/mL) and incubated at 70 °C, 90 min, in a volume of 100 μL (Nes, 1980). The reaction was terminated by extraction 3 times with 200 μL ether to remove unreacted osmium tetroxide. DNA samples were ethanol-precipitated and resuspended in 10–50 μL of TE buffer.

Preparation of Apurinic DNA Substrates. End-labeled DNA substrates containing apurinic sites were prepared as described (Lindhal & Nyberg, 1972) in a reaction mixture (100 μL) containing 0.1 M NaOAc, pH 5.1, incubated 30 min, 70 °C, and recovered by ethanol precipitation and resuspended in TE buffer.

HPLC Analysis of Enzyme-Released Modified Bases. The supernatants from ethanol precipitation of calf thymus endonuclease or *E. coli* endonuclease III digests were lyophilized, resuspended in 30 μL of water, and analyzed on a C_{18} reverse-phase column (Altex ODS). The enzyme-released, modified bases were eluted from the column with 10 mM KH_2PO_4 in 1% methanol, flow rate 1 mL/min. The radioactivity contained in each fraction was assayed by scintillation counting, and a background of 26 cpm was subtracted from each sample. Fraction volumes of 0.5 and 0.3 mL were assayed for osmium tetroxide damaged poly(dA-[^{3}H]dT) and UV-damaged poly(dG-[^{3}H]dC), respectively. The elution of base standards from the column was determined by monitoring absorbance at 220 nm.

DNA Sequencing Reactions and Gel Electrophoresis. The purine (G+A) and pyrimidine (CT) base specific DNA sequencing reactions (Maxam & Gilbert, 1980) were run alongside each set of samples analyzed on sequencing gels. Reprecipitated DNA samples from enzyme digestions and chemical treatments were loaded onto denaturing, 20% polyacrylamide–7 M urea gels and subjected to electrophoresis and autoradiography as described (Doetsch et al., 1985).

RESULTS

The purification of the calf thymus endonuclease is described in detail elsewhere (Helland et al., 1986). It is likely that this enzyme is similar to that initially described by Bachetti and Benne (1975) and later by Breimer (1983). In addition, it is similar to several other DNA repair enzymes isolated from human lymphoblasts, rat liver, and mouse plasmacytoma cells (Brent, 1983; van Lanker, 1974; Nes, 1980). The enzyme preparation used contains no contaminating endonuclease activities on undamaged DNA substrates. It is free of both 3′ and 5′ exonuclease and phosphatase activities (Helland et al., 1986). The calf thymus endonuclease possesses no divalent cation requirement, has a broad pH optimum, and requires reducing agents for maximum activity.

Recognition of Ultraviolet Light Base Damage by Calf Thymus Endonuclease. Initial studies with the calf thymus enzyme suggested that the UV photoproduct recognized was not a cyclobutane pyrimidine dimer, as the UV light dose required to produce a single endonuclease cleavage site per φX174 supercoil produces 30 dimers (Helland et al., 1986). To determine the sites of nucleotide incision by the calf thymus endonuclease, 3′ end labeled, double-stranded DNA fragments of defined sequence were irradiated with high doses (10 000 J/m^2) of UV light, incubated with the enzyme, and analyzed on DNA sequencing gels. Comparison of the enzyme-generated DNA cleavage fragments with the base-specific DNA sequencing fragments revealed the nucleotide location and extent of UV damage specific calf thymus endonuclease activity (Figure 1A). Incubation of 3′ end labeled, UV-irradiated DNA with the calf thymus endonuclease produced a pattern of DNA fragments that differed markedly from the patterns produced from *M. luteus* pyrimidine dimer specific endonuclease and hot alkali treatments (Figure 1A, lanes 3–5). *M. luteus* enzyme and hot alkali treatment of UV-irradiated DNA result in strand cleavage at sites of cyclobutane dimers and (6-4) photoproducts, respectively (Lippke et al., 1981). The calf thymus endonuclease incises UV-irradiated DNA primarily at sites of cytosine regardless of whether or not they are flanked on the 5′ or 3′ side with purine or pyrimidine bases. The enzyme also cleaves the DNA at some sites of isolated or adjacent thymine bases. Unirradiated DNA (Figure 1A, lane 2) and UV-damaged, single-stranded DNA (not shown) are not substrates for the enzyme. The calf thymus endonuclease produces DNA fragments that have the same electrophoretic mobility as those that correspond to cleavage at cytosine bases by the DNA sequencing chemical cleavage reactions.

The location and extent of incision by the calf thymus endonuclease suggests that the enzyme does not cleave UV-irradiated DNA at cyclobutane dimers or (6-4) photoproducts. This conclusion is supported by the finding that calf thymus endonuclease cleavage of UV-irradiated DNA is unaltered following the removal of cyclobutane dimers from the substrate DNA with *E. coli* photolyase. Figure 1B shows that photolyase treatment of UV-irradiated, 3′ end labeled DNA results in the removal of all *M. luteus* pyrimidine dimer endonuclease incision sites (lanes 4 and 5). In contrast, the UV-induced DNA damage recognized by the calf thymus endonuclease is unaffected by treatment with the photolyase (Figure 1B, lanes 1 and 2). It is unlikely that the calf thymus endonuclease cleaves DNA at sites of (6-4) photoproducts. The sites of endonuclease cleavage are different from the sites of (6-4) photoproduct formation as judged by hot alkali sensitive sites. Moreover, the calf thymus endonuclease cleaves UV-irradiated DNA at positions of isolated cytosines or thymines, sites at which (6-4) photoproducts do not occur. Additionally, the response for formation of the endonuclease-sensitive sites was not that anticipated for the (6-4) photoproducts. For these reasons, we conclude that the calf thymus endonuclease does not cleave DNA at sites of either cyclobutane pyrimidine dimers or (6-4) photoproducts. Furthermore, the calf thymus endonuclease does not cleave DNA damaged with *cis*-dichlorodiammineplatinum(II), psoralen, or *N*-acetoxy-2-(acetylamino)fluorene (not shown).

Similarity of Mammalian and Bacterial Repair Enzymes. Both the calf thymus endonuclease and *E. coli* endonuclease III cleave heavily UV irradiated DNA (Figure 1A; Helland et al., 1986; Radman, 1976; Gates, 1977). Figure 2A illustrates a side by side comparison of the cleavage products by these two enzymes on a heavily UV irradiated, 3′ end labeled DNA substrate. An identical set of cleavage products is produced by treatment of this substrate with either the calf thymus endonuclease or *E. coli* endonuclease III (Figure 2A, lanes 3 and 4). This result indicates that both enzymes recognize the same UV-induced cytosine and thymine base damage and that the cleavage events 3′ to the damaged base are the same. An identical experiment was performed with a UV-irradiated DNA substrate labeled at the 5′ terminus (Figure 2b). Comparison of the calf thymus endonuclease and *E. coli* endonuclease III cleavage products revealed that although there is a one to one correspondence of cleavage products for these two enzymes, they differ with respect to the nature of the 3′ terminus of the scission products. Such a difference is evidenced by the observed shift in the electro-

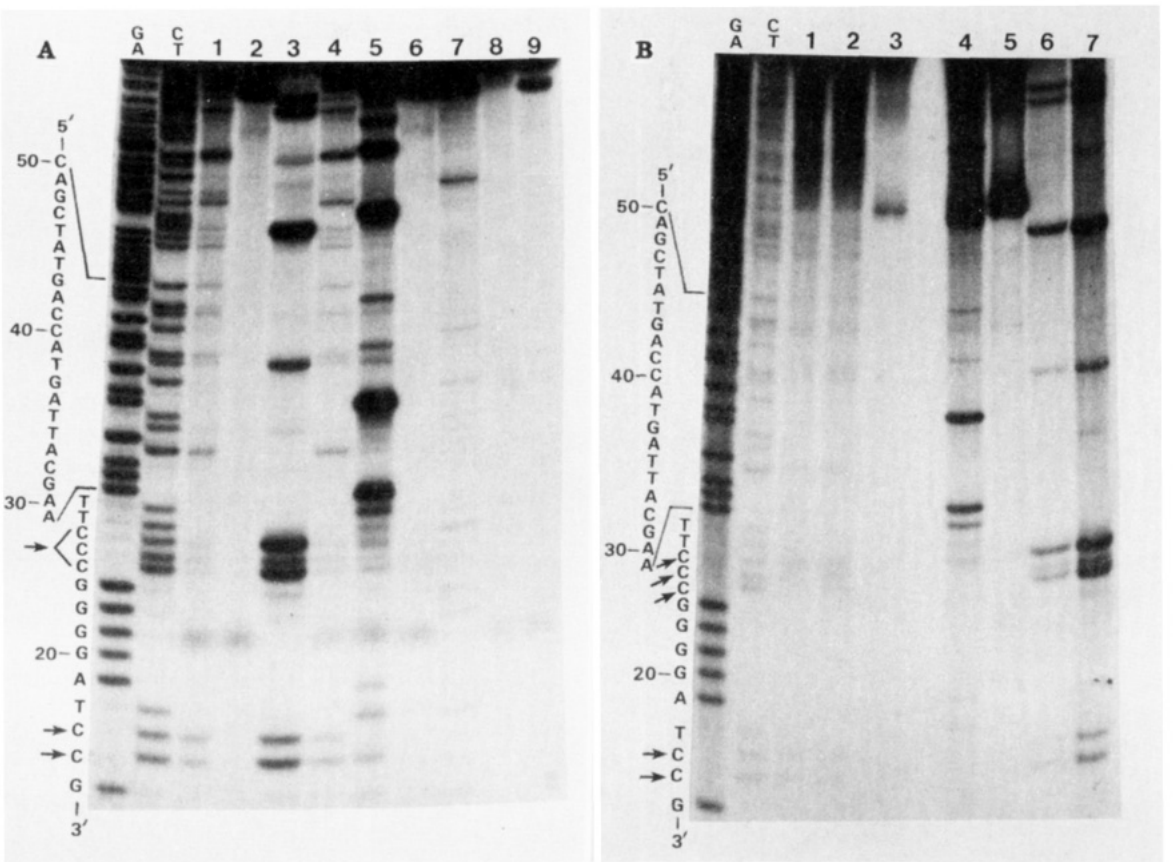


FIGURE 1: Base specificity of calf thymus endonuclease incision of UV-irradiated DNA. A 3′ end labeled restriction fragment A (Materials and Methods) was UV-irradiated with a dose of 10000 J/m^2. (A) Irradiated (lanes 1, 3, 4, 5, and 8) and unirradiated (lanes 2, 6, 7, and 9) DNA samples were treated with calf thymus endonuclease (lanes 1, 2, and 4), hot alkali (lanes 3 and 7), *M. luteus* pyrimidine dimer specific endonuclease (lanes 5 and 6), or no treatment (lanes 8 and 9). (B) Irradiated (lanes 1, 2, and 4–7) and unirradiated (lane 3) DNA samples were treated with DNA photolyase (lanes 2, 5, and 7) and/or calf thymus endonuclease (lanes 1–3), *M. luteus* pyrimidine dimer specific endonuclease (lanes 4 and 5), or hot alkali (lanes 6 and 7). The G+A- and C+T-specific DNA sequencing reactions were included in the far-left lanes. Arrows denote several of the cytosine and thymine sites of calf thymus endonuclease and *E. coli* endonuclease III DNA incision. Base numbering starts from the 3′ end labeled terminus. UV irradiation of DNA and analysis of treated samples on sequencing gels was as described under Materials and Methods.

phoretic mobilities of the mammalian and bacterial enzyme-generated scission products (Figure 2B, lanes 3 and 4). We conclude that cytosine photoproducts induced by high doses of UV light account for the major fraction of scission events that occur upon treatment with either of the two endonucleases.

What is the nature of the cytosine UV photoproduct? Candidates for such an adduct include derivatives of 6-hydroxy-5,6-dihydrocytosine (cytosine photohydrates) and, under conditions leading to deamination, uracil (Fisher & Johns, 1976; Kochetkov & Budovskii, 1972). Cytosine photohydrates were unstable, rapidly revert back to cytosine, and are not likely to be formed under our experimental conditions (Fisher & Johns, 1976; Kochetkov & Budovskii, 1972). The cytosine adduct recognized by the calf thymus and *E. coli* enzymes is stable. Enzyme incision of DNA held at room temperature remains unchanged 24 h following UV irradiation (not shown). Furthermore, HPLC analysis of the UV base adduct released by both enzymes indicates the photoproduct is neither uracil nor a uracil photoproduct (Figure 4). The predominance of cytosine as a target for these enzymes following UV irradiation also suggests the major adduct is not a thymine hydrate, which has been reported to occur in heavily UV irradiated DNA (Yamane et al., 1967). At present, we do not know the identity of the cytosine photoproduct. However, it is very likely that this novel cytosine photoproduct is the one routinely monitored on UV-damaged substrates during the purification of both the calf thymus endonuclease and *E. coli* endonuclease III (Helland et al., 1986; Radman, 1976; Gates & Linn, 1977).

N-Glycosylase Activity of the Calf Thymus Endonuclease. Previous studies indicate that the *E. coli* endonuclease III removes damaged bases from DNA via an *N*-glycosylase activity followed by a 3′ apurinic/apyrimidinic (AP) endonuclease activity (Demple & Linn, 1980). To determine whether or not the calf thymus endonuclease also removed damaged bases via an *N*-glycosylase activity, osmium tetroxide treated poly(dA-[^{3}H]dT) was incubated with either the calf thymus endonuclease or *E. coli* endonuclease III. The removal of damaged bases was monitored by HPLC analysis of the ethanol-soluble enzyme reaction products. *E. coli* endonuclease III released thymine glycol from the substrate DNA (Figure 3B). These results are in agreement with Demple and Linn (1980) and verify that endonuclease III removes thymine glycol from DNA via an *N*-glycosylase activity. Thymine glycol was also released from osmium tetroxide damaged DNA by the calf thymus endonuclease (Figure 3A). Two peaks of radioactivity flank the major thymine glycol peak in the HPLC elution profile. This material probably represents the isomers of thymine glycol (J. Cadet, personal communication). This observation demonstrates that the calf thymus endonuclease contains an *N*-glycosylase activity that recognizes thymine glycol in DNA. No products corresponding to release of the modified nucleoside thymidine glycol were detected in the DNA treated with either the calf thymus or *E. coli* enzyme.

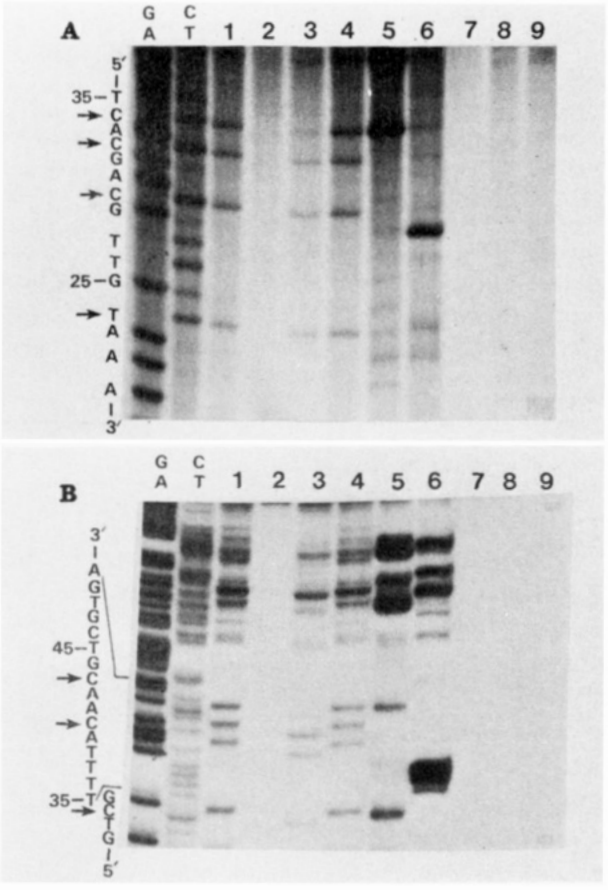


FIGURE 2: Comparison of calf thymus endonuclease and *E. coli* endonuclease III nucleotide incision on UV-damaged DNA. DNA substrates (panel A) 3′ end labeled restriction fragment D or (panel B) 5′ end labeled restriction fragment E were UV-irradiated with a dose of 10000 J/m^2 (lanes 1, and 3–6) or unirradiated (lanes 2, and 7–9) and teated with *E. coli* endonuclease III (lanes 1, 2, and 4), calf thymus endonuclease (lanes 3 and 7), hot alkali (lanes 5 and 8), or *M. luteus* pyrimidine dimer specific endonuclease (lanes 6 and 9). The *M. luteus* pyrimidine dimer specific endonuclease preparation used in these experiments contained a contaminating activity that cleaved some of the UV-irradiated substrates at nondimer sites. This activity appears to be directed against other types of UV photoproducts and is, at present, uncharacterized. Treated DNA samples were analyzed on DNA sequencing gels as described (Materials and Methods). Base numbering starts from the 3′ end labeled terminus (A) or the 5′ end labeled terminus (B).

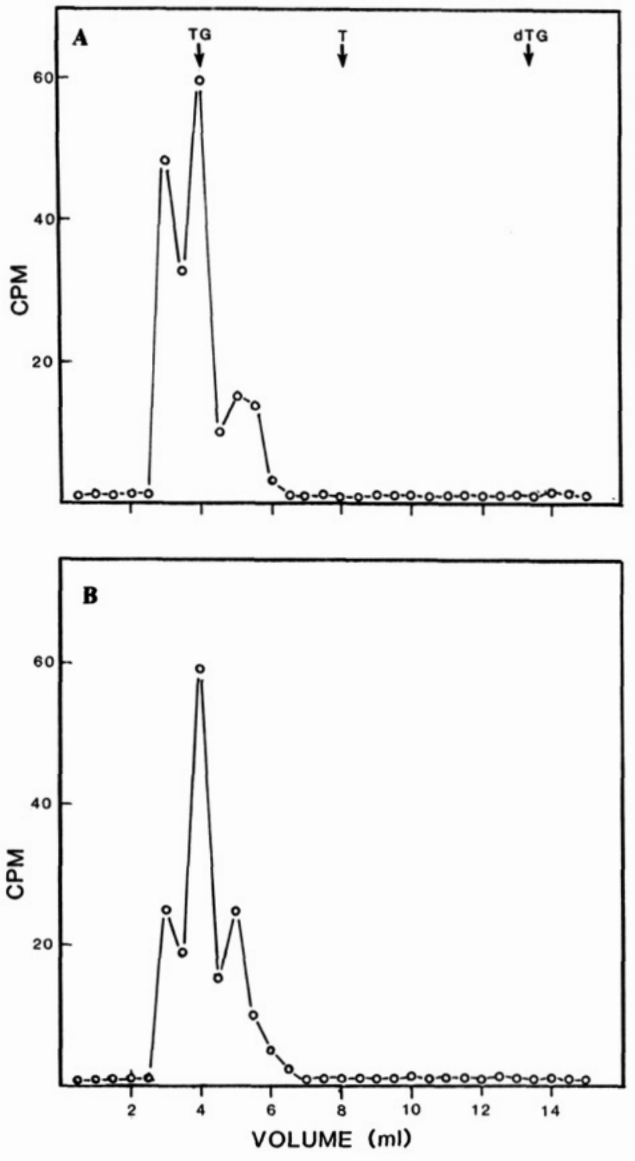


FIGURE 3: HPLC analysis of modified bases released from osmium tetroxide damaged DNA by calf thymus endonuclease and *E. coli* endonuclease III. Ethanol-soluble enzyme digestion products of osmium tetroxide damaged poly(dA-[^{3}H]dT) were subjected to HPLC analysis (C_{18} reverse-phase column). (A) Thymine glycol release by calf thymus endonuclease. (B) Thymine glycol release by *E. coli* endonuclease III. Arrows indicate the positions where authentic thymine glycol (TG), thymine (T), and thymidine glycol (dTG) are eluted from the column. Thymine glycol (26 cpm above background) was spontaneously released from DNA exposed to osmium tetroxide with no further treatment.

Do the calf thymus endonuclease and *E. coli* endonuclease III also contain *N*-glycosylase activities that recognize the cytosine UV photoproduct? To address this question, UV-irradiated poly(dG-[^{3}H]dC) was treated with either the calf thymus endonuclease or *E. coli* endonuclease III. The ethanol-soluble enzyme reaction products were analyzed by reverse-phase high-pressure liquid chromatography. Both enzymes remove the same photoproduct from DNA (Figure 4). Although the product has not been completely characterized, the position of elution from the reverse-phase column suggests that it is a modified cytosine base rather than a modified cytidine. Deoxycytidine, thymidine, and other modified pyrimidine nucleosides are eluted from the column at substantially later times (not shown).

Incision Mechanism 3′ to the Damaged Base. A series of experiments were performed to determine the mechanism of DNA strand scission by the calf thymus and *E. coli* enzymes. Experiments using 3′ end labeled DNA suggested that both the calf thymus endonuclease and *E. coli* endonuclease III cleave UV-irradiated DNA between the damaged base and the corresponding 3′-phosphoryl group (Figure 2A, lanes 3 and 4). Such an event produces a DNA fragment containing a phosphoryl group at the 5′ terminus and is identical with the corresponding Maxam and Gilbert base-specific sequencing fragment for the putative damaged pyrimidine (Maxam & Gilbert, 1980). The enzymatic removal of a 5′-terminal phosphate group from a DNA fragment results in a species that migrates more slowly in a DNA sequencing gel compared to the terminal phosphate-containing fragment (Gordon & Haseltine, 1981; Tapper & Clayton, 1981). Treatment of either calf thymus endonuclease or *E. coli* endonuclease III scission products with alkaline phosphatase produces DNA fragments with slower electrophoretic mobilities compared to the untreated scission products (Figure 5). These experiments demonstrate that the products of the calf thymus endonuclease and *E. coli* endonuclease III reactions contain 5′-terminal phosphate groups. We conclude that the calf thymus endo-

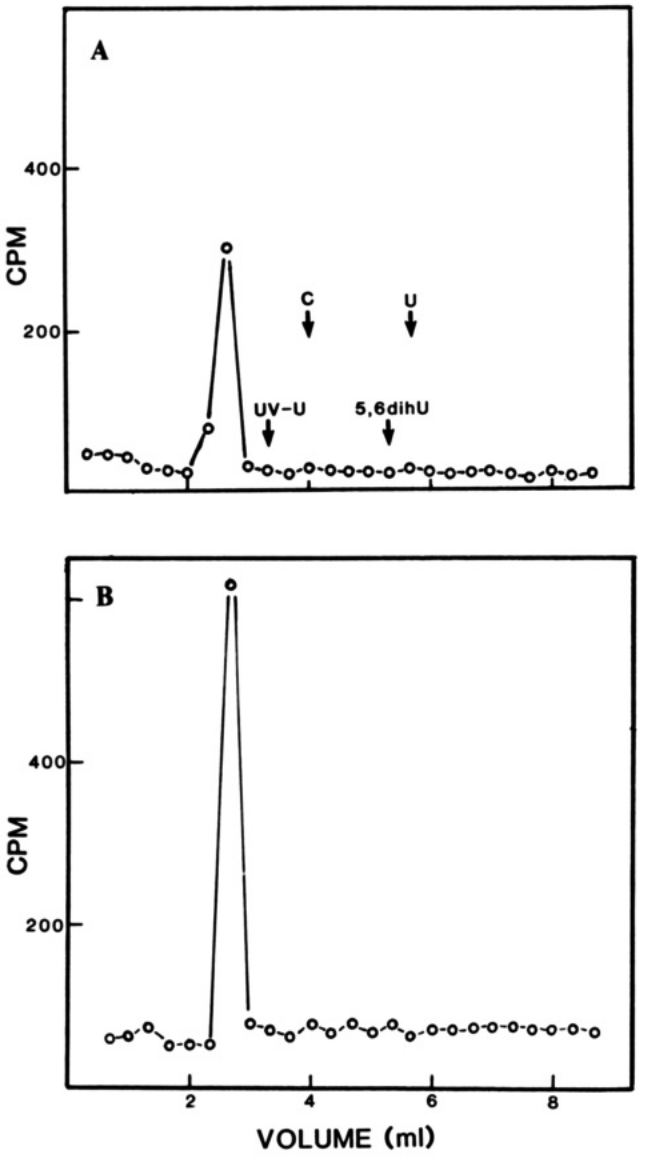


FIGURE 4: HPLC analysis of modified bases released from UV-damaged DNA by calf thymus endonuclease and *E. coli* endonuclease III. Ethanol-soluble enzyme digestion products of UV-irradiated (10 000 J/m^2) poly(dG-[^{3}H]dC) were subjected to HPLC analysis as described above. (A) Cytosine adduct release by calf thymus endonuclease or (B) *E. coli* endonuclease III. Arrows indicate the positions where authentic uracil (U), 5,6-dihydrouracil (5,6-dihU), cytosine (C), and a uracil photoproduct (UV-U) are eluted from the column.

nuclease, like the *E. coli* endonuclease III, cleaves the phosphodiester bond between the sugar and 3′-phosphate.

Incision Mechanism 5′ to the Damaged Base. To determine whether or not calf thymus endonuclease DNA cleavage events also occur 5′ to damaged bases, 5′ end labeled, UV-damaged DNA fragments were used as substrates. There is a one to one correspondence of cleavage products for the calf thymus and *E. coli* enzymes for 5′ end labeled DNA (Figure 2B). However, the electrophoretic mobilities of the cleavage products differ for the two enzymes. Cleavage products of the calf thymus endonuclease migrate more rapidly than those produced by cleavage of the same DNA substrate with *E. coli* endonuclease III (Figure 2B, lanes 3 and 4). The mobility of the calf thymus endonuclease fragments corresponds exactly to the mobility of the fragments produced by the pyrimidine base specific sequencing reactions, a procedure that yields fragments containing 3′-terminal phosphate groups (Maxam & Gilbert, 1980). The presence of a 3′-terminal phosphate

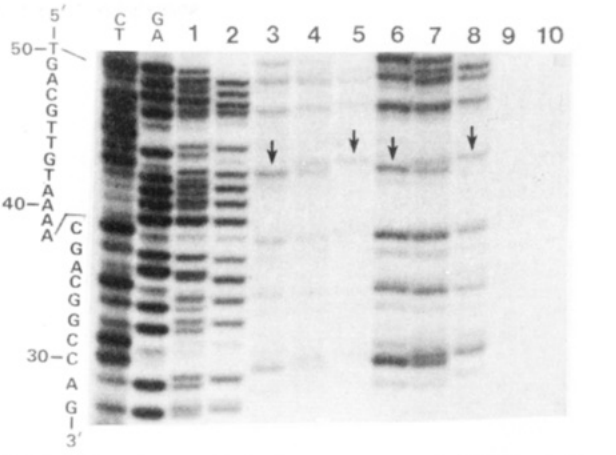


FIGURE 5: 5′ terminus analysis of enzyme-generated DNA scission products using UV-irradiated 3′ end labeled restriction fragment C. UV-irradiated DNA (lanes 3 and 8) was incubated with calf thymus endonuclease (lanes 3 and 5) or *E. coli* endonuclease III (lanes 6 and 8). A portion of the enzyme digests and the G+A sequencing reaction were treated separately with calf alkaline phosphatase, which removes 5′-terminal phosphate groups from DNA (lanes 2, 5, and 8). Lanes 1, 4, and 7 represent mixtures of the DNA products analyzed in lanes G+A and 2, lanes 3 and 5, and lanes 6 and 8 respectively. Undamaged DNA was incubated with the calf thymus (lane 9) or *E. coli* (lane 10) enzymes. Arrows indicate differences in mobilities of DNA fragments with (lanes 3 and 6) and without (lanes 5 and 8) 5′-terminal phosphate groups. Treated samples were analyzed on sequencing gels as described under Materials and Methods.

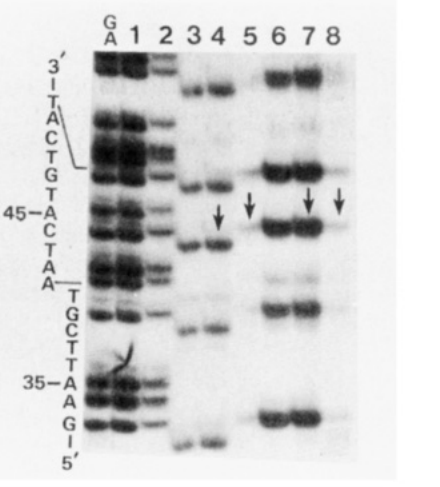


FIGURE 6: 3′ terminus analysis of enzyme-generated DNA scission products using UV-irradiated, 5′ end labeled restriction fragment F. UV-irradiated DNA (lanes 3–8) was incubated with calf thymus endonuclease (lanes 3 and 5) or endonuclease III (lanes 6 and 8). A portion of the enzyme digests and the G+A sequencing reaction were treated with 3′ phosphatase (associated with T4 polynucleotide kinase), which removes 3′-terminal phosphate groups from DNA (lanes 2, 5, and 8). Lanes 1, 4, and 7 represent mixtures of the DNA products in lanes G+A and 2, lanes 3 and 5, and lanes 6 and 8, respectively. Less radioactivity was loaded into lanes 5 and 8 compared to the rest of the lanes. Treated samples were analyzed on sequencing gels as described under Materials and Methods.

group on the calf thymus endonuclease scission products was confirmed by the observation that treatment with 3′-phosphatase (associated with T4 polynucleotide kinase) (Royer-Pokora et al., 1981; Cameron & Uhlenbeck, 1977) produced DNA fragments with decreased electrophoretic mobilities compared to untreated scission products (Figure 6, lanes 3–5). We conclude that the calf thymus endonuclease also cleaves UV-irradiated DNA between the damaged base and the 5′-phosphoryl group producing a scission product containing a 3′-terminal phosphate group.

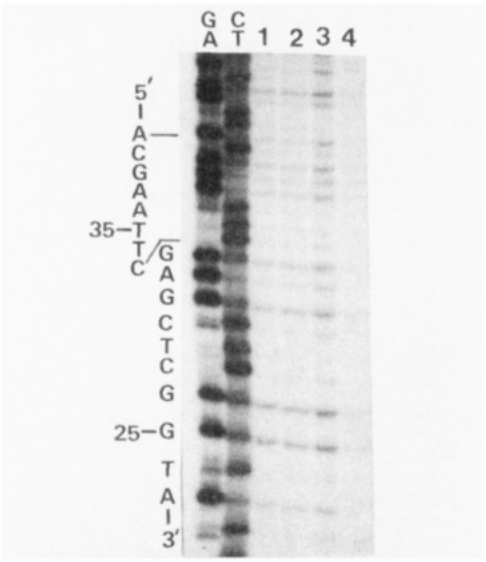


FIGURE 7: Comparison of calf thymus endonuclease and *E. coli* endonuclease III incision of 3′ end labeled, apurinic DNA. AP DNA (restriction fragment B) was treated with calf thymus endonuclease (lane 1), *E. coli* endonuclease III (lane 2), hot alkali (lane 3), or no treatment (lane 4). Treated samples were analyzed on sequencing gels ad described under Materials and Methods.

In contrast, the electrophoretic mobility of the fragments produced by *E. coli* endonuclease III correspond to cleavage events at pyrimidine bases that terminate with 3′ base-free sugars and, hence, migrate more slowly in sequencing gels (Figure 2B, lanes 1 and 4). The presence of a base-free sugar at the 3′ terminus of *E. coli* endonuclease III scission products (Demple & Linn, 1980) should render these fragments resistant to 3′ phosphatase. This prediction is supported by the observation that the mobility of such products is not altered by treatment with 3′ phosphatase (Figure 6, lanes 6–8). Mild alkaline hydrolysis of DNA fragments containing 3′-terminal base-free sugars should result in β-elimination of the deoxyribose and a subsequent increase in the electrophoretic mobility of the fragment (Haseltine et al., 1980). Treatment of endonuclease III scission products with mild alkali produced an increase in fragment mobility indicating elimination of the base-free sugar (not shown). These experiments confirm the previous finding (Demple & Linn, 1980) that *E. coli* endonuclease III contains both an *N*-glycosylase and a 3′ endonuclease activity but does not possess the 5′ endonuclease activity present in the calf thymus preparation.

These results provide insight regarding the *N*-glycosylase activity of the calf thymus enzyme and on the UV-damaged DNA substrates. The same modified cytosine product is released from UV-irradiated DNA by either the *E. coli* endonuclease III or the calf thymus enzyme as shown by HPLC analysis of the enzyme digestion products (Figure 4). Treatment of UV-irradiated DNA with *E. coli* endonuclease III produces 3′ base-free sugars at all sites of cytosine cleavage, an observation that demonstrates the *N*-glycosylase activity of the *E. coli* enzyme at these sites of modified cytosine. The *N*-glycosylase activity of *E. coli* endonuclease III has also been demonstrated for a variety of other pyrimidine base damage products (Breimer & Lindahl, 1984). The cytosine products released by the two enzymes are the same and, hence, suggest that the calf thymus enzyme also possesses an *N*-glycosylase activity that recognizes the cytosine UV photoproduct.

AP Endonuclease Activity. To determine whether the calf thymus enzyme also acted on apurinic/apyrimidinic (AP) DNA, 3′ end labeled DNA was treated under mild acidic

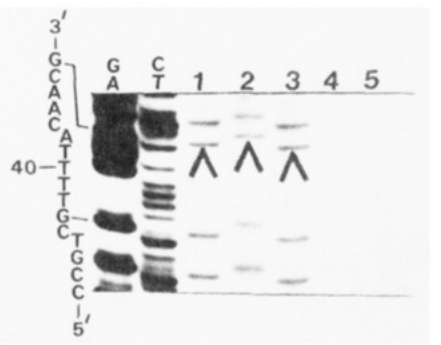


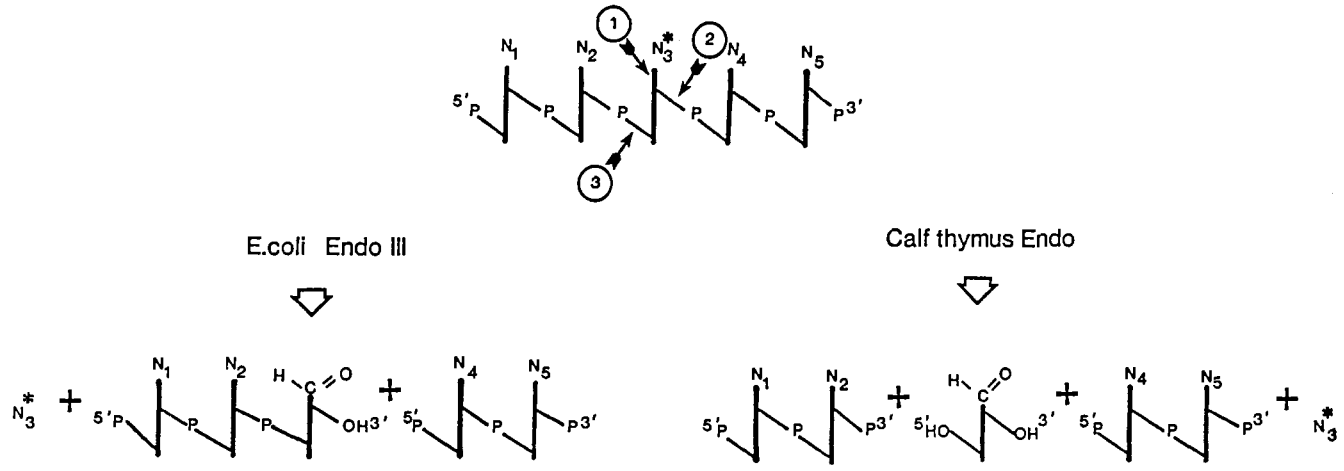
FIGURE 8: Comparison of calf thymus endonuclease and *E. coli* endonuclease III incision of 5′ end labeled, apurinic DNA. AP DNA (restriction fragment E) was treated with calf thymus endonuclease (lane 1), *E. coli* endonuclease III (lane 2), or hot alkali (lane 3). Undamaged DNA was treated with calf thymus endonuclease (lane 4) or *E. coli* endonuclease III (lane 5). Treated samples were analyzed on sequencing gels as described under Materials and Methods. Arrows indicate the difference in electrophoretic mobilities of the DNA scission products at AP site G47 generated by calf thymus endonuclease, *E. coli* endonuclease III, and hot alkali treatment.

conditions to produce apurinic sites (Lindahl & Nyberg, 1972). It was then incubated with either the calf thymus endonuclease or endonuclease III. Treatment of 3′ end labeled AP DNA with either the calf thymus or *E. coli* endonuclease produces similar cleavage products (Figure 7). Such cleavage products correspond to breakage of the DNA at sites of purine bases. Cleavage at sites of guanine bases occurs at a higher frequency compared to cleavage at sites of adenine bases. These results are in accord with previous studies demonstrating greater acid lability at sites of guanine as compared to sites of adenine (Lindahl & Nyberg, 1972). The electrophoretic mobilities of the cleavage products are those expected for DNA fragments terminated with 5′-phosphoryl groups. The electrophoretic mobility of cleavage products produced by calf thymus endonuclease treatment of 5′ end labeled DNA that contains AP sites is that expected for scission products containing 3′-terminal phosphate groups (Figure 8). We conclude that the calf thymus enzyme contains an AP endonuclease activity that cleaves the phosphodiester bonds on both sides of the AP sugar. In contrast, the electrophoretic mobility of cleavage products produced by *E. coli* endonuclease III cleavage of 5′ end labeled AP DNA is that expected for scission products containing a 3′-terminal base-free sugar (Figure 8).

Discussion

The calf thymus endonuclease and *E. coli* endonuclease III are similar with respect to both substrate specificity and mechanism of action. In addition to activities directed against UV-damaged and osmium tetroxide damaged DNA substrates, both enzymes recognize and cleave modified pyrimidines from DNA damaged with ionizing radiation, potassium permanganate, and hydrogen peroxide (unpublished results). The mechanism of incision by these enzymes includes removal of the damaged base via *N*-glycosylase and endonuclease scission events. Both endonucleases are relatively small and possess no divalent cation requirements (Helland et al., 1986; Radman, 1976). However, these two enzymes differ with respect to their endonuclease activities. A model for incision of damaged DNA is presented in Scheme I for both enzymes. The calf thymus enzyme has the ability to cleave both phosphodiester bonds that flank the modified base, thus completely excising the modified base and the corresponding sugar. The resultant DNA contains a gap flanked by both 5′- and 3′-phosphoryl groups. We cannot conclude that these three activities are all encoded by a single polypeptide, even though all three activities

Scheme I: Mechanism of Action of Calf Thymus Endonuclease and *E. coli* Endonuclease III[a]



[a] A damaged pyrimidine base (N_3*) is removed from DNA by both enzymes via an *N*-glycosylase activity (arrow 1). Both enzymes cleave the phosphodiester bond 3′ to the resulting apyrimidinic (AP) site between the base-free sugar and corresponding phosphate (arrow 2). The calf thymus endonuclease also cleaves the phosphodiester bond 5′ to the AP site between the deoxyribose and the corresponding phosphate (arrow 3). The calf thymus and *E. coli* enzyme cleavage products differ with respect to the nature of the 3′ terminus.

copurify throughout the entire enzyme isolation scheme. The ability of all three activities to function in the presence of 10 mM EDTA rules out the possibility that the endonuclease activities are attributable to contamination by the major mammalian AP endonucleases. Such AP endonucleases are divalent cation dependent (Linn, 1982) and do not copurify with the calf thymus endonuclease (Helland et al., 1986). The associated 5′ AP endonuclease of the calf thymus enzyme is a novel activity producing a scission product containing a 3′-terminal phosphate. This property distinguishes the calf thymus AP endonuclease from the other previously described class II 5′ AP endonucleases (Linn, 1982).

The removal of a modified base and the corresponding sugar by the calf thymus endonuclease should result in the release of deoxyribose. Experiments to detect enzyme-generated deoxyribose or a modified deoxyribose are in progress. The identification of enzyme-released free deoxyribose and the presence of the *N*-glycosylase and 3′ and 5′ AP endonuclease activities in a homogeneous enzyme preparation should confirm the proposed three-step mechanism. A similar three-step mechanism has been found for a *M. luteus* enzyme that recognizes oxidative and radiation-induced DNA damage (W. D. Henner, personal communication).

In contrast to the mechanism of cleavage by the calf thymus endonuclease, *E. coli* endonuclease III lacks the 5′ AP endonuclease activity. Hence, the resultant scission products differ as the endonuclease III scission product contains a 3′ base-free sugar. We suggest that subsequent repair steps acting on DNA fragments terminated with either a 3′-phosphate or a 3′ base-free sugar involve trimming of the DNA by action of a phosphatase and certain AP endonuclease activities to yield a 3′-hydroxyl group that is subsequently acted upon by a repair DNA polymerase.

The similarity of the calf thymus endonuclease and *E. coli* endonuclease III and their role in the repair of damaged oxidized bases prompt us to propose that they be called redoxy *N*-glycosylase AP endonucleases or more simply redoxy endonucleases. This terminology emphasizes the oxidative and radiation-induced pathways leading to pyrimidine ring saturation, fragmentation, or contraction. Such modified pyrimidines are the substrates for these enzymes. The mechanisms leading to the loss of pyrimidine ring planarity are the result of either oxidation or reduction reactions (Cadet & Teoule, 1978).

The conservation of DNA repair mechanisms between bacterial and mammalian cells emphasizes the fundamental nature for the reversal of oxidative DNA damage. A similar conservation of substrate specificity and mechanism of action exists for bacterial and mammalian *O*-^{6}-methylguanine-DNA methyltransferase (Karran et al., 1979; Bogden et al., 1981). The identification and characterization of other highly conserved DNA repair enzymes should reveal which types of DNA damage are the most biologically relevent. Such information should provide an indication of the role of certain DNA repair pathways in maintaining the biological integrity of an organism.

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Registry No. Mammalian DNA repair endonuclease, 52906-91-9; endonuclease III, 60184-90-9; DNA *N*-glycosylase, 70356-40-0; AP endonuclease, 65742-70-3.

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Amino Acid Sequence of Human Histidine-Rich Glycoprotein Derived from the Nucleotide Sequence of Its cDNA†

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ABSTRACT: A λgt11 library containing cDNA inserts prepared from human liver mRNA has been screened with an affinity-purified antibody to human histidine-rich glycoprotein (HRG) and then with a restriction fragment isolated from the 5′ end of the largest cDNA insert obtained by antibody screening. A number of positive clones were identified and shown to code for HRG by DNA sequence analysis. A total of 2067 nucleotides were determined by sequencing 3 overlapping cDNA clones, which included 121 nucleotides of 5′-noncoding sequence, 54 nucleotides coding for a leader sequence of 18 amino acids, 1521 nucleotides coding for the mature protein of 507 amino acids, a stop codon of TAA, and 352 nucleotides of 3′-noncoding sequence followed by a poly(A) tail of 16 nucleotides. The length of the noncoding sequence of the 3′ end differed in several clones, but each contained a polyadenylylation or processing sequence of AATAAA followed by a poly(A) tail. More than half of the amino acid sequence of HRG consisted of five different types of internal repeats. Within the last 3 internal repeats (type V), there were 12 tandem repetitions of a 5 amino acid segment with a consensus sequence of Gly-His-His-Pro-His. This repeated portion, referred to as a "histidine-rich region", contained 53% histidine and showed a high degree of similarity to a histidine-rich region of high molecular weight kininogen.

Histidine-rich glycoprotein (HRG)[1] has been isolated and characterized from human serum (Heimburger et al., 1972; Morgan, 1978), plasma (Lijnen et al., 1980; Koide et al., 1982, 1985), and platelets (Leung et al., 1983) as well as from rabbit serum (Morgan, 1981). Although the physiological function of HRG has not yet been established, a number of biological properties have been reported including interaction with heparin (Heimburger et al., 1972; Koide et al., 1982; Lijnen et

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[1] Abbreviations: HRG, histidine-rich glycoprotein; HMW kininogen, high molecular weight kininogen; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; kDa, kilodalton(s).

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