Characterization of the Nuclease Activity of *Drosophila* Rrp1 on Phosphoglycolate- and Phosphate-Modified DNA 3′-Termini

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ABSTRACT: *Drosophila* Rrp1 includes a carboxy-terminal region homologous to *Escherichia coli* exonuclease III which is sufficient to repair both oxidative and alkylation damage to DNA. An apurinic/apyrimidinic endonuclease activity intrinsic to Rrp1 was characterized previously. In this work, the 3′-phosphodiesterase and 3′-phosphatase activities of Rrp1 are demonstrated and characterized. Phosphoglycolate- and phosphate-modified DNA 3′-termini are formed by oxygen radical induced DNA cleavage. To demonstrate the 3′-phosphodiesterase activity of Rrp1, a 3′-phosphoglycolate-terminated oligonucleotide substrate was generated by site-specific cleavage of a unique GpC dinucleotide by iron(II) bleomycin. Removal of the terminal phosphoglycolate is detected by mobility shift on a DNA sequencing gel. Rrp1 cleaves the phosphoglycolate and releases a product with a 3′-hydroxyl terminus. Phosphoglycolate is removed more readily than the 3′-terminal dGMP residue. Rrp1 phosphodiesterase activity is not inhibited by 120 mM NaCl, while the 3′-exonuclease is reduced 25-fold. Using a 3′-phosphate-terminated oligonucleotide, the phosphatase activity of Rrp1 is at least 25-fold lower than its phosphodiesterase or apurinic endonuclease, and 56-fold lower than exonuclease III activity on the identical substrate. Rrp1 3′-phosphatase is reduced 25-fold by 80 mM NaCl. These results were confirmed using an assay that measures the ability of Rrp1 to stimulate DNA synthesis on circular DNA substrates nicked by various DNA damage treatments. In that assay, Rrp1 poorly repairs 3′-phosphate-terminated nicks introduced by micrococcal nuclease. The significance of these enzymatic properties for the biological role of Rrp1 is discussed.

Reactive oxygen species accumulate in cells due to environmental stress and normal metabolic processes (Farr et al., 1986; Greenberg & Demple, 1988). Their ability to cause damage to cellular components is well-known, and their interactions with nucleic acids have been studied extensively (Hutchinson, 1985; von Sonntag, 1987). Such damage has been connected to processes that include mutagenesis, carcinogenesis, and aging (Greenberg & Demple, 1988; Orr & Sohal, 1994; Ames et al., 1993). Molecular effects on nucleic acids include strand breaks, abasic sites, and base damage (Hutchinson, 1985; Takeshita et al., 1978; von Sonntag, 1987). Cells have defenses against such damage that include enzymatic machinery that removes the damaging oxygen species (Greenberg & Demple, 1988) as well as enzymatic machinery capable of recognizing and repairing many lesions caused by oxidative processes (Lindahl, 1982; Sancar & Sancar, 1988).

Strand breaks with terminal blocking groups are among the lesions that accumulate in oxidatively damaged cells and nucleic acids (Friedberg, 1985; von Sonntag, 1987). Repair DNA synthesis at these strand breaks is known to require prior processing by repair enzymes acting as 3′-phosphodiesterases or 3′-phosphomonoesterases. Two major *Escherichia coli* enzymes (Demple et al., 1986; Bernelot-Moens & Demple, 1989), one yeast enzyme (Johnson & Demple, 1988a,b), and several distinct mammalian and human enzymes (Chen et al., 1991; Seki et al., 1991; Robson et al., 1991; Winters et al., 1992; Seki & Oda, 1988) are known to have 3′-phosphodiesterase repair activity toward oxidatively damaged DNA. The *in vivo* relevance of these activities is demonstrated in *E. coli* deficient in the enzymes exonuclease III and endonuclease IV. Such strains are highly sensitive to various types of oxidative damage, including ionizing radiation, hydrogen peroxide, and bleomycin (Cunningham et al., 1986).

Drosophila Rrp1 has several enzymatic activities, including dsDNA 3′-exonuclease, apurinic/apyrimidinic (AP)[1] endonuclease, DNA strand transferase, and ssDNA binding (Sander et al., 1993; Nugent et al., 1993). The carboxy-terminal third of Rrp1 is homologous to *E. coli* exonuclease III and several eukaryotic AP endonucleases (Sander et al., 1991a). We previously reported that the nuclease region of Rrp1 can facilitate the repair of DNA lesions caused by different DNA damaging agents, including hydrogen peroxide and *tert*-butyl hydroperoxide, when expressed in repair-deficient strains of *E. coli* (Gu et al., 1993). In this work, we have characterized the 3′-phosphodiesterase and 3′-phosphatase activities of Rrp1 using two novel assays.

Two assays that are used in most studies of 3′-phosphodiesterases measure the removal of 3′-blocking groups indirectly. One assay measures the ability of a repair enzyme to stimulate polymerase activity on damaged DNA containing 3′-blocking groups (Demple et al., 1986; Seki & Oda, 1988;

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[1] Abbreviations: AP, apurinic/apyrimidinic; dsDNA, double-strand DNA; ssDNA, single-strand DNA; scDNA, supercoiled DNA; rcDNA, relaxed circular DNA; ncDNA, nicked circular DNA; PG, phosphoglycolate; ExoIII, exonuclease III.

Robson et al., 1991). In addition, a chemically modified DNA polymer containing isotopically labeled 3′-phosphoglycoaldehyde has been used extensively as a model substrate (Johnson & Demple, 1988b). To extend the methodology available for study of this reaction, a new type of substrate was developed exploiting the sequence-specific cleavage of DNA by bleomycin (Takeshita et al., 1978) to create an oligonucleotide with a unique terminal phosphoglycolate group. This substrate allows direct measurement of the removal of phosphoglycolate. Winters et al. (1994) recently reported the development of a 3′-phosphoglycolate-terminated oligonucleotide substrate similar to the one reported here. Rrp1 phosphodiesterase removes terminal phosphoglycolate, leaving a 3′-hydroxyl terminus that is stable under conditions that inhibit Rrp1 3′-exonuclease. Direct measurement of phosphate removal from a 3′-phosphate-terminated oligonucleotide also demonstrates a phosphatase activity 2 orders of magnitude lower than the Rrp1 3′-phosphodiesterase. Additional characteristics of the Rrp1 phosphodiesterase and phosphatase activities are presented and discussed.

MATERIALS AND METHODS

Reagents and enzymes were obtained from the following sources: hydrogen peroxide (H_2O_2), *tert*-butyl hydroperoxide (*t*-BuO_2H), iron ammoniumsulfate, and bleomycin from Sigma; deoxyribonucleotides from Pharmacia LKB Biotechnology, Inc.; uracil *N*-glycosylase and DNA polymerase I Klenow fragment from United States Biochemical Corp.; T4 polynucleotide kinase and exonuclease III from New England Biolabs; topoisomerase I from Life Technologies, Inc. Oligonucleotides were purchased from Oligo's, etc., and purified by preparative polyacrylamide gel electrophoresis prior to use. Plasmid DNA was purified by an alkaline lysis procedure followed by chromatography on Qiagen columns (Qiagen, Inc.).

Recombinant Rrp1 was prepared as previously described (Sander et al., 1993), and stored at −20 °C in buffer G (50% glycerol, 50 mM Hepes, pH 7.5, 50 mM NaCl, 0.1 mM Na_3-EDTA, 0.2 mM dithiothreitol, 0.2 mM phenylmethanesulfonyl fluoride, and 0.25 μg/mL each of the peptides pepstatin A, leupeptin, chymostatin, antipain, and aprotinin) at a concentration of 0.3 mg/mL or higher. The enzyme shows no detectable loss of activity stored under these conditions. Immediately prior to use, dilutions were made using the following diluent: 10% glycerol, 50 mM Hepes, pH 7.5, 50 mM NaCl, 0.1 mM EDTA, and 1 mg/mL BSA. The preparation and storage of Rrp1 in the presence of 0.1 mM EDTA result in an enzyme preparation devoid of nuclease activity in the absence of exogenous divalent metal cations. T4 endonuclease V was a generous gift of R. S. Lloyd (University of Texas).

Preparation of Nicked Circular Damaged DNA Substrates. A 5.4 kb plasmid, pRrp1C259, which has an 800 bp insert in the vector pET3d (Novagen, Inc.) was used to prepare all nicked circular DNA substrates. The conditions used for DNA damage treatments were as follows: (1) 25 mM sodium phosphate, pH 7.2, 10 mM NaCl, 5 mM $MgCl_2$, 10 μM iron ammonium sulfate, 0.05 μM bleomycin, and 80 ng/μL relaxed circular plasmid DNA incubated for 5 min at 37 °C; (2) 50 mM sodium phosphate, pH 7.2, 20 mM NaCl, 10 μM iron ammonium sulfate, 1 mM sodium ascorbate, and 100 ng/μL plasmid DNA incubated for 5 min at room temperature; (3) 50 mM sodium phosphate, pH 7.2, 20 mM NaCl, 1 mM sodium ascorbate, 0.03% hydrogen peroxide, and 30 ng/μL plasmid DNA incubated for 5 min at room temperature; (4) 150 mM Tris-HCl, pH 8.8, 1 mM $CaCl_2$, 50 mM NaCl, 40 ng/μL plasmid DNA, and micrococcal nuclease incubated 15 min at 37 °C; (5) 0.1 M NaCl, 0.02 M sodium acetate, pH 5.0, and 200 ng/μL plasmid DNA incubated 15 min at 65 °C; (6) 50 mM Hepes, pH 7.4, 20 mM NaCl, 1 mM DTT, 25 μg/mL BSA, 50 ng/μL apurinic plasmid DNA (as prepared in treatment 5 above), and T4 endonuclease V incubated 15 min at 37 °C. After treatment, samples were extracted with phenol/chloroform once and precipitated with ethanol (1, 4, 6) or were precipitated with ethanol (2, 3, 5). The number of nicks per molecule was estimated by electrophoresis in the presence of ethidium bromide followed by quantitative densitometry, essentially as described by Kuhnlein et al. (1976). Since the bleomycin and micrococcal nuclease treated substrates contain small amounts of linear DNA, the number of nicks per molecule was estimated using the method described by Hertzberg and Dervan (1984).

Reactivation Assay. Seventy-five nanograms of plasmid DNA was incubated 4 min at 30 °C in the presence of Rrp1 or exonuclease III or in the absence of enzyme in a 10 μL volume containing 50 mM sodium phosphate, pH 7.2, 120 mM NaCl, 0.2 mM Na_3EDTA, 5 mM $MgCl_2$, and 25 μg/mL BSA, followed by 10 min at 65 °C. Samples were cooled on ice. The reaction was adjusted to yield final conditions of 50 mM sodium phosphate, pH 7.2, 120 mM NaCl, 0.2 mM Na_3EDTA, 5 mM $MgCl_2$, 100 μM each of dATP, TTP, and dGTP, 1 μM dCTP, 1 mM DTT, 0.13 μCi/μL [α-^{32}P]dCTP, and 0.01 unit of Klenow polymerase in a volume of 15 μL, and incubation was for 30 min at 37 °C. Control experiments show that incorporation of radiolabel is linear with respect to time of polymerization and amount of polymerase under the conditions used (not shown). Reactions were terminated by addition of SDS, Na_3EDTA, and proteinase K to concentrations of 0.5%, 12 mM, and 15 μg/mL, respectively. Samples were analyzed by agarose gel electrophoresis and dried, and the radioactivity was quantitated using a Molecular Dynamics phosphorimager. Relative activity (Figure 2) was calculated in the following manner. All values were corrected by subtracting two types of background: (1) synthesis occurring in the presence of repair enzyme but using an undamaged control DNA, and (2) synthesis occurring in the absence of Rrp1 using the damaged DNA substrate (see Figure 1). Relative activity is the ratio of enzyme-dependent damage-dependent synthesis to enzyme-independent damage-independent synthesis [(signal − background)/background]. The undamaged control DNA used to determine the first type of background was the same DNA stock that was used for preparation of the damaged DNA substrates (Figure 1). Results were reproducible with at least three preparations of each DNA substrate. In addition, results were similar over a broad pH range from 6.2 to 9.5 and with either sodium phosphate or Tris-HCl as buffer (data not shown).

Preparation of DNA Substrates for AP Endonuclease, Phosphodiesterase, and Phosphatase Assays. AP endonuclease and phosphatase substrates: A 37 base oligonucleotide containing a unique uracil residue was 5′-^{32}P-end-labeled. The specific activity of the labeled oligonucleotide was

determined by running an aliquot of the kinase reaction on a 20% polyacrylamide gel and quantitating the amount of incorporated radioactivity [(5−10) × 10^4 cpm/ng]. The oligonucleotide was incubated at 30 °C overnight in 25 μL of 10 mM Tris-HCl, pH 8.0, and 50 mM EDTA containing 3 units of uracil- *N*-glycosylase. For the AP endonuclease substrate, the reaction was terminated by addition of 8 μL of formamide gel loading buffer (95% formamide, 20 mM EDTA, 0.05% Bromphenol Blue, and 0.05% Xylene Cyanol FF). For the phosphatase substrate, the sample was adjusted to 50 μL and 1.0 M piperidine, incubated at 90 °C for 30 min, lyophilized to dryness, resuspended in 50 μL of H_2O, lyophilized again, and resuspended in formamide gel loading buffer. Phosphodiesterase: A 31 base oligonucleotide containing a unique GpC dinucleotide was 5′-^{32}P-end-labeled, and specific activity was determined as described above. The oligonucleotide was precipitated by 6 volumes of ethanol and resuspended in 50 mM $NaHPO_4$, pH 7.2, and 50 mM NaCl and annealed with an equimolar amount of the complementary oligonucleotide by heating at 70 °C and slow cooling to room temperature. The sample was chilled on ice, and additions were made to the following final conditions: 0.4 mM DTT, 100 μM bleomycin, 100 μM iron(II) ammonium sulfate. Incubation proceeded on ice for 10 min. The sample was concentrated using a microcon-3 device, and 0.5 volume of formamide loading buffer was added. The substrates were purified on 12% (AP endonuclease and phosphatase) or 16% (phosphodiesterase) denaturing polyacrylamide gels and eluted using an Elutrap (Schleicher & Schuell). The recovery of oligonucleotide was determined, and a 2.5-fold molar excess of unlabeled complementary 35 base oligonucleotide was added. Annealing was carried out at 70 °C for 1 min in 100 mM Tris-HCl, pH 8.0, and 20 mM NaCl, containing 50 μg/mL BSA, followed by slow cooling to room temperature. [Some unlabeled complementary oligonucleotide remains present in the substrate as ssDNA; this ssDNA does not inhibit the Rrp1 reaction (Nugent et al., 1993).] The annealing efficiency was determined by comparing the electrophoretic mobility of the ssDNA oligonucleotide and the annealed dsDNA substrate on a nondenaturing 18% polyacrylamide gel.

Nuclease Assays. AP endonuclease, 3′-phosphodiesterase, 3′-phosphatase, and 3′-exonuclease reactions were carried out in buffer containing 50 mM Tris-HCl, pH 8.0, 0.2 mM EDTA, 5 mM $MgCl_2$, 50 μg/mL BSA, and 4−9 nM dsDNA oligonucleotide substrate in a final volume of 10 μL. NaCl was added as follows (or as indicated): AP endonuclease, 50 mM; 3′-phosphodiesterase, 150 mM; 3′-phosphatase, 10 mM; and 3′-exonuclease, 10 mM. Incubations were carried out at 30 °C for 5 min, or for 15 min for the 3′-phosphatase assay, and stopped by the addition of proteinase K to 0.1 mg/mL and EDTA to 25 mM. Proteinase K digestion was carried out at 42 °C for 15 min. Samples were prepared for gel electrophoresis by addition of 6 μL of formamide gel loading buffer (95% formamide, 20 mM EDTA, 0.05% Bromphenol Blue, and 0.05% Xylene Cyanol FF). Reaction products were analyzed on 16% or 12% (3′-phosphatase) denaturing polyacrylamide gels. The reaction products were quantitated using a Molecular Dynamics phosphorimager. The detection limit of this assay is estimated to be ≈1% of input substrate. One unit of activity produces 1 pmol of product in 1 min at 30 °C.

Rrp1 3′-exonuclease activity was measured with the oligonucleotide BL1-17. The amount of exonuclease activity with this oligonucleotide is significantly higher than the amount observed with other oligonucleotide substrates under similar conditions (Nugent et al., 1993) or with larger dsDNA substrates (Sander et al., 1993). Comparison of oligonucleotides with varying sequence suggests the difference between oligonucleotides is attributable to the sequence of the oligonucleotide (data not shown). The difference between the oligonucleotide assay and the values obtained by measuring soluble radioactivity released from a uniformly labeled dsDNA substrate reflects the degree to which the assay is sensitive to enzyme processivity.

Extension of BL1-17PG(Rrp1) with Klenow Polymerase. The DNA substrates [BL1-17PG(Rrp1) or BL1-17PG] were incubated for 20 min at 20 °C in the presence of 20 mM Tris-HCl, pH 8.0, 10 mM NaCl, 0.5 mM Na_3EDTA, 2 mM DTT, 5 mM $MgCl_2$, and 200 μM dNTPs and the presence or absence of 1 unit of Klenow polymerase and 0.5 ng of Rrp1. Samples were analyzed by electrophoresis on a 16% denaturing polyacrylamide gel.

RESULTS

Rrp1 was shown previously to display tightly associated high specific activity AP endonuclease activity. In this report, we have characterized the 3′-phosphodiesterase and 3′-phosphatase activities associated with Rrp1 that may play a role in DNA repair.

As an initial characterization of Rrp1 3′-phosphodiesterase, five ncDNA substrates were generated with reagents that cause strand breaks with 3′-blocking lesions. These substrates, as well as positive control (AP scDNA) and negative control (undamaged DNA) substrates, were used in an assay that links DNA repair with subsequent DNA synthesis. This assay measures indirectly the ability of the repair enzyme to remove 3′-blocking damage by measuring the stimulation of DNA synthesis after pretreatment of the substrate with repair enzyme. The following reagents were used to create 3′-blocked ssDNA breaks: bleomycin, iron(II) ammonium sulfate, hydrogen peroxide, depurination followed by endonuclease V, and micrococcal nuclease. In each case, the conditions chosen favor ssDNA breaks over dsDNA breaks and result in substrates with on average 1−4 nicks per molecule (data not shown). The 3′-terminal groups blocking DNA synthesis represented with these substrates include phosphate, phosphoglycolate, and 3′-terminal base-free deoxyribose. Rrp1 effectively removes 3′-blocking groups from the damaged DNA substrates, as shown in Figure 1, with the exception that nicked DNA generated by micrococcal nuclease is poorly activated for DNA synthesis. For the remaining substrates (including the positive control substrate AP scDNA), approximately 5-fold (on a weight basis) or 2-fold (on a molar basis) more Rrp1 than exonuclease III protein is required to stimulate a comparable amount of DNA synthesis (Figure 2). A 10-fold higher amount of Rrp1 is required to stimulate low levels of DNA synthesis from the phosphate-blocked substrate generated with micrococcal nuclease. In contrast, exonuclease III stimulates DNA synthesis much more efficiently with this substrate. To further characterize the 3′-DNA repair activities of Rrp1, oligonucleotide substrates were developed with either 3′-terminal phosphoglycolate or 3′-terminal phosphate

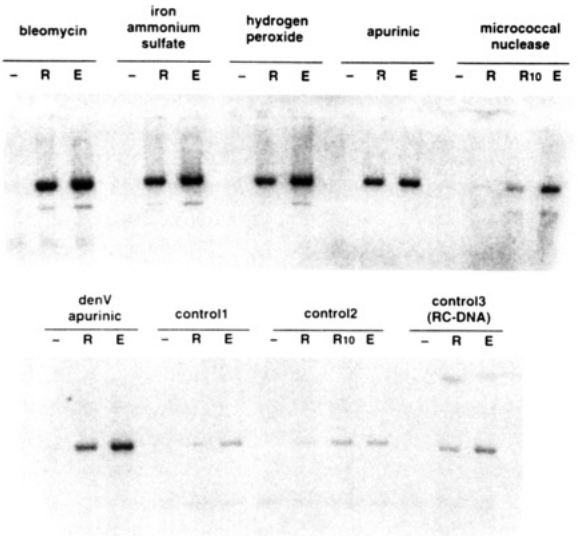

FIGURE 1: Reactivation assay. Each of the five damaged nicked circular DNA substrates, AP scDNA, or three undamaged control substrates was incubated in the absence of DNA repair enzyme (−), in the presence of 1 ng of Rrp1 protein (R) or 10 ng of Rrp1 (R_{10}), or in the presence of 0.2 ng of exonuclease III (E) during the first incubation for 4 min at 30 °C. Following heat inactivation of the repair enzyme (10 min, 65 °C), DNA synthesis by Klenow polymerase was carried out for all samples in the presence of [α-^{32}P]-dCTP. A phosphorimager scan of the dried gel is shown.

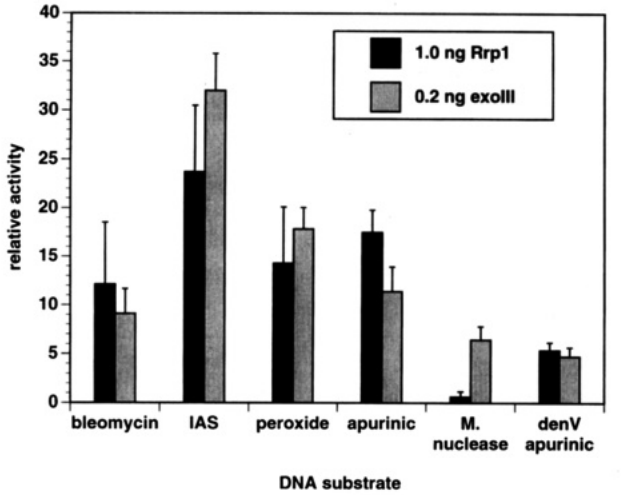

FIGURE 2: Graphic analysis of Rrp1 and exonuclease III activity in the reactivation assay. Values shown are the average and standard deviation (error bars) of at least three (Rrp1) or two (exonuclease III) data points.

groups. Bleomycin was used as a site-specific DNA cleaving agent to generate an oligonucleotide substrate for 3′-phosphodiesterase activity assays. As shown previously, bleomycin cleaves preferentially at GpC or GpT dinucleotides (Takeshita et al., 1978) and leaves 3′-termini carrying phosphoglycolate (Giloni et al., 1981). A dsDNA oligonucleotide was formed by hybridization of the two complementary oligonucleotides BL1 (31mer) and BL2 (35mer) (Figure 3A). Preparative cleavage of the dsDNA oligonucleotide shown in Figure 3A produces one major cleavage product, a 17mer oligonucleotide with a 3′-terminal phosphoglycolate (data not shown); as expected, and as shown below, this cleavage occurs at the unique GpC dinucleotide. The cleaved 5′-^{32}P-labeled oligonucleotide, BL1-17PG, was purified by preparative gel electrophoresis and annealed to BL2 (Figure 3A,B). As shown in Figure 4, BL1-17PG has

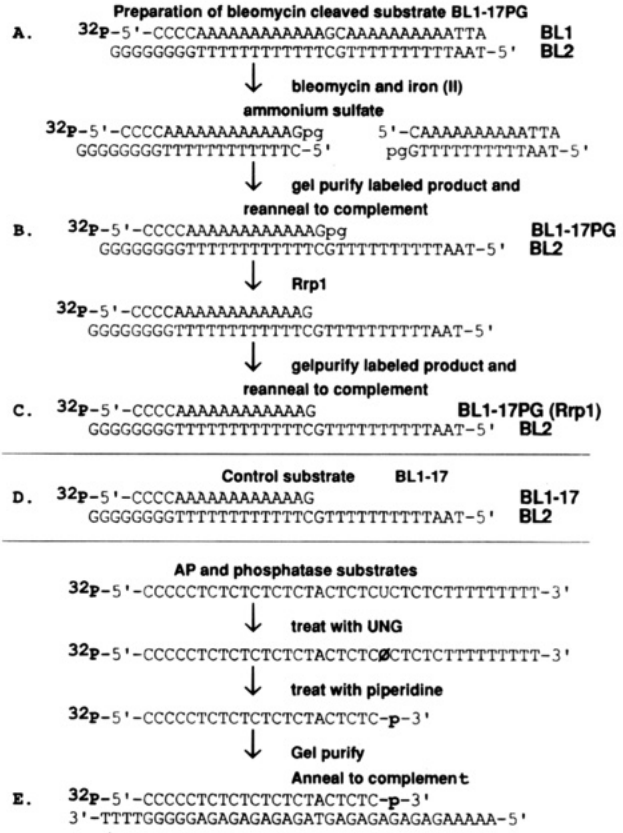

FIGURE 3: DNA sequences and procedures for oligonucleotide substrates. Oligonucleotide sequences used and the procedures followed to generate the substrates in Figures 4−7 are shown above. (A−D) Substrates for 3′-phosphodiesterase assay. PG stands for phosphoglycolate. (E) Substrate for 3′-phosphatase assay. UNG stands for uracil *N*-glycosylase; p is for phosphate.

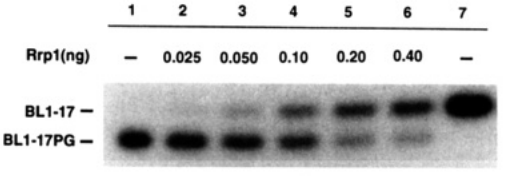

FIGURE 4: Rrp1 removes phosphoglycolate from the 3′-end of BL1-17PG. The partially dsDNA substrate shown in Figure 3B was incubated with varying amounts of Rrp1 for 5 min at 30 °C and analyzed on a 16% denaturing acrylamide gel. Rrp1 3′-exonuclease activity is suppressed by the addition of 100 mM NaCl. BL1-17 is the control oligonucleotide shown in Figure 3D. The mobilities of BL1-17PG and BL1-17 are indicated.

an electrophoretic mobility slightly faster than the 17mer identical to the first 17 nucleotides of BL1 (BL1-17, Figure 3D), and treatment of BL1-17PG with Rrp1 causes the oligonucleotide to undergo a mobility shift and comigrate with the 17mer BL1-17. This is consistent with removal of the 3′-phosphoglycolate end group by Rrp1, as is expected from previous studies of class II AP endonucleases with phosphoglycolate or phosphoglycoaldehyde terminated substrates (Levin & Demple, 1990; Bernelot-Moens & Demple, 1989; Demple et al., 1986; Winters et al., 1994). Rrp1 requires a dsDNA substrate and exogenous Mg^{2+} for the phosphoglycolate removal (not shown) as has been found for other nuclease functions of Rrp1 (Nugent et al., 1993).

As shown previously, Rrp1 is a 3′-exonuclease that cleaves the 3′-terminal phosphodiester bond between dNMP residues in undamaged dsDNA (Sander et al., 1991b, 1993). The exonuclease is not active on the product of Rrp1 phospho-

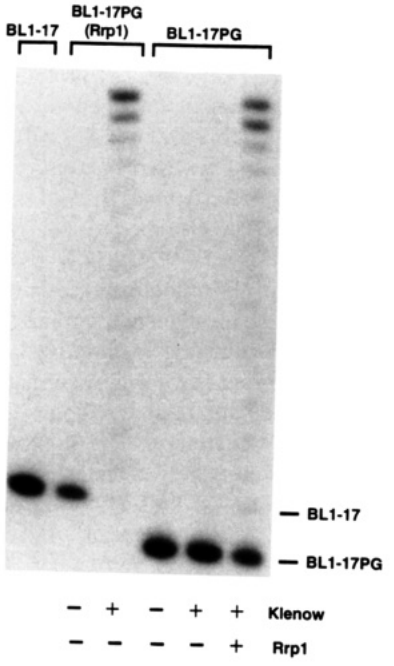

FIGURE 5: Product of Rrp1 phosphodiesterase activity on the bleomycin cleavage product has a 3′-hydroxyl terminus. The Rrp1 cleavage product of BL1-17PG [BL1-17PG (Rrp1)] was purified from a denaturing acrylamide gel. The structures of the two substrates [BL1-17PG/BL2 or BL1-17PG (Rrp1)/BL2] are shown in Figure 3B,C. Rrp1 (0.5 ng) or Klenow polymerase (1 unit) was incubated with each substrate as indicated, and analyzed on a 16% denaturing polyacrylamide gel. The mobilities of BL1-17PG and BL1-17 are indicated.

diesterase under the conditions shown here (Figure 4) since it is suppressed by addition of 100 mM NaCl (see below). The product of Rrp1 phosphodiesterase was gel-purified and annealed to the 35mer oligonucleotide BL2 to form a template-primer for DNA synthesis by Klenow DNA polymerase (Figure 3B,C). As shown in Figure 5, DNA synthesis occurs when the purified Rrp1 cleavage product is used as a primer, or when the primer BL1-17PG is treated with Rrp1 and Klenow polymerase simultaneously. The 17mer is extended to a 31mer against the template 35mer BL2. Therefore, Rrp1 cleaves the phosphoglycolate terminated oligonucleotide and forms a 3′-hydroxyl terminus.

The relative ability of Rrp1 to remove 3′-phosphoglycolate and undamaged 3′-terminal dGMP differs significantly when the two substrates are utilized under low ionic strength conditions (Figure 6). The rate of terminal base removal is reflected in the measurement of uncleaved substrate remaining in the presence of variable amounts of Rrp1. The specific activity of Rrp1 for these two reactions differs approximately 4-fold (see below).

The phosphatase activity of Rrp1 is likely to be important for repair of oxidative lesions, as well as lesions processed by other repair AP lyases that utilize a concerted $\beta-\delta$ elimination mechanism (Bailly et al., 1989). We developed a phosphatase substrate to directly examine the formation of a phosphate-free product by Rrp1. The preparation of this substrate is summarized in the bottom part of Figure 3. A 37mer oligonucleotide with a unique uracil residue 22 nucleotides from the 5′-end was treated with uracil *N*-glycosylase, cleaved with piperidine, and purified by gel electrophoresis. The 3′-phosphate terminated 22mer was annealed to a complementary 37mer (Figure 3E). As shown in Figure 7, Rrp1 is a class II AP endonuclease and cleaves

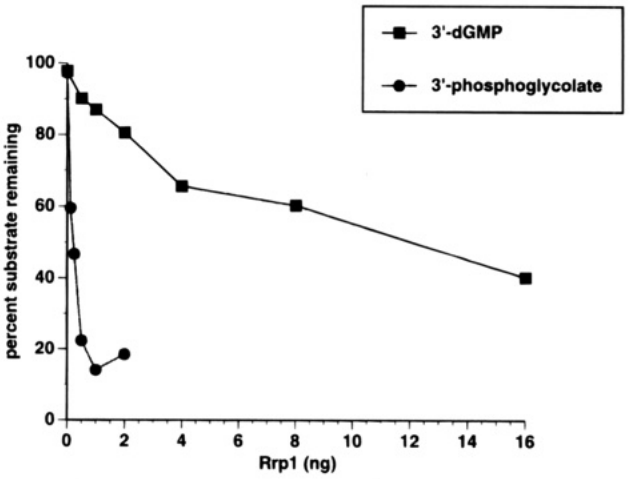

FIGURE 6: Rrp1 nuclease activity on 3′-phosphoglycolate and 3′-hydroxyl termini. The 3′-phosphoglycolate terminated (BL1-17PG, Figure 3B) or a 3′-dGMP terminated control substrate (BL1-17, Figure 3D) was incubated with increasing amounts of Rrp1 protein for 5 min at 30 °C. The reactions were carried out in buffer containing 10 mM NaCl. The amount of uncleaved DNA substrate remaining is shown.

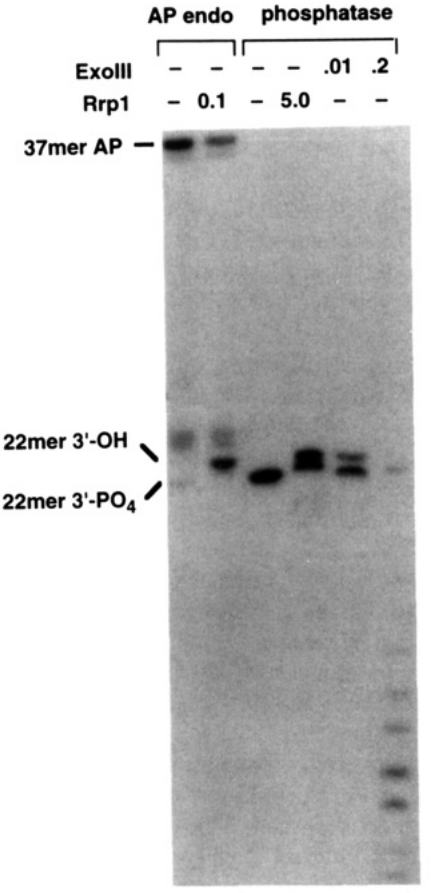

FIGURE 7: Rrp1 inefficiently removes 3′-terminal phosphate. AP endonuclease or phosphatase assays were carried out in the presence or absence of the indicated enzyme. The AP substrate (37mer AP) contains a unique AP site 23 nucleotides from its 5′-end (Figure 3, lower section). The AP product (22mer 3′-OH) is a 22mer with a 3′-hydroxyl end. The phosphatase substrate is identical in sequence to the AP product and has a 3′-phosphate end (Figure 3E). Exonuclease III digests the phosphatase substrate exonucleolytically, indicating that the substrate is dsDNA (a requirement for 3′-phosphatase activity and exonuclease activity).

the abasic oligonucleotide, forming a 3′-hydroxyl terminated 22mer. The 3′-phosphate 22mer oligonucleotide has an

Table 1: Relative Activity Values for Enzymatic Functions of Rrp1[a]

	Rrp1sp act., units/mg (SD)[b]	rel sp act.[c] ExoIII/Rrp1
phosphodiesterase	5.47×10^4 (0.75)	2.41
exonuclease[d]	1.35×10^4 (0.07)	3.34
phosphatase	1.08×10^3 (0.37)	55.87
AP endonuclease	4.75×10^4 (0.49)	0.37

[a] All values should be considered substrate-specific. Strong nucleotide sequence effects have only been observed to date for the exonuclease reaction (M. Sander, unpublished results). [b] One unit of activity produces 1 pmol of product per minute at 30 °C under the standard assay conditions. The average of three values is shown with the standard deviation in parentheses. [c] (Specific activity$_{ExoIII}$)(ExoIII monomer mol wt)/(specific activity$_{Rrp1}$)(Rrp1 monomer mol wt). [d] This value for the A-rich oligonucleotide BL1-17 is higher than the value observed for pyrimidine-rich sequences (M. Sander, unpublished results).

electrophoretic mobility slightly greater than the 3′-hydroxyl terminated 22mer; treatment with Rrp1 (5 ng) or exonuclease III (0.01 ng) results in a product that comigrates with the 3′-hydroxyl 22mer, as expected for phosphate removal. Higher amounts of exonuclease III degrade the substrate exonucleolytically; this indicates that the phosphatase substrate is fully double-stranded, since exonuclease III requires a dsDNA substrate for exonuclease activity (Rogers & Weiss, 1980). The specific activity (adjusted for the different size of the Rrp1 and exonuclease III monomers) of the Rrp1 phosphatase is 56-fold lower than the specific activity of exonuclease III, and 50-fold lower than the Rrp1 phosphodiesterase specific activity. This result confirms the data presented above indicating poor stimulation of DNA synthesis using the micrococcal nuclease nicked substrate (Figures 1 and 2).

The relative specific activities of Rrp1 for 3′-phosphodiesterase, 3′-exonuclease, 3′-phosphatase, and AP endonuclease are shown in Table 1. By comparison to exonuclease III, a lower relative activity is observed for all three reactions requiring cleavage of a terminal phosphoester bond (diester or monoester); the relative specific activity of exonuclease III/Rrp1 is 2.41, 3.34, and 55.87 for 3′-phosphodiesterase, 3′-exonuclease, and 3′-phosphatase, respectively. Relative to exonuclease III, the phosphatase function of Rrp1 appears to be reduced significantly more than the 3′-phosphodiesterase or 3′-exonuclease. All values reported here should be considered substrate-specific, since the DNA oligonucleotide sequence may affect the specific activity. To date we have observed strong sequence affects only for the 3′-exonuclease assay; recent observations indicate that purine-rich oligonucleotides, such as the one used here, are cleaved by Rrp1 much more efficiently than pyrimidine-rich sequences (Nugent et al., 1993; M. Sander, unpublished results).

Previous studies indicated that the ssDNA binding and exonuclease activities of Rrp1 are sensitive to inhibition by NaCl (M. Sander, unpublished results). Rrp1 3′-phosphatase demonstrates similar NaCl inhibition (Figure 8A). In the presence of 80 mM NaCl, the activity is reduced to either 30% or 5% for the exonuclease or phosphatase reaction, respectively. In contrast, Rrp1 AP endonuclease and 3′-phosphodiesterase are resistant to inhibition by 120 mM NaCl. Phosphodiesterase and AP endonuclease activities are reduced only to 50% or 40%, respectively, in the presence of 160 mM NaCl. The divalent metal ion requirements for these activities are compared in Figure 8B. After purification in the presence of 0.1 mM EDTA, Rrp1 protein is devoid of nuclease activity in the absence of exogenous divalent metal cations. Earlier work demonstrated that Ca^{2+} supports the Rrp1 AP endonuclease but not the 3′-exonuclease activity (Nugent et al., 1993). To extend that observation, the specific activity was compared for the four nuclease functions in the presence of either 5 mM $MgCl_2$ or 5 mM $CaCl_2$. The results demonstrate that phosphodiesterase is not inhibited by Ca^{2+}, AP endonuclease is only slightly inhibited, and both 3′-exonuclease and 3′-phosphatase are strongly inhibited. The ratios of the Ca^{2+}-supported to the Mg^{2+}-supported enzyme specific activities are 0.87 and 0.20 for 3′-phosphodiesterase and AP endonuclease, respectively. The 3′-exonuclease and 3′-phosphatase activities are barely detectable in the presence of Ca^{2+} and are reduced to less than 1% of the activity level in the presence of Mg^{2+}. However, Ca^{2+} does not inhibit Mg^{2+}-dependent 3′-exonuclease activity when both divalent ions are included in a single reaction (data not shown).

DISCUSSION

The ability of *Drosophila* Rrp1 to repair 3′-terminal damage to DNA has been demonstrated using three distinct activity assays: a linked repair/DNA synthesis assay, a 3′-phosphodiesterase assay that directly measures removal of terminal phosphoglycolate, and a 3′-phosphatase assay that directly measures removal of terminal phosphate. While Rrp1 efficiently repairs oxidative damage that includes AP sites or phosphoglycolate-blocked 3′-termini (Figures 2, 4, 6, and 8), it poorly repairs 3′-phosphate-blocked termini and is less efficient in removing undamaged terminal dNMP groups than phosphoglycolate termini (Figures 6 and 7). The 3′-phosphatase and 3′-exonuclease activities of Rrp1 are also sensitive to inhibition by NaCl and $CaCl_2$ (Figure 8).

The carboxy-terminal third of Rrp1 is homologous to the repair nuclease exonuclease III. Although the ability to perform each of the four nuclease functions, 3′-phosphodiesterase, 3′-exonuclease, 3′-phosphatase, and AP endonuclease, is conserved between the two proteins, the ratio of these activities is not conserved. Interestingly, several human AP endonucleases differ both from exonuclease III and from Rrp1 in the ratio of these activities. Chen et al. (1991) describe two human 3′-repair diesterases, one of which has approximately equal 3′-phosphodiesterase and AP endonuclease activity (1:7 ratio of specific activities, 3′-phosphodiesterase to AP endonuclease) and a second with much greater AP endonuclease than 3′-diesterase (1:160 ratio of specific activities). The latter protein is proposed to be identical to hAPE, also known as HAP1 and REF1, and both enzymes are reported to have similar levels of 3′-phosphatase and 3′-phosphodiesterase activity (Chen et al., 1991). hAPE is also known to be deficient in 3′-exonuclease (Demple et al., 1991). Therefore, the most distinctive similarity for the members of this protein family is an efficient AP endonuclease activity. The conservation of this one function suggests that it is important for the biological role of the eukaryotic homologues. It is possible that additional enzymes unrelated to exonuclease III have an important role in repairing strand breaks with 3′-blocking groups. A precedent for altered biological roles for eukaryotic AP endonucleases is the APN1 protein from *Saccharomyces cerevisiae*. APN1 is a homologue of endonuclease IV, the

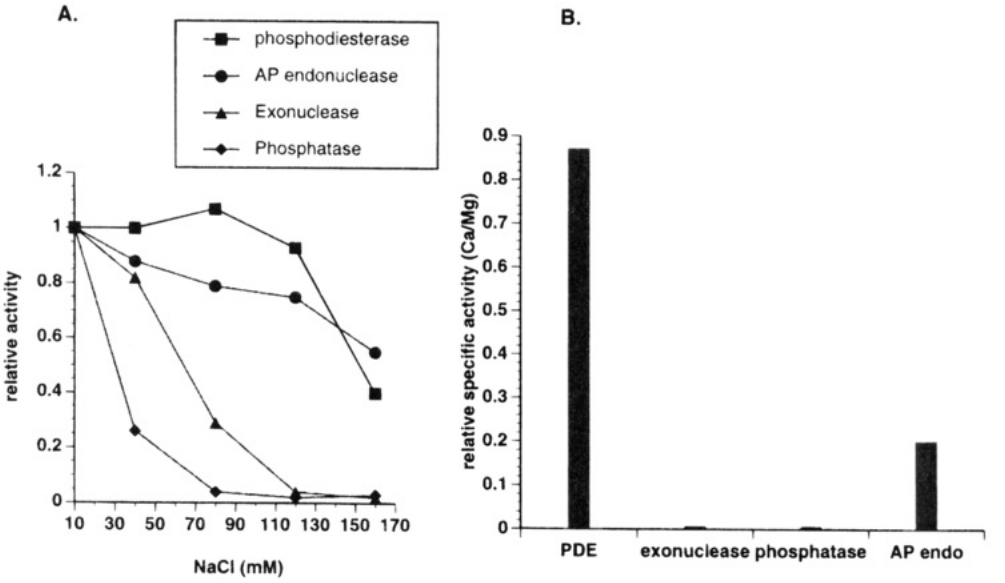

FIGURE 8: NaCl and $CaCl_2$ inhibition of Rrp1 nuclease activities. (A) Nuclease assays were carried out in the presence of variable amounts of NaCl. Activity is normalized to the amount of activity in the presence of 10 mM NaCl, the lowest concentration tested. Values shown are the average of four determinations. (B) The specific activity of Rrp1 was measured in the presence of 5 mM $MgCl_2$ and 5 mM $CaCl_2$. The ratio of the Ca^{2+}-dependent activity to the Mg^{2+}-dependent activity is shown. PDE is for phosphodiesterase.

enzyme present at low but inducible levels in *E. coli* (Chan & Weiss, 1987). In contrast, APN1 is a constitutive enzyme and is thought to be the only AP endonuclease and 3′-phosphodiesterase active in yeast cells (Popoff et al., 1990; Johnson & Demple, 1988a).

We report here that the 3′-phosphatase activity of Rrp1 is very weak with a relative specific activity 56-fold lower than exonuclease III. It is possible that both 3′-phosphate and 3′-diester terminated strand breaks result as *in vivo* products of oxidative DNA damage (von Sonntag, 1987). Therefore, the low 3′-phosphatase activity of Rrp1 may be related to the observation that Rrp1 confers variable levels of protection against reagents that cause oxidative damage. Studies of Rrp1 complementation of repair-deficient *E. coli* demonstrate a significant but low level of protection against hydrogen peroxide-induced lethality, but a higher level of protection against DNA damage induced by *t*-BuO_2H (Gu et al., 1993). In addition, in a recent study of Rrp1 mutants in which the 3′-phosphatase and 3′-phosphodiesterase activities are mutationally separated, a specific correlation between enzymatic deficiency in 3′-phosphatase and a loss of the ability to complement hydrogen peroxide-induced DNA damage is demonstrated (Gu et al., 1994). Mutagenesis of Rrp1 protein also reveals that the 3′-phosphatase activity is more readily inactivated by mutation than either the AP endonuclease or the 3′-phosphodiesterase activity (Gu et al., 1995). It may be possible to select Rrp1 mutants with increased phosphatase activity by screening for alterations that increase the ability of Rrp1 to confer resistance to oxidative DNA damage *in vivo*, and such mutants might identify amino acid residues in Rrp1 that result in its poor 3′-phosphatase activity.

It will be of interest to determine the *in vivo* relevance of the 3′-phosphodiesterase and 3′-phosphatase functions of Rrp1 in *Drosophila*. Other repair nucleases from *Drosophila* are known but not well characterized (Grabowski et al., 1991; Spiering & Deutsch, 1986). It is likely that other *Drosophila* enzymes exist to repair 3′-blocking damage, as is known for *E. coli* and human cells (Chen et al., 1991; Demple et al., 1986; Bernelot-Moens & Demple, 1989). In light of the poor repair of 3′-phosphate-blocked ends by Rrp1, a more efficient 3′-phosphatase might be required for a high level of resistance to oxidative DNA damage. However, it remains possible that stimulatory factors present *in vivo* in *Drosophila* enhance the 3′-phosphatase activity of Rrp1.

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REFERENCES

Ames, B. N., Shinegawa, M. K., & Hagen, T. M. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 7915.
Bailly, V., Verly, W. G., O'Connor, T., & Laval, J. (1989) *Biochem. J. 262*, 581.
Bernelot-Moens, C., & Demple, B. (1989) *Nucleic Acids Res. 17*, 587.
Chan, E., & Weiss, B. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 3189.
Chen, D. S., Herman, T., & Demple, B. (1991) *Nucleic Acids Res. 19*, 5907.
Cunningham, R. P., Saporito, S. M., Spitzer, S. G., & Weiss, B. (1986) *J. Bacteriol. 168*, 1120.
Demple, B., Johnson, A., & Fung, D. (1986) *Proc. Natl. Sci. U.S.A. 83*, 7731.
Demple, B., Herman, T., & Chen, D. S. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 11450.
Farr, S. B., D'Ari, R., & Touati, D. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 8268.
Friedberg, E. C. (1985) in *DNA Repair*, W. H. Freeman, New York.
Giloni, L., Takeshita, M., Johnson, F., Iden, C., & Grollman, A P. (1981) *J. Biol. Chem. 256*, 8608.
Grabowski, D. T., Deutsch, W. A., Derda, D., & Kelley, M. R. (1991) *Nucleic Acids Res. 19*, 4297.
Greenberg, J. T., & Demple, B. (1988) *EMBO J. 7*, 2611.
Gu, L., Huang, S.-M., & Sander, M. (1993) *Nucleic Acids Res. 21*, 4788.
Gu, L., Huang, S.-M., & Sander, M. (1994) *J. Biol. Chem. 269*, 32685.
Hertzberg, R. P., & Dervan, P. B. (1984) *Biochemistry 23*, 3934.
Hutchinson, F. (1985) *Prog. Nucleic Acid Res. Mol. Biol. 32*, 115.

Johnson, A. W., & Demple, B. (1988a) *J. Biol. Chem. 263*, 18017.
Johnson, A. W., & Demple, B. (1988b) *J. Biol. Chem. 263*, 18009.
Kuhnlein, U., Penhoet, E. E., & Linn, S. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 1169.
Levin, J. D., & Demple, B. (1990) *Nucleic Acids Res. 18*, 5069.
Lindahl, T. (1982) *Annu. Rev. Biochem. 51*, 61.
Nugent, M., Huang, S.-M., & Sander, M. (1993) *Biochemistry 32*, 11445.
Orr, W. C., & Sohal, R. S. (1994) *Science 263*, 1128.
Popoff, S. C., Spira, A. I., Johnson, A. W., & Demple, B. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 4193.
Robson, C. N., Milne, A. M., Pappin, J C., & Hickson, I. D. (1991) *Nucleic Acids Res. 19*, 1087.
Rogers, S. G., & Weiss, B. (1980) *Methods Enzymol. 65*, 201.
Sancar, A., & Sancar, G. B. (1988) *Annu. Rev. Biochem. 57*, 29.
Sander, M., Lowenhaupt, K., Lane, W. S., & Rich, A. (1991a) *Nucleic Acids Res. 19*, 4523.
Sander, M., Lowenhaupt, K., & Rich, A. (1991b) *Proc. Natl. Acad. Sci. U.S.A. 88*, 6780.
Sander, M., Carter, M., & Huang, S.-M. (1993) *J. Biol. Chem. 268*, 2075.
Seki, S., & Oda, T. (1988) *Carcinogenesis 9*, 2239.
Seki, S., Ikeda, S., Watanabe, S., Hatsushika, M., Tsutsui, K., Akiyama, K., & Zhang, B. (1991) *Biochim. Biophys. Acta 1079*, 57.
Spiering, A. L., & Deutsch, W. A. (1986) *J. Biol. Chem. 261*, 3222.
Takeshita, M., Grollman, A. P., Ohtsubo, E., & Ohtsubo, H. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 5983.
von Sonntag, C. (1987) in *The chemical basis of radiation biology*, pp 221−294, Taylor & Francis, London.
Winters, T. A., Weinfeld, M., & Jorgensen, T. J. (1992) *Nucleic Acids Res. 20*, 2573.
Winters, T. A., Henner, W. D., Russell, P. S., McCullough, A., & Jorgensen, T. J. (1994) *Nucleic Acids Res. 22*, 1866.

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