Nuclease SP: a novel enzyme from spinach that incises damaged duplex DNA preferentially at sites of adenine

Paul W.Doetsch*, William H.McCray Jr, Keunmyoung Lee, Donald R.Bettler
and Mario R.L.Valenzuela

Department of Biochemistry, Emory University School of Medicine, Atlanta, GA 30322, USA

Received March 24, 1988; Revised and Accepted June 10, 1988

ABSTRACT

A novel endonuclease has been isolated from extracts of spinach leaves (*Spinacia oleracea*). The enzyme has been purified by a series of column chromatography steps and has a molecular size of approximately 43,000 daltons. The spinach endonuclease cleaved double stranded DNA damaged by ultraviolet light or cis-diamminedichloroplatinum (II) primarily at sites of adenine when end-labelled DNA fragments of defined sequence were employed as substrates. The nature of the structural distortion contained in damaged, duplex DNA appears to be an important determinant for endonuclease cleavage. DNA helical distortions produced by UV light-induced (6-4) pyrimidine-pyrimidone photoproducts, but not cyclobutane pyrimidine dimers are recognized by the enzyme. The DNA cleavage products generated by the enzyme contain 3'-hydroxyl and 5'-phosphoryl termini. Single stranded DNA and RNA are hydrolyzed by the spinach endonuclease. This enzyme, which we call nuclease SP, is similar in several respects to other single-strand-specific nucleases such as *N. crassa* and mung bean nucleases and may function in DNA repair and/or recombination events in spinach cells. Nuclease SP should be a useful tool for the analysis of (6-4) photoproducts occurring in duplex DNA.

INTRODUCTION

DNA and RNA nucleases are a diverse group of enzymes that participate in a variety of biological processes including replication, transcription, genetic recombination, DNA repair, RNA processing and bacterial restriction (for a review, see ref. 1). One group of enzymes referred to as single-strand-specific nucleases, catalyze the hydrolysis of single stranded DNA and RNA. Several of these single-strand-specific nucleases such as S1, *N. crassa*, and *Alteromonas* BAL 31 nucleases are capable of recognizing structural alterations in duplex DNA damaged by UV light and other carcinogens (2-6). Although the biological functions of these enzymes remain unclear, evidence exists that they may mediate certain events involved in DNA repair and recombination (1, 7). The biological roles of these enzymes notwithstanding, a number of single-strand-specific nucleases have been employed as enzymatic tools for a wide variety of nucleic acid structural manipulations including analysis of DNA and RNA secondary structure and mapping the 5' and 3' ends of RNA transcripts.

We report here the characterization of a novel enzyme, nuclease SP, from spinach leaves that is able to recognize helical distortions introduced into duplex DNA by ultraviolet light or the antitumor agent cis-diamminedichloroplatinum (II). The major photoproducts present in UV

irradiated DNA, cyclobutane pyrimidine dimers and (6-4) pyrimidine-pyrimidone photoproducts [(6-4) photoproducts] may elicit different biological consequences depending on the system investigated (8-10) and may induce substantially different types of helical distortions in duplex DNA (11-14). Endonucleases from *M. luteus* and T4 phage-infected *E. coli* have been isolated and characterized that cleave duplex DNA in response to cyclobutane pyrimidine dimers, but not (6-4) photoproducts (15-20). However, to date, there are no reports of endonucleases that will recognize and cleave duplex DNA in response to (6-4) photoproducts, but not cyclobutane pyrimidine dimers. We have investigated the activity of nuclease SP on DNA substrates of defined sequence containing both (6-4) photoproducts and cyclobutane pyrimidine dimers or (6-4) photoproducts only in order to determine the base specificity and mode of phosphodiester bond cleavage mediated by the enzyme and to gain insight into the nature of the helical distortion(s) recognized by the enzyme.

MATERIALS AND METHODS

Enzymes and Chemicals

M. luteus UV-specific endonuclease was prepared as described (19). *E. coli* endonuclease III was a gift of Dr. Richard Cunningham, SUNY, Albany. Deoxyribodipyrimidine photolyase (DNA photolyase) was provided by Dr. Aziz Sancar, University of North Carolina. Restriction enzymes, DNA polymerase I (Klenow fragment), T4 DNA polymerase, T4 polynucleotide kinase, and Φ X174(+) single stranded DNA were purchased from Bethesda Research Laboratories. Calf alkaline phosphatase was from Boehringer-Mannheim. [γ - 32 P]ATP (sp. act. 5000 Ci/mmol) and [α - 32 P]dNTPs (sp. act. 800 Ci/mmol) were purchased from Amersham. Calf liver rRNA (18S, 28S) was purchased from Pharmacia. Cis-diamminedichloroplatinum (II) was purchased from Aldrich. All other chemicals were of the highest commercially available purity.

Purification of Nuclease SP

The purification of nuclease SP was carried out at 4°C and will be described in detail elsewhere (P.W. Doetsch, W. H. McCray, and M. R. L. Valenzuela, submitted). Briefly, homogenized spinach leaves (*Spinacia oleracea*) in buffer A (10 mM KH₂PO₄, pH 7.5, 1 mM EDTA, 10 μ M phenylmethylsulfonyl flouride) were filtered through cheese cloth and brought to 35% saturation with ammonium sulfate. Nuclease SP activity was monitored using the base-specific DNA endonuclease assay described below. Following centrifugation, the pellet was discarded and the pooled supernatants were brought to 70% ammonium sulfate saturation. The resulting precipitate was resuspended in buffer A minus EDTA and extensively dialyzed against the same buffer. The dialyzed preparation was then brought to 45% saturation with acetone and centrifuged. The pooled supernatants were brought to 75% acetone saturation and the resulting precipitate was resuspended in 50 mM KH₂PO₄, pH 7.5 (buffer B) dialyzed

against the same buffer and constituted fraction I. Fraction I was applied to a DEAE cellulose column equilibrated in buffer B and nuclease SP was eluted with 0.2 M KH_2PO_4 , pH 7.4 (fraction II) following a column wash with buffer B to remove unbound proteins. Fraction II was dialyzed against buffer B, applied to an Affi-gel blue column equilibrated with the same buffer, and was eluted stepwise with 50 mM, 0.15 M, and 0.4 M KH_2PO_4 , pH 7.3. Nuclease SP activity was contained in the 0.4 M KH_2PO_4 fractions (fraction III). Fraction III was further purified by gel filtration (Ultrogel AcA 44, equilibrated in 0.2 M KH_2PO_4) and eluted as a broad peak of activity (fraction IV) in the molecular size range of 30-45 kD. Fraction IV (specific activity 2.7×10^5 units/mg) was the source of nuclease SP in the experiments described below (unless otherwise indicated) and 0.5 μg of enzyme was used in each experiment. One unit of nuclease SP activity is defined as the amount of enzyme required to produce 1 fmol of UV-specific nicks in irradiated pUC19 plasmid DNA in 30 min at 37° C.

Size Fractionation of Nuclease SP

Partially purified (fraction I) nuclease SP (0.3 mg) was applied to a calibrated, Sephadex G-100 (superfine) column (Pharmacia, 1.5 X 94 cm) equilibrated with buffer B plus 0.5 M NaCl. The column was eluted with the equilibration buffer (1 ml fraction volumes) and aliquots (10 μl) of various fractions were incubated with UV-irradiated (2 kJ/m²), 3' end-labelled DNA fragment 1 substrates. The incubation samples were processed and analyzed on a DNA sequencing gel as described below for nuclease SP base-specific DNA endonuclease activity.

DNA Nicking Assay

The reaction mixture (25 μl final volume) contained 50 mM KH_2PO_4 , pH 7.4, 85 fmol of pUC19 supercoiled DNA and various amounts of nuclease SP preparations. DNA supercoils (5 μl droplets on ice) were UV-irradiated (254nm) with a dose of 1 kJ/m² from a mineralight shortwave lamp. Following incubation at 37° C for 30 min, the reaction was terminated by the addition of 3 μl of stop solution (10% SDS, 0.25% bromphenol blue, 25% ficoll), loaded onto a 0.9% agarose mini-gel and electrophoresed for 90 min at 80 V. The amounts of supercoiled (form I) and nicked (form II) DNA in a given sample were determined by densitometric scans (Bio-Rad model 620 video densitometer) of the photographic negative of the UV light-illuminated, ethidium bromide-stained gel. The number of nicks per pUC19 molecule was determined by the relation $n = -\ln(\text{form I fraction})$ as described previously (21).

Single Stranded DNA and RNA Hydrolysis Assay

The reaction mixture (25 μl final volume) was identical to that used for the DNA nicking assays except that $\phi\text{X174}(+)$ DNA (0.3 μg) or calf liver 18S and 28S rRNA (0.5-5.0 μg) was used in place of DNA. The RNA substrates were incubated with 0.5 μg of nuclease SP for 30 min at 37° C. The reactions were terminated and analyzed on agarose gels as described above for the DNA nicking assays.

Preparation of Defined Sequence DNA Damage Substrates

A 125 bp Sal I-Pvu II restriction fragment was generated from pUC19 and was either 3' end-labelled (fragment 1) with [α - 32 P]dNTPs and DNA polymerase I (Klenow fragment) or 5' end-labelled (fragment 2) with [γ - 32 P]ATP and polynucleotide kinase as previously described (16, 22). The end-labelled, defined sequence DNA fragments [sp. act. (2-10) $\times 10^6$ cpm/ μ g DNA] were isolated on preparative, non-denaturing polyacrylamide gels as previously described (22) and, following recovery, were suspended in TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA). These defined sequence DNA fragments were damaged with UV light as described above and used as substrates for the various enzymes used in this study.

Platinum Damage of DNA Substrates

3' End-labelled DNA fragment 1 was reacted with 7.5 μ M cis-diamminedichloroplatinum (II) in an incubation mixture (50 μ l) containing 5 mM KH_2PO_4 , pH 7.3, 100 ng salmon sperm carrier DNA for 8 hr, 37 $^\circ$ C. Unreacted platinum was removed by phenol extraction of the incubation mixture and the DNA was ethanol precipitated, and resuspended in TE buffer. Following incubation with nuclease SP and prior to electrophoresis on DNA sequencing gels, the platinum-damaged DNA sample was incubated with 0.1M NaCN at 37 $^\circ$ C for 6 hr to remove bound platinum (23).

Nuclease SP Base-Specific DNA Endonuclease Assay

The base specificity of DNA cleavage mediated by nuclease SP was monitored by incubating aliquots of column fractions (from various purification steps) or other nuclease SP preparations with UV-damaged 3' end-labelled fragment 1 in a reaction volume of 50 μ l, in 50 mM KH_2PO_4 , pH 7.4, for 30 min, 37 $^\circ$ C. Following incubation, DNA samples were extracted 3 times with phenol- chloroform-isoamyl alcohol (20:19:1 v/v/v), ethanol precipitated, and analyzed on sequencing gels.

M. luteus Cyclobutane Pyrimidine Dimer-Specific Endonuclease Assay

UV-irradiated 3' end-labelled fragment 1 DNA substrates were incubated with 0.2 μ g *M. luteus* cyclobutane pyrimidine dimer-specific endonuclease in an incubation mixture (25 μ l) containing 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 50 mM NaCl for 30 min, 37 $^\circ$ C. Following incubation, the DNA samples were processed as described above for the nuclease SP base-specific DNA endonuclease assay.

Photoreactivation of Cyclobutane Pyrimidine Dimers

UV-irradiated 3' end-labelled fragment 1 DNA substrates were incubated with 4000 units (saturating amounts) of *E. coli* DNA photolyase to remove cyclobutane dimers as previously described (24). Samples were processed as described above for the nuclease SP endonuclease assay and the resulting ethanol-precipitated, photoreactivated DNA samples were subjected to further treatments with other enzymes or chemicals as described in the text.

Hot Alkali Treatment of UV-Damaged DNA

UV-irradiated, 3' end-labelled fragment 1 DNA substrates were treated with 1M piperidine (30 μ l) at 90° C for 30 min, for cleavage of DNA at sites of UV-induced (6-4) photoproducts (18). Following incubation, the samples were lyophilized and analyzed on DNA sequencing gels.

Quantitation of Nuclease SP and Hot Alkali Cleavage of UV-Damaged DNA

The dose response for induction of nuclease SP cleavage and (6-4) photoproduct formation on UV-irradiated DNA was determined from densitometric scans (Bio-Rad model 620) of the gel autoradiogram lanes from the experiments depicted in Figure 4. The optical density values (relative cleavage) obtained for a set of gel bands (cleavage products) were summed for each lane and normalized to account for differences in the amounts of radioactivity loaded in the gel lanes.

E. coli Endonuclease III Cleavage of UV-Damaged DNA

E. coli endonuclease III cleaves UV-damaged, 5' end-labelled DNA at sites of monobasic pyrimidine photoproducts to produce scission fragments containing 3' terminal, base-free, modified sugar residues (25-27). UV-irradiated, 5' end-labelled DNA fragment 2 was incubated with *E. coli* endonuclease III as described previously (27).

5' Terminal Analysis of DNA Scission Products

3' End-labelled DNA scission products generated by enzyme or chemical treatments were ethanol-precipitated, resuspended in TE buffer and incubated with 28 units of calf alkaline phosphatase (20 μ l reaction volume) at 37° C for 4 hr. Following incubation, the samples were processed as above for the nuclease SP endonuclease assays and analyzed on a DNA sequencing gel.

3' Terminal Analysis of DNA Scission Products

5' End-labelled DNA scission products generated by enzyme or chemical treatments were ethanol-precipitated, resuspended in TE buffer and subjected to one of the following procedures. Alkaline hydrolysis to remove 3' base-free sugars or modified sugars was carried out by incubating DNA substrates in 30 μ l of 0.1 N NaOH at 37° C for 30 min followed by the addition of 15 μ l of 0.2 N HCl to neutralize the reaction mixture. 3' Phosphatase digestions using T4 polynucleotide kinase to remove 3' terminal phosphoryl groups were performed as previously described (27). Incubations with T4 DNA polymerase (3'-5') exonuclease to degrade scission products containing 3' terminal hydroxyl groups were carried out as described (22). In each case, the resulting incubation products were processed as described for the nuclease SP endonuclease assays and analyzed on DNA sequencing gels.

DNA Sequencing Reactions and Gel Electrophoresis

The purine (G+A) and pyrimidine (C+T) base-specific DNA sequencing (28) were run alongside each set of samples in all experiments. Processed DNA samples were loaded onto denaturing, 20% polyacrylamide-7M urea gels and subjected to electrophoresis and autoradiography as described (22).

Figure 1. Nuclease SP cleavage of UV-damaged, supercoiled pUC19 DNA. UV-damaged (lanes 1-4) or undamaged (lanes 5-8) DNA substrates were incubated with 0.2 μ g (lanes 2 and 6), 0.6 μ g (lanes 3 and 7), or 2.0 μ g (lanes 4 and 8) of nuclease SP or reaction buffer alone (lanes 1 and 5) and subjected to agarose gel electrophoresis as described in Materials and Methods. Arrow S denotes the position of uncleaved, supercoiled form I molecules. Arrow R denotes nicked, relaxed form II molecules. The bands migrating between the arrows correspond to linear (form III) duplex molecules and the faint bands observed above arrow R correspond to supercoiled and relaxed pUC19 concatamers.

RESULTS

General Properties of Nuclease SP

Nuclease SP nicks UV-irradiated, supercoiled pUC19 DNA (form I), converting the damaged substrates to relaxed circular (form II) and linear (form III) duplex molecules (Fig. 1). A small amount of background cleavage on undamaged DNA occurs, however, the overall extent of cleavage is UV damage-dependent. Although added divalent cations are not required for activity, the enzyme lost more than 90% of its activity following dialysis against EDTA and could be reactivated following the addition of Zn^{2+} or other divalent metal cations (not shown). Fraction I was size-fractionated on a Sephadex G-100 (superfine) column. Nuclease SP activity was monitored by incubating an aliquot of each fraction with UV-damaged, 3' end-labelled DNA fragment 1 followed by the analysis of the incubation mixtures on a DNA sequencing gel (Fig. 2). Nuclease SP-mediated DNA cleavage was centered around fraction 86 and corresponded to an endonuclease activity eluting at a molecular size of approximately 43 kD. Nuclease SP-specific cleavage of UV-damaged DNA substrates of defined sequence occurs primarily at positions of adenine, less frequently at positions of thymine, and, in rare instances, at positions of cytosine. The extent of cleavage at particular adenine sites within the DNA sequence shown in Figure 2 varies considerably and ranges from extensive cleavage (A31) to little, if any, cleavage (A53). The electrophoretic mobilities of the nuclease SP-generated DNA scission products coincide with those produced by the base-specific, chemical cleavage reactions (Pu and Py lanes). This result suggests that the enzyme-generated cleavage products contain 5' terminal phosphoryl groups as do the chemical cleavage products (28). The DNA sequencing assay is

Figure 2. Size fractionation of nuclease SP. Partially purified (fraction I) nuclease SP was size-fractionated on Sephadex G-100 (superfine). Aliquots of each fraction were incubated with UV-irradiated 3' end-labelled DNA fragment 1 and the reaction products were analyzed on a DNA sequencing gel. Approximately 35 kcpm of processed incubation sample were loaded into each lane. The purine (Pu)- and pyrimidine (Py)-specific DNA sequencing reactions were included in the far-left lanes. Arrows denote nuclease SP-specific cleavages at sites of adenine and thymine. The majority of radioactivity in each lane corresponded to uncleaved, full-length DNA fragments and were located at the top of the gel and are not shown in the photograph (Materials and Methods). Base numbering starts from the 3' end labelled terminus.

specific for detecting nuclease SP activity under these conditions and can be utilized as a method to follow nuclease SP purification as the adenine-specific endonucleolytic properties of the enzyme can be precisely monitored. It is relevant to point out that this method can be modified for monitoring the endonucleolytic specificities of other types of DNA endonucleases and should be useful as a general tool for monitoring the purification of such enzymes.

Nuclease SP Activity on DNA Containing Cyclobutane Dimers or (6-4) Photoproducts

Initial experiments with nuclease SP suggested that adenine-specific cleavage of duplex DNA was dependent on DNA damage. Because UV light damage of DNA produces several different types of photoproducts, we wished to determine which types were responsible for nuclease SP cleavage of damaged DNA. We also considered the possibility that nuclease SP recognized a novel adenine UV photoproduct. The major photoproducts occurring in UV-damaged DNA are cyclobutane pyrimidine dimers and (6-4) photoproducts (29-31). For these experiments, 3'

Figure 3. Nuclease SP cleavage of UV-damaged DNA containing cyclobutane pyrimidine dimers and (6-4) photoproducts or (6-4) photoproducts alone. 3' End-labelled DNA fragment 1 was UV-irradiated with a dose of 0.5 kJ/m². UV-damaged (lanes 1-6) and undamaged (lanes 7-9) DNA substrates were treated with DNA photolyase (lanes 2, 4, 6) to remove cyclobutane pyrimidine dimers or left untreated (lanes 1, 3, 5) and incubated with nuclease SP (lanes 1, 2, 7) or *M. luteus* cyclobutane dimer-specific endonuclease (lanes 3, 4, 8) or hot alkali (lanes 5, 6, 9). The incubation products were analyzed on a DNA sequencing gel. The purine (Pu) and pyrimidine (Py) DNA sequencing reactions were run alongside the enzyme reaction lanes. The amounts of radioactivity loaded into the sample lanes were 111 kcpm, lane 1; 138 kcpm, lane 2; 83 kcpm, lane 3; 84 kcpm, lane 4; 88 kcpm, lane 5; 95 kcpm, lane 6; 165 kcpm, lane 7; 25 kcpm, lane 8; 94 kcpm, lane 9. Base numbering is as in the Figure 2 legend.

end-labelled DNA fragments of defined sequence containing both major photoproducts or only (6-4) photoproducts were utilized as substrates for nuclease SP followed by analysis of the DNA scission products on a DNA sequencing gel. DNA substrates were treated with either the *M. luteus* UV-specific endonuclease or hot alkali for determining the location and extent of formation of cyclobutane pyrimidine dimers or (6-4) photoproducts, respectively. Removal of cyclobutane pyrimidine dimers from the DNA substrates was achieved by incubating such substrates with DNA photolyase and resulted in completely abolishing DNA cleavage by the *M. luteus* UV-specific endonuclease (Fig. 3, lanes 3 and 4). In contrast, hot alkali cleavage of

UV-damaged DNA at sites of (6-4) photoproducts was unchanged following photolyase treatment (Fig. 3, lanes 5 and 6) and is in agreement with previous reports (27, 32). Nuclease SP cleavage of UV-damaged DNA at sites of adenine was also unchanged following photolyase removal of cyclobutane pyrimidine dimers indicating that, under these conditions, the enzyme does not cleave DNA in response to the presence of these UV photoproducts (Fig. 3, lanes 1 and 2). Under these conditions, unirradiated DNA was not cleaved by nuclease SP (Fig. 3, lane 7).

The notion that nuclease SP cleaves UV-damaged DNA in response to the presence of (6-4) photoproducts, but not cyclobutane pyrimidine dimers was further supported by a comparison of the UV dose response for the induction of (6-4) photoproducts with the induction of nuclease SP incisions at sites of adenine. The extent of hot alkali-induced cleavage of DNA damaged with UV light in a dose range of 0-10,000 J/m² coincided with the extent of nuclease SP-induced cleavage of UV-damaged substrates (Fig. 4). In general, the induction of (6-4) photoproducts increases with increasing UV dose, whereas the induction of cyclobutane pyrimidine dimers reaches a maximum level around 1000 J/m² and does not increase with higher UV doses (18, 33). These results suggest that nuclease SP nicks duplex DNA at positions of adenine in the vicinity of (within 2-7 nucleotides) and on the same strand that contains (6-4) photoproducts.

To address the possibility that nuclease SP was recognizing and cleaving a novel adenine UV photoproduct that possessed the same UV induction characteristics as (6-4) photoproducts, DNA damaged with cis-diamminedichloroplatinum (II) was employed as a substrate. Such damaged DNA substrates contain platinum modifications primarily at sites of guanine and particularly, within runs of adjacent guanines (23). Nuclease SP cleaved platinum-damaged DNA at positions of adenine and the majority of adenines within the DNA sequence examined were cleaved as was the case for UV-damaged DNA (Fig. 5). In addition, nuclease SP cleaved DNA damaged with N-acetoxy-N-acetyl-2-aminofluorene at positions of adenine (not shown). These results support the notion that nuclease SP is not cleaving DNA in response to specific adenine modifications, but rather incises DNA at positions of adenine in the vicinity of helical distortions produced by (6-4) photoproducts, platinum, and other modifications, but not cyclobutane pyrimidine dimers.

Mechanism of DNA Cleavage

To determine the nature of the 5' termini produced following nuclease SP cleavage of damaged DNA, 3' end-labelled, UV-damaged DNA substrates of defined sequence were incubated with the enzyme and subjected to further treatment with calf alkaline phosphatase, to remove putative 5' terminal phosphoryl groups followed by analysis on a sequencing gel (Fig. 6A). The purine-specific (G+A) chemical cleavage products contain 5'-terminal phosphoryl groups (28), and upon removal of such phosphoryl groups with alkaline phosphatase, result in DNA fragments containing 5'-hydroxyl groups with a slower electrophoretic mobility than untreated counterpart fragments (7) containing 5'-phosphoryl groups (Fig. 6A, lanes Pu, 1, and 2). Treatment of nuclease SP-generated DNA scission products with alkaline phosphatase resulted in the generation of altered fragments with slower electrophoretic mobilities compared to

untreated scission products (Fig. 6A, lanes 3-5). These experiments demonstrate that the DNA cleavage products produced by nuclease SP contain 5'-terminal phosphoryl groups.

A second set of experiments was performed to determine the nature of the 3' termini produced by nuclease SP cleavage of damaged DNA. The electrophoretic mobilities of 5' end-labelled DNA scission products generated by nuclease SP migrated more slowly compared with the corresponding purine-specific chemical cleavage products that contain 3'-terminal phosphoryl groups (Fig. 6B). This result suggested that nuclease SP-generated DNA scission products may be one nucleotide longer than the corresponding G+A chemical cleavage product and may contain 3'-terminal hydroxyl groups or modified sugar moieties. DNA cleavage fragments containing 3'-terminal hydroxyl groups are generated by a number of single-strand-specific nucleases (1) and cleavage fragments containing 3'-terminal base-free, modified sugars are produced by certain DNA repair enzymes such as *E. coli* endonuclease III that cleave damaged DNA via a combined N-glycosylase/apurinic-apyrimidinic endonuclease

Figure 4. UV dose response for induction of nuclease SP cleavage and (6-4) photoproduct formation in duplex DNA. 3' End-labelled DNA fragment 1 substrates were UV-irradiated with doses of (panel A) 0, 0.01, 0.05, 0.2, 1.0, 5.0, and 10 kJ/m² (lanes 1-7, respectively); and (panel B) 0, 0.01, 0.05, 0.2, 1.0, 2.0, 5.0, and 10 kJ/m² (lanes 1-8, respectively). UV-damaged DNA substrates were incubated with nuclease SP (panel A) or treated with hot alkali to produce cleavages at sites of (6-4) photoproducts (panel B) and the resulting cleavage products were analyzed on DNA sequencing gels. The amount of radioactivity loaded into the gel lanes shown in panel A varied from 66 to 85 kcpm, and for panel B varied from 52 to 93 kcpm. Pu and Py lanes and base numbering are as in the Figure 2 legend. A comparison (panel C) of the dose response for induction of nuclease SP cleavage (□) and (6-4) photoproduct formation (■) on UV-irradiated DNA was determined from densitometric scans of the autoradiograms in panels A and B (Materials and Methods).

mechanism (25-27). Mild alkali treatment of 5' end labelled DNA fragments containing a base-free, modified sugar results in the release of the sugar moiety and exposure of a 3' phosphoryl group (25). The net effect of such an event is a slight increase in the electrophoretic mobilities of the fragments containing the 3' phosphoryl groups (Fig. 6B, lanes 4-6). Mild alkali treatment of nuclease SP-generated DNA scission products had no effect on the electrophoretic mobilities of such fragments and indicated that they do not contain base-free, modified sugars (Fig. 6B, lanes 1-3).

A subsequent experiment was performed to determine whether or not nuclease SP-generated DNA scission products contained 3'-hydroxyl groups. T4 DNA polymerase (3'-5') exonuclease requires 3' hydroxyl groups to hydrolyze duplex DNA (34). We have previously established that T4 DNA polymerase (3'-5') exonuclease hydrolysis of UV damaged, 5' end-labelled DNA is blocked at the sites of photoproducts (22). The base-specific chemical cleavage products contain 3'-phosphoryl groups (28) and are resistant to hydrolysis by T4 DNA polymerase (3'-5') exonuclease (Fig. 6C, lane 1). Removal of such 3'-terminal phosphoryl groups with the 3'-phosphatase associated with T4 polynucleotide kinase results in products with slightly decreased electrophoretic mobilities (Fig. 6C, lane 3) and which are subsequently hydrolyzed by

T4 DNA polymerase (3'-5') exonuclease (Fig. 6C, lane 2). Digestion of nuclease SP DNA scission products with T4 DNA polymerase (3'-5') exonuclease resulted in the disappearance of adenine cleavage products and the appearance of exonuclease termination products at sites of UV photoproducts (Fig. 6C, lane 5). We conclude that nuclease SP produces DNA scission products containing 3'-terminal hydroxyl groups. Hence, the cleavage mechanism mediated by nuclease SP on damaged duplex DNA involves an incision of the DNA phosphodiester linkage between the 3'-hydroxyl of an adenine nucleotide and the 5'-phosphoryl of the adjacent nucleotide.

Activity on Single-Stranded DNA and RNA

Nuclease SP cleavage of UV or platinum-damaged duplex DNA suggested that certain helical distortions are recognized and acted upon by the enzyme. The most likely candidates for such distortions are local regions of single-strandedness induced by (6-4) photoproducts and platinum

Figure 5. Nuclease SP cleavage of DNA containing platinum adducts. 3' End-labelled DNA fragment 1 was damaged with cis-diamminedichloroplatinum (II) (lane 2) or left undamaged (lane 1) and treated with nuclease SP (lanes 1 and 2). The reaction products were analyzed on a DNA sequencing gel as described in the Figure 2 legend. Arrows denote sites of nuclease SP cleavage of duplex DNA at the indicated base numbers (from the 3' labelled end).

adducts. Nuclease SP completely hydrolyzed single-stranded Φ X174(+) DNA under conditions where little or no cleavage occurs on undamaged duplex DNA. No ethidium bromide-stained material was detected following agarose gel electrophoresis of Φ X174(+) DNA incubated with nuclease SP (Fig. 7A). In addition, nuclease SP degraded 18S and 28S rRNA into shorter fragments (Fig. 7B). These results indicate that an activity associated with nuclease SP possesses single-strand nuclease activity, although the possibility that these activities are contaminants cannot be ruled out since a homogeneous preparation of enzyme was not used in these experiments.

DISCUSSION

Nuclease SP possesses a number of properties similar to several single-strand-specific nucleases. In general, such enzymes require divalent cations for activity and produce cleavage products containing 3'-hydroxyl groups and 5'-phosphoryl groups (1). Among this group of enzymes, the P1 and mung bean nucleases preferentially cleave DNA between the 3'-hydroxyl of adenine and the 5'-phosphoryl of the adjacent nucleotide on undamaged, single and double stranded DNA (1, 35) as does nuclease SP on damaged, duplex DNA. In addition, the molecular sizes for P1 (44 kD) and mung bean (39 kD) nucleases are similar to that of nuclease SP (1, 35, 36). However, preliminary experiments with P1 and mung bean nuclease indicate that these enzymes cleave UV-damaged, end-labelled DNA at positions other than adenine

Figure 6. (Panel A) 5' Terminal analysis of nuclease SP DNA scission products. 3' End-labelled DNA fragment 1 was UV-irradiated (2 kJ/m²) and incubated with nuclease SP (Materials and Methods). Nuclease SP DNA scission products were treated with calf alkaline phosphatase (lane 3) or were left untreated (lane 5). Phosphatase-treated and untreated DNA

scission products were also mixed (lane 4). Arrows denote positions of adenine cleavage sites. The purine-specific DNA chemical cleavage products (lane Pu) containing 3'-phosphoryl groups were also incubated with phosphatase (lane 2) or mixed together (lane 1). (Panel B) Resistance of 3' termini of nuclease SP DNA scission products to alkaline hydrolysis. 5' End-labelled DNA fragment 2 was UV-irradiated and incubated with nuclease SP or *E. coli* endonuclease III. *E. coli* endonuclease III-generated DNA scission products containing 3' base-free, modified sugars were subjected to alkaline hydrolysis (lane 6), left untreated (lane 4), or mixed together (lane 5). Nuclease SP-generated DNA scission products were subjected to alkaline hydrolysis (lane 3), left untreated (lane 1) or mixed together (lane 2). Arrow s denotes a nuclease SP-generated DNA cleavage product at A39. Arrow e denotes endonuclease III-generated DNA cleavage product at C38 and arrow h denotes the C38 alkaline hydrolysis product. (Panel C) Test for presence of 3'-hydroxyl groups on nuclease SP DNA scission products. 5' End-labelled DNA fragment 2 was UV-irradiated and incubated with nuclease SP (lane 4) or incubated with nuclease SP and subsequently treated with T4 DNA polymerase (3'-5') exonuclease (lane 5). Purine-specific chemical cleavage products (lane Pu) treated with exonuclease only (lane 1), 3' phosphatase only (lane 3) or treated with 3' phosphatase and subsequently incubated with exonuclease (lane 2). Arrows a-c denote nuclease SP cleavages at A54, A39, and A34-35, respectively. Arrows d and e denote exonuclease termination sites at UV photoproducts occurring at the T50-T53 and T36-C38 pyrimidine clusters, respectively. Base numbering for panels A-C are from the labelled termini and the analysis of reaction products on DNA sequencing gels was as described for the Figure 2 legend.

(unpublished results). Nuclease SP is also similar in size to a 42 kD endonuclease purified from pea seeds, but differs with respect to the effect of EDTA on endonuclease activity (37).

Previous investigations with S1, *N. crassa*, and BAL 31 single-strand-specific nucleases

Figure 7. Nuclease SP degradation of single stranded DNA and RNA. (Panel A) Φ X174(+) DNA was incubated with nuclease SP (lane 1) or left untreated (lane 2) and analyzed by agarose gel electrophoresis as described in Materials and Methods. (Panel B) 0.5 μ g (lanes 1 and 5), 1.0 μ g (lanes 2 and 6), 2.0 μ g (lanes 3 and 7), and 5.0 μ g (lanes 4 and 8) of calf liver 18S and 28S rRNA were left untreated (lanes 1-4) or incubated with nuclease SP (lanes 5-8) and analyzed on a 1% agarose gel as described in Materials and Methods. The lower and upper bands observed in lanes 1-4 correspond to 18S and 28S rRNA, respectively. Species migrating further into the gel compared with the 18S and 28S bands correspond to rRNA degradation products.

indicate that these enzymes recognize DNA structural distortions induced by UV light and the carcinogen N-acetoxy-N-acetyl-2-aminofluorene (NAAAF) (2-6). The base specificities of cleavage mediated by these enzymes and the nature of the UV photoproducts responsible for nuclease activity were not determined in those studies. However, S1 and *N. crassa* nucleases differentially excise NAAAF-induced adducts present in duplex DNA indicating that these single-strand-specific nucleases can discriminate amongst the helical distortions induced by at least two different types of DNA adducts (6). It appears that the extent of helical disruption caused by a particular adduct is the major determinant for producing sites which are acted upon by these enzymes. Molecular modelling studies of the conformations of DNA containing (6-4) photoproducts and cyclobutane pyrimidine dimers have attempted to elucidate the helical distortions produced by these two photoproducts (13, 14, 38). Such studies have so far failed to reveal any significant differences between (6-4) photoproducts and cyclobutane pyrimidine dimers with respect to defining the helical disruptions extending beyond the local area surrounding these adducts (14, 38). Molecular mechanical simulations suggest that the helical disruption produced by a cyclobutane pyrimidine dimer does not appear to extend beyond a localized area surrounding the dimer and results in a minor "kink" in the DNA helix (13, 14, 38). However, it has been determined that the (6-4) photoproduct of the dinucleotide TpT fits a B-DNA TpT structural unit less well than does the corresponding *cis-syn* cyclobutane dimer suggesting that (6-4) photoproducts might cause a greater distortion of duplex DNA (J.-S. Taylor, personal communication). The finding that nuclease SP is responsive to (6-4) photoproducts but not to cyclobutane pyrimidine dimers under our experimental conditions suggests that substantial differences may exist between the helical disruptions caused by these two types of photoproducts.

The properties of nuclease SP indicate that it may mediate a role in DNA repair and/or recombination in spinach. The ability of nuclease SP to cleave DNA strands containing (6-4) photoproducts and platinum adducts suggests that this enzyme may function to initiate the repair of bulky, stable DNA lesions for which no specific DNA repair enzymes exist. A possible mechanism by which nuclease SP could initiate repair would be to nick DNA at positions of adenine on either side (5' or 3') of the lesion. The repair of the nicked strand containing the damage could be completed by the subsequent activities of a DNA exonuclease, repair polymerase, and ligase. Nucleases possessing the properties of nuclease SP might also be expected to function during recombination events such as in the generation of heteroduplexes and in the removal of mismatches that might arise in such structures (1, 7). A 42 kD nuclease from *Ustilago maydis* possesses properties broadly similar to nuclease SP and is present in reduced amounts in *Ustilago* mutants defective in allelic recombination (1, 39). In addition, the *Ustilago* nuclease recognizes base mismatches (1) and it is also conceivable that nuclease SP could function in mismatch repair processes by incising duplex DNA in the vicinity of mispaired bases.

This is the first report, to our knowledge, of an endonuclease activity that recognizes and

cleaves UV-damaged DNA in response to the presence of (6-4) photoproducts, but not cyclobutane pyrimidine dimers. In this regard and, under the conditions of our assay, nuclease SP may be useful as a tool for the detection and quantitation of (6-4) photoproducts in UV-damaged DNA. Nuclease SP may also find utility as a probe for the detection of helical distortions present in duplex DNA produced by bulky, stable adducts induced by a variety of DNA damaging agents.

ACKNOWLEDGMENTS

We thank Dag Helland, Evelyne Sage, and John-Stephen Taylor for helpful discussions. This work was supported by PHS grant CA42607 from the National Cancer Institute, DHHS. M.R.L.V. was a recipient of an Emory University Medical Student Summer Research Fellowship.

*To whom correspondence should be addressed

REFERENCES

1. Shishido and Ando, T. (1982) In Linn, S. M. and Roberts, R.J. (eds), *Nucleases*, Cold Spring Harbor Laboratory, Cold Spring Harbor, pp.155-185.
2. Kato, A.C. and Fraser, M.J. (1973) *Biochim. Biophys. Acta* **312**, 545-655.
3. Legerski, R.J., Gray, H.B., and Robberson, D.L. (1977) *J. Biol. Chem.* **252**, 8740-8745.
4. Shishido, K. and Ando, T. (1974) *Biochem. Biophys. Res. Comm.* **59**, 1380-1388.
5. Wiegand, R.C., Godson, G.N., and Radding, C.M. (1975) *J. Biol. Chem.* **250**, 8848-8855.
6. Yamasaki, H., Pulkrabek, P., Grunberger, D., and Weinstein, I.B. (1977) *Cancer Res.* **37**, 3756-3760.
7. Sadowski, P.D. (1982) In Linn, S. M. and Roberts, R.J. (eds), *Nucleases*, Cold Spring Harbor Laboratory, Cold Spring Harbor, pp.155-185.
8. Franklin, W.A. and Haseltine, W.A. (1986) *Mutat. Res.* **165**, 1-7.
9. Hutchinson, F. (1987) *Photochem. Photobiol.* **45**, 897-903.
10. Tang, M.S., Hrcir, J., Mitchell, D., Ross, J., and Clarkson, J. (1986) *Mutat. Res.* **59**, 1380-1388.
11. Franklin, W.A., Doetsch, P.W., and Haseltine, W.A. (1985) *Nucleic Acids Res.* **13**, 5317-5325.
12. Kemmink, J., Boelens, T., van der Marel, G.A., van Boom, J.H., and Kaptein, R. (1987) *Nucleic Acids Res.* **15**, 4645-4652.
13. Pearlman, D.A., Holbrook, S.R., Pirkle, D.H., and Kim, S.-H. (1985) *Science* **227**, 1304-1308.
14. Rao, S.N. and Kollman, P.A. (1985) *Photochem. Photobiol.* **42**, 465-475.
15. Carrier, W.L. and Setlow, R.B.J. (1970) *J. Bacteriol.* **102**, 178-186.
16. Chan, G.L., Doetsch, P.W., and Haseltine, W.A. (1985) *Biochemistry* **24**, 5723-5728.
17. Friedberg, E.C. and King, J.J. (1971) *J. Bacteriol.* **106**, 500-507.
18. Lippke, J.A., Gordon, L.K., Brash, D.E., and Haseltine, W.A. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 3388-3392.
19. Riazuddin, S. and Grossman, L. (1977) *J. Biol. Chem.* **252**, 6280-6286.
20. Yasuda, S. and Sekiguchi, M. (1970) *Proc. Natl. Acad. Sci. U.S.A.* **67**, 1839-1845.
21. Jorgensen, T.J., Kow, Y.W., Wallace, S.S., and Henner, W.D. (1987) *Biochemistry* **26**, 6436-6443.
22. Doetsch, P.W., Chan, G.L., and Haseltine, W.A. (1985) *Nucleic Acids Res.* **13**, 3285-3304.
23. Tullius, T.D. and Lippard, S.J. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 3489-3492.

24. Sancar, A. and Sancar, G.B. (1984) *J. Mol. Biol.* 172, 223-227.
25. Bailly, V. and Verly, W.G. (1987) *Biochem. J.* 242, 565-572.
26. Demple, B. and Linn, S. (1980) *Nature* 287, 203-208.
27. Doetsch, P.W., Helland, D.E., and Haseltine, W.A. (1986) *Biochemistry* 25, 2212-2220.
28. Maxam, A. M. and Gilbert, W. (1980) *Methods. Enzymol.* 65, 499-560.
29. Patrick, M.H. and Gray, D. (1976) *Photochem. Photobiol.* 42, 507-513.
30. Patrick, M.H. (1977) *Photochem. Photobiol.* 25, 357-372.
31. Patrick, M.H. and Rahn, R.O. (1977) In Wang, S.-Y. (ed.) *Photochemistry and Photobiology of Nucleic Acids*, Academic Press, New York, pp.35-91.
32. Brash, D.E., Franklin, W.A., Sancar, G.B., Sancar, A., and Haseltine, W.A. (1985) *J. Biol. Chem.* 260, 11438-11441.
33. Gordon, L.K. and Haseltine, W.A. (1982) *Radiat. Res.* 89, 99-112.
34. Huang, W.M. and Lehman, I.R. (1972) *J.Biol. Chem.* 247, 3139-3146.
35. Johnson, P.H. and Laskowski, M. (1970) *J. Biol. Chem.* 245, 891-898.
36. Kowalski, D. Kroeker, W.D., and Laskowski, M. (1976) *Biochemistry* 15, 4457-4462.
37. Wani, A.A. and Hadi, S.M. (1979) *Arch. Biochem. Biophys.* 196, 138-146.
38. Rao, S.N., Keepers, J.W., and Kollman, P.A. (1984) *Nucleic Acids Res.* 12, 4789-4807.
39. Holloman, W.K. and Holliday, R. (1973) *J. Biol. Chem.* 248, 8107-8113.