Characterization of the Apurinic Endonuclease Activity of *Drosophila* Rrp1

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ABSTRACT: *Drosophila* Rrp1 (Recombination repair protein 1) belongs to a family of DNA repair nucleases that includes *Escherichia coli* exonuclease III, *Streptococcus pneumoniae* exonuclease A, bovine BAP, mouse APEX endonuclease, and human APE. Within a 252 amino acid region, collinear homology is shared between all members. Rrp1 is unique in that it includes a 427 amino acid N-terminal region not related to any known sequence. The protein copurifies with an apurinic endonuclease and a double-stranded DNA 3′-exonuclease. In this study, a 5′-end-labeled 37 base pair oligonucleotide substrate containing a single apurinic site was used to characterize the endonuclease activity of Rrp1. This substrate is utilized efficiently by Rrp1: the specific activity observed is 1×10^5 units/mg. The abasic double-stranded DNA oligonucleotide is cleaved only at the abasic site to create a single-strand break. Strand breaks are not detected in the complementary strand, in the single-stranded DNA oligonucleotide, or in the base-paired control substrate. After endonucleolytic cleavage at the abasic site, exonucleolytic processing at the nick is slow and requires a molar excess of Rrp1, while exonuclease III degrades the nicked substrate more efficiently. The Rrp1 cleavage product comigrates with a DNaseI cleavage product, and the newly formed terminus supports DNA synthesis by DNA polymerase. Therefore, Rrp1 cleaves the phosphodiester backbone at one position 5′ to the apurinic site and leaves a 3′-hydroxyl terminus. Rrp1 is a class II apurinic endonuclease and is likely to be important in DNA repair in *Drosophila.*

Abasic sites in DNA are estimated to accumulate spontaneously in the genome at a rate of approximately 10^4 lesions/cell/day (Lindahl & Nyberg, 1972). In addition, abasic DNA sites are induced by both oxidative and alkylation damage (Friedberg, 1985), and they are lethal lesions that can block a DNA replication fork *in vitro* (Loeb, 1985; Strauss et al., 1982; Boiteux & Laval, 1982). Repair of these common lesions is facilitated by apurinic/apyrimidinic (AP)[1] endonucleases and AP lyases, a ubiquitous group of enzymes that recognize this form of DNA damage and cleave the phosphodiester backbone, thus preparing for polymerase-catalyzed replacement of the missing base. The importance of this group of enzymes for maintaining genomic integrity is confirmed by the increase in spontaneous mutation rate in yeast and *Escherichia coli* mutants that are deficient in AP endonuclease function (Ramotar et al., 1991; Robson & Hickson, 1991; Cunningham et al., 1986). These mutants also demonstrate large increases in lethality after exposure to agents that induce DNA damage (Cunningham et al., 1986).

At least five AP endonucleases and AP lyase activities are known in *E. coli* (Kornberg & Baker, 1992). Two of these are class II AP endonucleases, which cleave DNA by a hydrolytic mechanism on the 5′-side of the abasic site, leaving 3′-hydroxyl termini at the single-strand breaks (Linn et al., 1981). These two proteins, endonuclease IV and exonuclease III, are both members of a conserved family of proteins and have been extensively studied (Popoff et al., 1990; Sander et al., 1991a; Demple et al., 1991). Exonuclease III is a constitutive activity that is abundant in *E. coli*, representing 80–90% of the total measurable AP endonuclease activity in a crude extract. Endonuclease IV is an inducible activity: it represents either 5% or up to 50% of the activity in uninduced or induced cells, respectively (Chan & Weiss, 1987).

Exonuclease III is a multifunctional enzyme that is a 3′-exonuclease specific for dsDNA, a class II AP endonuclease, a 3′-phosphatase, and a 3′-phosphodiesterase. The AP endonuclease activity of exonuclease III is essential for the repair of abasic sites, as indicated by the fact that a *dut* (dUTPase) *xth* (exonuclease III) double-mutant cell has a conditional lethal phenotype that can be rescued by a third mutation in the *ung* gene (uracil-*N*-glycosylase) (Taylor & Weiss, 1982). *xth* mutants are hypersensitive to hydrogen peroxide (Demple et al., 1983) and *xth nfo* double mutants, deficient in both exonuclease III and endonuclease IV, show large increases in lethality in comparison to the *nfo* single mutants after exposure to DNA-damaging agents (Cunningham et al., 1986).

Recently, a family of proteins related to exonuclease III was characterized. Exonuclease A from *Streptococcus pneumoniae* is similar to exonuclease III both at the amino acid sequence level and in its enzymatic properties (Puyet et al., 1989). Four eukaryotic homologues belonging to this protein family are known, including human APE, mouse APEX, bovine BAP, and *Drosophila* Rrp1 (Demple et al., 1991; Robson et al., 1991; Seki et al., 1991a; Sander et al., 1991a). All members of the family share a homologous carboxy-terminal region 252 amino acids in length, in which regions of tandem conserved amino acids are found. Each of these proteins is an efficient AP endonuclease. However, only exonuclease III, exonuclease A, mouse APEX, and *Drosophila* Rrp1 are active on undamaged DNA (3′-exonuclease); the human and bovine proteins have lost this enzymatic capability (Demple et al., 1991; Robson et al., 1991).

Drosophila Rrp1 is an unusual member of the exonuclease III protein family since it is unique in its enzymatic properties and its physical structure. The Rrp1 protein sequence includes a 427 amino acid N-terminal region not found in other

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[1] Abbreviations: AP, apurinic/apyrimidinic; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; bp, base pair; BSA, bovine serum albumin.

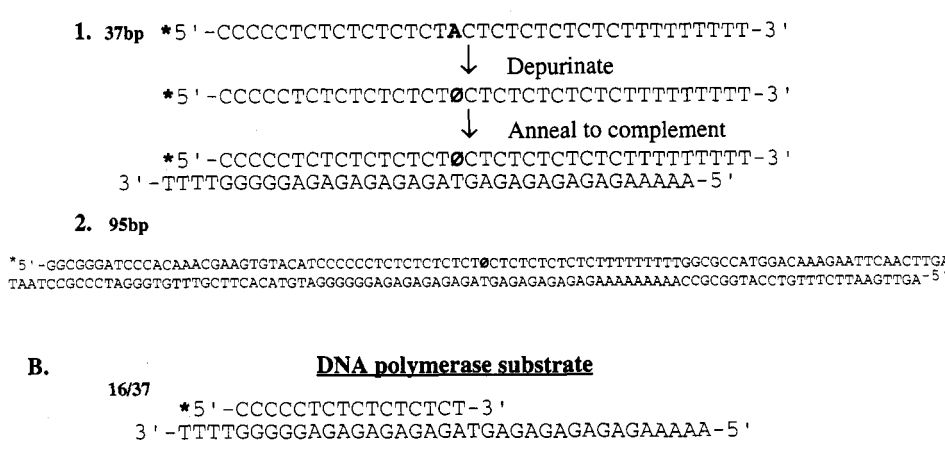

FIGURE 1: DNA substrates. The sequences of the DNA substrates are shown. (A) Part 1 summarizes the protocol for preparation of the dsDNA 37-bp apurinic substrate (see Materials and Methods). Part 2 shows the sequence of the 95-bp dsDNA apurinic substrate. (B) The primer template used as a T4 DNA polymerase substrate is shown. The 16-nucleotide primer is the primary product of the Rrp1 cleavage reaction using the 37 bp oligonucleotide substrate.

members of the protein family or in other known protein sequences (Sander et al., 1991a). Two enzymatic functions, ssDNA renaturation and DNA strand transfer, are associated with this region of Rrp1 (Sander et al., 1991b, 1993). The nuclease activities of Rrp1 were characterized previously and shown to be similar to those of exonuclease III: Rrp1 has a high specific activity AP endonuclease and a dsDNA specific-3′-exonuclease (Sander et al., 1991b, 1993). The biological function(s) of Rrp1 in *Drosophila* is not yet known since Rrp1 mutants have not yet been isolated. However, recent work establishes that Rrp1 is active in complementing the repair deficiencies of *E. coli* strains that are deficient in both exonuclease III and endonuclease IV (Gu et al., 1993). Thus, Rrp1 is likely to be important in DNA repair in *Drosophila*.

In this study, the AP endonuclease activity of Rrp1 is examined using a 37-bp dsDNA oligonucleotide substrate containing a unique AP site. The specificity of this reaction is characterized with respect to substrate requirements, the cleavage site, the structures of the cleaved termini, and the specific activity of the reaction. A comparison of Rrp1 and exonuclease III catalyzed cleavage of this substrate is also presented. This work demonstrates that Rrp1 is a class II AP endonuclease. This enzymatic capability and the 3′-exonuclease activity of Rrp1 are likely to be important to its function *in vivo*.

MATERIALS AND METHODS

Proteins and DNA. T4 polynucleotide kinase and T4 DNA ligase were purchased from New England Biolabs. Klenow DNA polymerase and Exo⁻Klenow were purchased from USB. Oligonucleotides were purchased from Oligo's, Etc., Inc. (Oregon) and purified by preparative polyacrylamide gel electrophoresis prior to use.

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 EDTA, 0.2 mM dithiothreitol, 0.2 mM phenylmethane sulfonyl chloride, 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. Unless otherwise indicated, recombinant Rrp1 purified from *E. coli* cells was used in all experiments.

Exonuclease III was purchased from New England Biolabs. No variations in enzyme characteristics were observed when several lots were tested. The enzyme stock was stored at −20 °C in a buffer containing 50% glycerol, 200 mM KCl, 5 mM KPO_4 (pH 6.5), 0.05 mM EDTA, 5 mM β-mercaptoethanol, and 0.2 mg/mL BSA. Working dilutions were made as described for Rrp1.

Preparation of an Oligonucleotide Substrate Containing a Unique Apurinic Site. A 37-base oligonucleotide containing a unique purine residue was [5′-^{32}P]-end-labeled (oligonucleotide sequences are shown in Figure 1). 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 37 °C in 40 mM HCl for 20 h, resulting in partial depurination, purified on a 20% denaturing polyacrylamide gel, and eluted from the gel using an Elutrap (Schleicher and Schuell). The recovery of oligonucleotide was determined, and a 2-fold molar excess of an unlabeled complementary 37-base oligonucleotide was added. Annealing was carried out at 37 °C for 30 min in 100 mM Tris-HCl (pH 8.0) and 20 mM NaCl containing 50 μg/mL BSA. The sample was cooled to room temperature and stored at 4 °C. (Some unlabeled complementary oligonucleotide remains present in the substrate as ssDNA. As shown in Figure 4, this ssDNA does not inhibit the Rrp1 reaction.) The annealing efficiency was determined by comparing the electrophoretic mobility of the ssDNA oligonucleotide and the annealed dsDNA substrate on a nondenaturing 20% polyacrylamide gel. The efficiency of depurination (30–50%) was determined by quantitative cleavage with 1 M piperidine for 30 min at 90 °C followed by electrophoretic separation of the cleaved and uncleaved oligonucleotide. The final substrate contains variable amounts (less than 1% of the total labeled substrate) of a fast migrating labeled material that has not been characterized (see, for example Figures 2 and 3).

Preparation of a 95-bp Substrate Containing a Unique Apurinic Site. The apurinic oligonucleotide was prepared as described above, omitting the labeling step. Prior to electrophoresis, the depurinated oligonucleotide was mixed with four other unlabeled oligonucleotides and one [5′-^{32}P]-end-

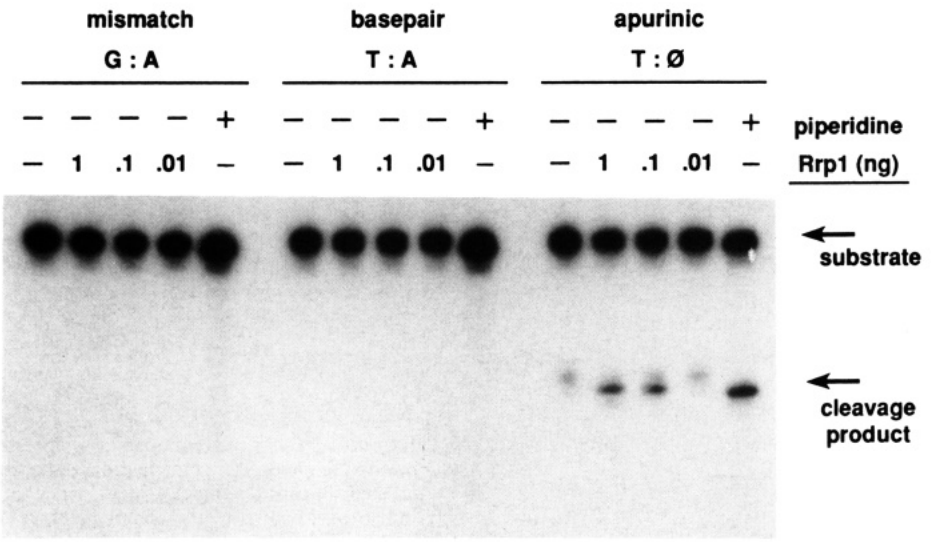

FIGURE 2: Rrp1 AP endonuclease cleavage of the apurinic oligonucleotide substrate. Rrp1 cleavage reactions were incubated at 30 °C for 5 min with the indicated dsDNA oligonucleotide substrate and variable amounts of Rrp1 protein. In the presence of the apurinic substrate (T:Ø), a unique Rrp1 cleavage product (arrow) is seen that migrates slightly slower than the piperidine marker. (Piperidine cleaves apurinic and apyrimidinic sites quantitatively, leaving 3′-phosphoryl termini.) No cleavage of either the T:A Watson–Crick base-paired or the G:A mismatched substrate is observed.

labeled oligonucleotide in the presence of T4 DNA ligase. These six oligonucleotides can be ligated to form the unique dsDNA 95 bp substrate with one labeled strand shown in Figure 1A, part 2. The oligonucleotide sequences are as follows: ^{32}P-GGCGGGATCCCACAAACGAAGTGTACATC, AAAGAGAGAGAGAGTAGAGAGAGAGA, GGCGCCATGGACAAAGAATTCAACTTGAA, AGTTGAATTCTTTGTCCATGGCGCCAAAAAA and GGGGGGATGTACACTTCGTTTGTGGGATCCCGCCTAAT. The sample was ligated for 5 h and then loaded onto a 12% denaturing polyacrylamide gel. The labeled and unlabeled strands comigrate during this electrophoresis. They were coeluted from the gel and renatured. Renaturation was confirmed by electrophoretic separation and was close to maximal efficiency.

AP Endonuclease Assay Using the 37-bp Oligonucleotide Substrate. Apurinic endonuclease reactions were carried out in buffer containing 50 mM Tris-HCl (pH 8.0), 10 mM NaCl, 0.2 mM EDTA, 5 mM $MgCl_2$, 50 μg/mL BSA, and 4 nM dsDNA oligonucleotide substrate in a final volume of 10 μL. Incubations were carried out at 30 °C 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 the addition of 6 μL of formamide gel loading buffer (95% formamide, 20 mM EDTA, 0.05% Bromphenol blue, 0.05% Xylene Cyanol FF). Reaction products were analyzed on 20% denaturing polyacrylamide gels either 0.8 or 0.4 mm thick. One unit of AP endonuclease activity cleaves 1 pmol of AP sites per minute at 30 °C. When required, the reaction products were quantitated using a Molecular Dynamics phosphorimager. The detection limit of this assay is estimated to be ≈1% of input substrate.

AP Endonuclease Assay Using the 95-bp Oligonucleotide Substrate. Reactions were carried out as for the 37-bp substrate with the following changes: The substrate was preincubated with or without Rrp1 for 20 min at 12 °C in reaction buffer lacking $MgCl_2$. Cleavage was initiated by the addition of one-tenth volume of 10 mM $MgCl_2$ and 20 mM $CaCl_2$. Incubation was continued for 6 min at 12 °C. The reactions were stopped and analyzed by gel electrophoresis on a 12% denaturing polyacrylamide gel as described above.

Other Methods. Protein concentrations were determined by the Coomassie blue dye binding assay of Bradford (1976).

RESULTS

The Rrp1 AP endonuclease was previously characterized using a partially depurinated plasmid DNA substrate (Sander et al., 1991b). In this assay, a high specific activity endonuclease is detected both for native Rrp1 purified from *Drosophila* tissue and for recombinant Rrp1 purified from *E. coli* (Sander et al., 1993). In many other studies, oligonucleotide substrates have been successfully used to characterize enzymes involved in DNA repair (Takeshita et al., 1987; Naser et al., 1988; Houten et al., 1986; Sanderson et al., 1989). To further study the AP endonuclease activity of Rrp1, a synthetic 37-bp oligonucleotide substrate containing a single abasic site was constructed. The oligonucleotide sequence is shown in Figure 1A, along with a summary of the method used for substrate preparation. A polypyrimidine oligonucleotide containing a single adenine residue was [5′-^{32}P]-end-labeled, partially depurinated by acid treatment, and then annealed with its complement to produce a double stranded 37-bp substrate containing one centrally located AP site and four-base, 3′-protruding ends. The ssDNA 3′-protruding ends prevent exonucleolytic degradation of the substrate, since Rrp1 only poorly degrades ssDNA termini. The degree of substrate depurination was determined by cleavage to completion with piperidine and varied from ≈30 to 50%.

The specificity of the Rrp1 AP endonuclease was tested using three substrates (Figure 2), including the depurinated 37 bp substrate (T:Ø), a control substrate containing a T:A base pair in place of the AP site, and a substrate containing a G:A mismatch in place of the AP site. Rrp1 cleavage reactions were incubated with the indicated substrates and variable amounts of Rrp1. No cleavage product is evident for either the substrate containing a G:A mismatch or the T:A Watson–Crick base-paired substrate. However, a unique Rrp1 cleavage product is seen when Rrp1 is coincubated with the AP substrate (T:Ø; see arrow in Figure 2). The Rrp1 product has a mobility very similar to that of the piperidine marker. This result indicates that the Rrp1 endonuclease recognizes and cleaves AP sites with high specificity. We estimate a

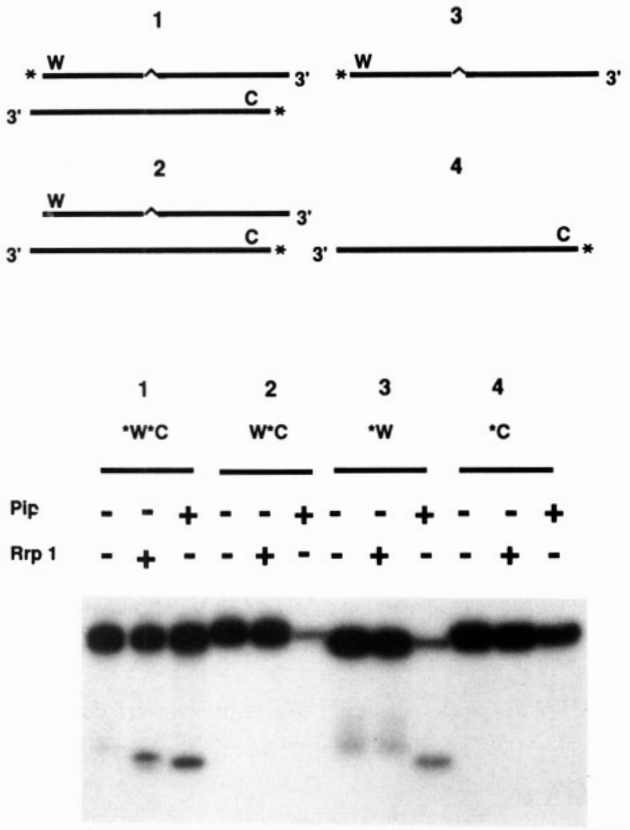

FIGURE 3: Single-strand cleavage occurs only on the apurinic DNA strand of the dsDNA substrate. The non-apurinic ssDNA oligonucleotide (C) was ^{32}P-end-labeled and hybridized to an either labeled or unlabeled complementary Watson strand (W) containing a unique apurinic site (∧) to form substrates 1 and 2, respectively. Substrates 3 and 4 are the ^{32}P-end-labeled apurinic (W) or non-apurinic (C) ssDNA oligonucleotides. Rrp1 cleavage reactions were carried out in the presence of excess enzyme (3 ng) for 8 min. Cleavage product is observed only for substrate 1, indicating that no dsDNA breaks are made and that Rrp1 requires a dsDNA substrate.

minimum of 10–20-fold preferential cleavage at the abasic site, on the basis of the sensitivity limit of this assay. The depurinated 37-bp substrate contains a small amount (less than 1% of the total labeled substrate) of preexisting fast migrating material (Figures 2 and 3, lanes without enzyme added). Quantitative analysis of additional cleavage reactions with Rrp1 indicate that the Rrp1 product derives from the intact 37-nucleotide oligonucleotide containing an abasic site, and not from this small amount of contaminant in the substrate.

Four additional substrates were designed to test whether Rrp1 makes dsDNA endonucleolytic cleavages and whether Rrp1 requires a dsDNA substrate (Figure 3). For substrate 1, the 37-bp dsDNA oligonucleotide is [5′-^{32}P]-end-labeled on both the apurinic "Watson" strand (W) and on its native complement (C). Substrate 2 is the same dsDNA oligonucleotide, but it is labeled only on the strand complementary to the abasic strand. Substrate 3 is single-stranded and is identical to the labeled apurinic strand of substrate 1. Substrate 4, also single-stranded, is identical to the labeled complementary strand in substrate 2. Rrp1 cleavage products are observed only for substrate 1. Cleavage activity is not detectable on either of the ssDNA substrates, whether it includes an AP site (substrate 3) or lacks an AP site (substrate 4). Furthermore, the labeled cleavage product of substrate 2 is not detected, indicating that Rrp1 cleaves only the abasic strand when present in a dsDNA substrate. Taken together, these results indicate that Rrp1 cleaves the abasic strand of a dsDNA substrate and cleaves ssDNA with a much lower

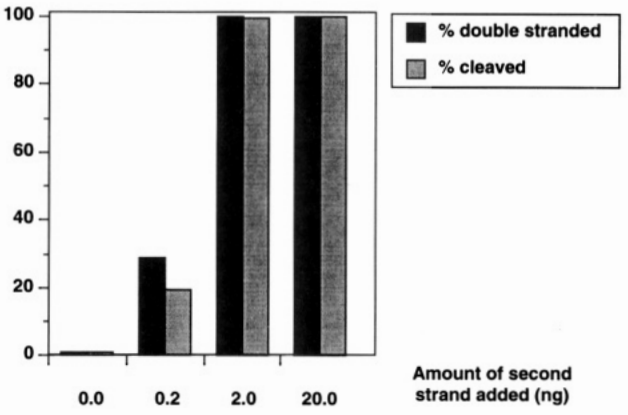

FIGURE 4: Cleavage reactions in the presence of varying amounts of complementary oligonucleotide. A constant amount of ^{32}P-end-labeled apurinic oligonucleotide was incubated in the presence of the indicated amounts of the complementary oligonucleotide or in its absence. The amount of dsDNA substrate present was determined (black bars), and these samples were used in reactions with 1.6 ng of Rrp1 protein for 5 min. The amount of Rrp1 cleavage product (gray bars) increases with increasing amounts of dsDNA substrate.

efficiency. Two other eukaryotic AP endonucleases from calf thymus (Sanderson et al., 1989) and mouse (Haukanes et al., 1989) are known to have either an absolute requirement for ssDNA or a strong preference for ssDNA, respectively.

A more quantitative analysis of the Rrp1 requirement for dsDNA is presented in Figure 4. Increasing amounts (0, 0.2, 2.0, and 20 ng) of the complementary strand were annealed with a constant amount of the labeled abasic strand of the oligonucleotide to form substrates that had different ratios of double-stranded and single-stranded molecules. In the absence of the complementary oligonucleotide, no Rrp1 cleavage product is detected (gray bars), indicating the poor ability of Rrp1 to cleave a single-stranded abasic substrate. At 0.2 ng of second strand, approximately 30% of the labeled AP strand was in a double stranded form (black bars), yielding a cleavage efficiency of approximately 20% relative to that observed in the presence of excess second strand. At 2.0 and 20.0 ng of second strand, all of the abasic strand was annealed with its complement. Under these conditions, relative cleavage efficiency is at its maximal level.

Rrp1 possesses a dsDNA-specific 3′-exonuclease activity. However, in earlier studies it was noted that the ratios of the 3′-exonuclease and the AP endonuclease specific activities of Rrp1 and its homologue exonuclease III are dramatically different: with a linear dsDNA substrate, at least a 2 order of magnitude lower specific activity is observed for Rrp1 (Sander et al., 1993). Both of the 3′-ends of the dsDNA oligonucleotide substrate described above are protected from exonucleolytic degradation by their ssDNA character: the rate of terminal base removal by Rrp1 from four-base 3′-protruding ends is approximately 10-fold slower than the rate of removal from a blunt dsDNA end (Sander et al., 1991b; and data not shown). After endonucleolytic cleavage of the dsDNA AP oligonucleotide substrate at the AP site, the newly generated 3′-end is a potential substrate for the Rrp1 3′-exonuclease. The newly generated 5′-end is not expected to be sensitive to removal by Rrp1 (Sander et al., 1991b). In a separate experiment the stability of the 5′-end at the cleaved AP site was confirmed by using a 3′-endlabeled oligonucleotide substrate (data not shown).

We examined the 3′-exonuclease activity of Rrp1 at the nicked AP site under conditions of varying enzyme concentration and reaction time. Standard Rrp1 cleavage reactions

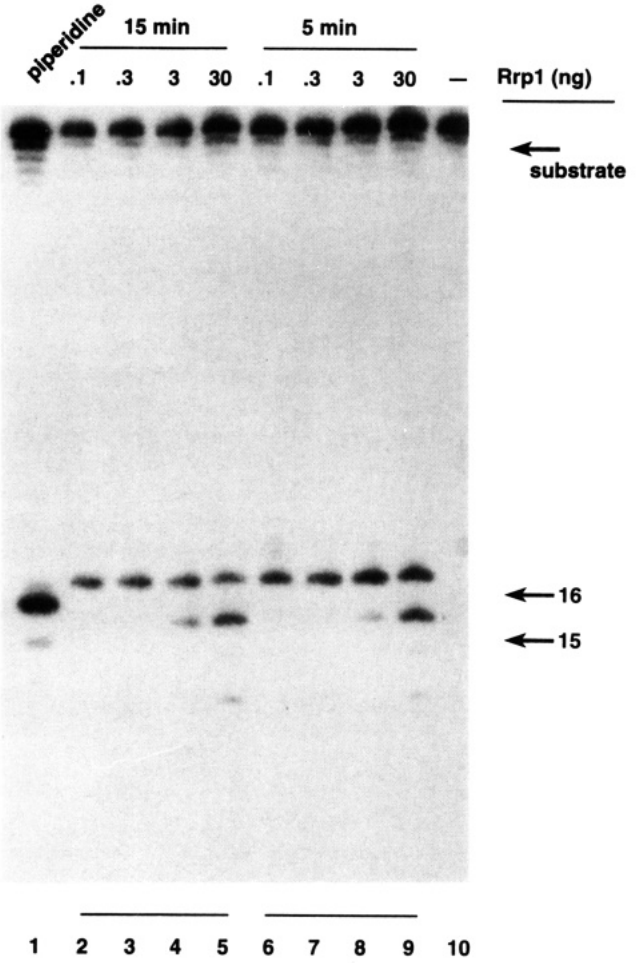

FIGURE 5: Appearance of the 15-nucleotide cleavage product. Appearance of the 15-nucleotide cleavage product is slow and requires excess Rrp1 protein. Cleavage reactions are shown in the presence of varying amounts of Rrp1 protein as indicated. Aliquots of the reactions were removed after 5 or 15 min of incubation. The mobilities of the 16- and 15-nucleotide products are indicated.

were carried out using 0.1, 0.3, 3, and 30 ng of Rrp1, and aliquots of the reaction were removed at 5 or 15 min (Figure 5). The molar ratio of Rrp1 monomer to substrate oligonucleotide is varied from 0.03 to 10 in this experiment. Products of the reaction were analyzed on a DNA sequencing gel. Appearance of the 16-nucleotide endonucleolytic cleavage product is rapid, with the reaction being complete after a 5-min incubation in the presence of 0.1 ng of Rrp1 (lane 6). Under these conditions, Rrp1 is acting catalytically, since there is a 31:1 molar excess of substrate molecules to Rrp1 monomers. Degradation by Rrp1 of the primary cleavage product to a 15 nucleotide product is slow, however, and requires a greater than equimolar amount of Rrp1 monomer (lanes 4, 5, 8, and 9). Rrp1 3′-exonuclease activity on the 15-nucleotide product occurs only with the highest amount of enzyme (10-fold molar excess), but appreciable amounts of 14- and 13-nucleotide products do not accumulate. Indeed, Rrp1 3′-exonuclease activity appears to be limited to an inefficient removal of one base from the initial 16-nucleotide cleavage product. Incubation with 30 ng of Rrp1 results in the removal of an average of 0.62 base from the primary cleavage product in 5 min and 0.86 base in 15 min.

We compared the exonuclease and AP endonuclease activities of Rrp1 and exonuclease III (Figure 6). Cleavage reactions were carried out for 2 min at 30 °C with 0.03, 0.3, 3, or 30 ng of Rrp1 (panel A) or with 0.02, 0.2, 2, or 20 ng of exonuclease III (panel B) in the presence of either 5 mM $MgCl_2$ or 5 mM $CaCl_2$. The molar ratio of Rrp1 monomer to substrate oligonucleotide is varied from 0.01 to 10 in this experiment. In the presence of $CaCl_2$, the exonuclease activity of exonuclease III is dramatically lower than that in the presence of $MgCl_2$. Therefore, a comparison of the endonuclease activity of the two proteins can be made in the presence of this divalent cation. By quantitation of the data shown in Figure 6, we determined that exonuclease III demonstrates an AP endonuclease specific activity that is 7-fold higher than the specific activity of Rrp1. In the presence of $MgCl_2$, the Rrp1 AP endonuclease specific activity is 1×10^5 units/mg (Figure 6 and data not shown). A reliable exonuclease III AP endonuclease specific activity is difficult to determine in the presence of $MgCl_2$, due to the enzyme's rapid degradation of the primary cleavage product (Figure 6). However, the average number of bases each enzyme removes from its primary cleavage product can be calculated in order to compare their exonuclease function using this substrate. By quantitating the representation of each exonuclease product produced in an experiment similar to the one shown in Figure 6, we found that 2 ng of exonuclease III removes an average of 7.2 bases from the primary cleavage product in 2 min. As seen in Figure 6, exonuclease III can degrade the apurinic substrate to six nucleotides in the presence of $MgCl_2$. As described above, a molar excess of Rrp1 is required for the inefficient removal of one base in 5 min. Clearly, exonuclease III utilizes this 3′-terminus more efficiently than Rrp1.

To confirm that the weak exonuclease activity present in the Rrp1 fraction is intrinsic to Rrp1, an exonuclease-deficient mutant that lacks the 142 carboxy-terminal amino acids was characterized (Sander et al., 1993). This mutant protein was overexpressed and purified by the same protocol as intact Rrp1. Exonuclease assays, including assays similar to those shown in Figures 5 and 6, using primer–template substrates and a molar excess of the mutant protein demonstrate no residual exonuclease assay associated with this protein fraction (data not shown). Thus, the exonuclease activity observed in Figures 5 and 6 is not due to a contaminating exonuclease but is intrinsic to the Rrp1 protein.

The structure of the newly generated DNA 3′-terminus after Rrp1 endonucleolytic cleavage at the AP site was determined in the following experiments. First, the mobility of the reaction product was compared to markers with known terminal structures: a DNaseI cleavage product and a piperidine cleavage product. The major Rrp1 cleavage product comigrates with the DNase I product and migrates slightly slower than the piperidine product, as shown in Figure 7A. Since DNaseI is known to produce 3′-hydroxyl termini, this suggests that Rrp1 cleaves the phosphodiester backbone at one position 5′ to the AP site, leaving a 3′-hydroxyl terminus. The piperidine cleavage product is known to have a 3′-terminal phosphate group. Since the piperidine marker migrates slightly faster than the Rrp1 product, this is consistent with a 3′-hydroxyl group at the terminus generated by Rrp1.

A DNA polymerase assay was used to confirm the 3′-terminal structure of the major Rrp1 product, since polymerase activity on the Rrp1 cleavage product requires a 3′-hydroxyl structure. The 16-nucleotide product of the Rrp1 cleavage reaction was purified and annealed to the unlabeled 37-nucleotide complement to form a primer–template for the polymerase reaction (Figure 1B). This substrate was incubated with the indicated dNTPs in the presence of either Klenow DNA polymerase or exonuclease-free Klenow DNA polymerase. Reaction products were analyzed on a DNA sequencing gel. A single adenine residue can be added to the

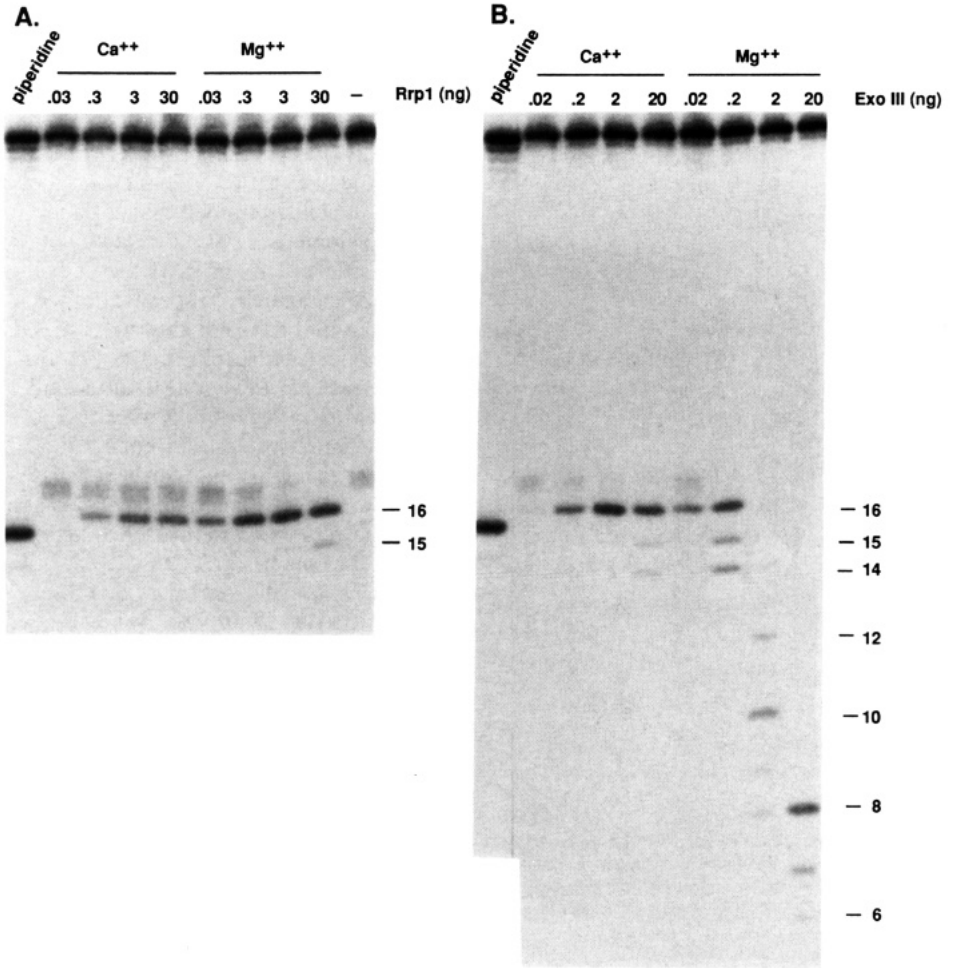

FIGURE 6: Comparison of the exonuclease and AP endonuclease activities of Rrp1 and exonuclease III. Cleavage reactions were carried out for 2 min at 30 °C with the indicated amounts of either Rrp1 (A) or exonuclease III (B). For reactions including $MgCl_2$, the buffer contained 50 mM Tris-HCl (pH 8.0), 10 mM NaCl, 0.2 mM EDTA, 5 mM $MgCl_2$, and 50 μg/mL BSA (lanes marked Mg^{2+}). For reactions including $CaCl_2$, the $MgCl_2$ was omitted and 5 mM $CaCl_2$ was substituted (lanes marked Ca^{2+}). The AP endonuclease specific activities of Rrp1 and exonuclease (exoIII) III differ by less than 7-fold in the presence of $CaCl_2$, which suppresses the exonuclease of exoIII. In the presence of $MgCl_2$, exoIII degrades the nicked substrate to a 6-nucleotide product, while Rrp1 inefficiently removes one nucleotide. The size of cleavage products is indicated in nucleotides.

16 nucleotide primer to form a 17-nucleotide product (Figure 7B, dA lanes). Minor bands indicating misincorporation of adenine in positions 18 and 19 are seen only in the presence of exonuclease-deficient Klenow DNA polymerase. In the presence of dGTP, dCTP, and dTTP, no significant polymerization by Klenow DNA polymerase occurs, due to the absence of dATP to incorporate opposite the unique T residue in the template (Figure 7B, dGCT lane). In the presence of dATP, dCTP, and dTTP, however, all of the 16-nucleotide primer can be extended to 33 bases in length (dACT lanes) by Klenow or Exo$^-$Klenow. We conclude that Rrp1 cleaves at the 5′-side of the abasic site in this substrate and leaves a 3′-hydroxyl terminus. This defines Rrp1 as a class II AP endonuclease.

The Rrp1 monomer (M_r = 75 000) is significantly larger than other well characterized AP endonucleases, including exonuclease III (M_r = 29 000). If the 37-bp oligonucleotide substrate used in this study were shorter than the preferred Rrp1 DNA substrate, an aberrant reaction might be observed. Therefore, the properties of Rrp1 AP endonuclease activity on a 95-bp substrate containing a single AP site flanked by random sequence DNA were examined. The central 37 bp of this DNA fragment is a polypyrimidine region similar to the 37-bp substrate described above. Using asymmetric protruding ends to assure a unique 95-bp product, oligonucleotides containing random sequence DNA were ligated to the apurinic fragment to form the substrate shown in Figure 1A, part 2. Rrp1 cleavage of this substrate results in a 45-nucleotide cleavage product with a mobility slightly slower than that of the piperidine cleavage product (Figure 8). Quantitation of this experiment shows that the fraction of cleaved molecules is identical for Rrp1 and piperidine, indicating the efficient utilization of this substrate and the high specificity of the endonuclease. In addition, the newly formed 3′-hydroxyl terminus is poorly utilized by the Rrp1 3′-exonuclease, since the 45-nucleotide primary cleavage product persists. A similar cleavage pattern was observed when several reactions conditions were varied, including temperature, $MgCl_2$ concentration, and Rrp1 concentration (data not shown). We conclude that Rrp1 acts similarly on the 37- and 95-bp substrates.

DISCUSSION

Drosophila Rrp1 belongs to a family of proteins that is conserved from *E. coli* to humans. The structural similarity between these proteins suggests conservation of biological and enzymatic functions. We have characterized the AP endonuclease activity of Rrp1 using an oligonucleotide DNA substrate and found it to be similar to previously studied class II AP endonucleases. Rrp1 makes a ssDNA nick adjacent to abasic sites in dsDNA substrates. The newly generated 3′-

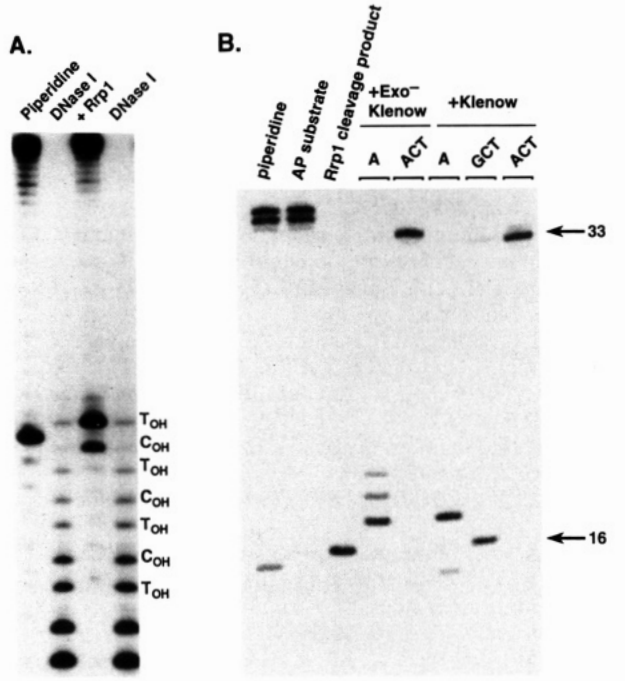

FIGURE 7: Rrp1 cleavage production of a 3′-hydroxyl DNA terminus. The product of the Rrp1 cleavage reaction comigrates with a DNaseI cleavage product and has a 3′-terminus that can be utilized by Klenow DNA polymerase. (A) The mobilities of the cleavage products produced by Rrp1 are compared to the mobilities of DNaseI cleavage products on a 20% DNA sequencing gel. (B) The 16-nucleotide product of the Rrp1 cleavage reaction was purified and annealed to the 37-nucleotide complement to form a primer–template for a polymerase reaction (see Figure 1B). This substrate was incubated with either Exo$^-$ Klenow polymerase or Klenow polymerase and the indicated dNTPs (125 μM) for 5 min at 30 °C. Reaction products were analyzed on a 20% sequencing gel.

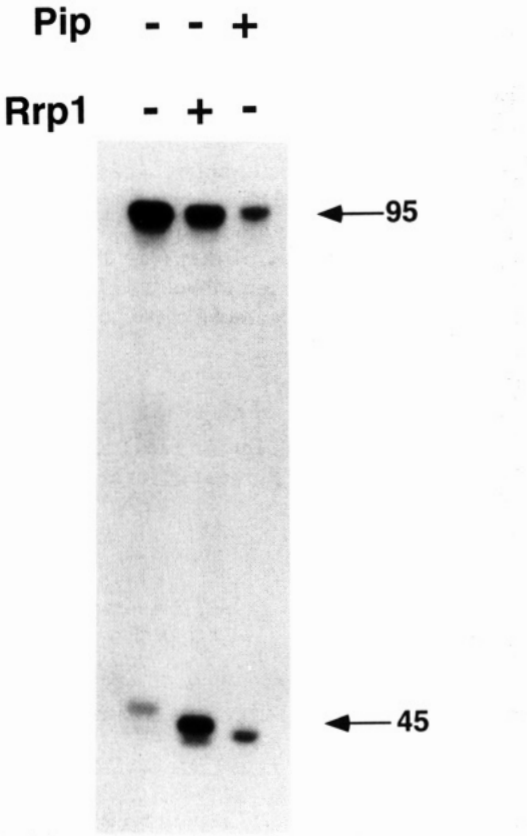

FIGURE 8: Rrp1 cleavage of a 95-bp AP substrate. Cleavage reactions were carried out in the presence or absence of 6 ng of Rrp1 as indicated. Piperidine cleaved substrate is shown as a marker. The mobilitiesof the substrate (95) and the product (45) bands are marked with arrows.

end is on the 5′-side of the abasic site and is a hydroxyl terminus that can be utilized by DNA polymerase.

It was shown previously, with a partially depurinated plasmid DNA substrate, that Rrp1 has a high specific activity AP endonuclease (10^5 units/mg; Sander et al., 1993). The specific activity is similar when the oligonucleotide is used as a substrate. In the presence of Mg^{2+}, it is difficult to compare the specific activities of Rrp1 and exonuclease III. However, in the presence of Ca^{2+}, Rrp1 and exonuclease III AP endonucleases differ in specific activity by less than 7-fold with this DNA substrate. Since Rrp1, like its protein homologues, prepares AP sites for repair polymerase with a high catalytic efficiency, it seems likely that this conserved *in vitro* function represents a conserved *in vivo* function as well.

In contrast to the AP endonuclease, the exonuclease function of exonuclease III is not conserved among the members of this protein family, since it is not present in purified fractions from either human or bovine cells and is attenuated for Rrp1 (Sander et al., 1993) and mouse APEX proteins (Seki et al., 1991b). The Rrp1 3′-exonuclease activity has a low specific activity and does not extensively degrade dsDNA (Sander et al., 1991b, 1993). At the nicked AP site, molar excess is required in order to observe the slow removal of a single nucleotide from the newly generated 3′-end (Figures 5 and 6). When a primer–template substrate similar to the substrate shown in Figure 1B is used in an Rrp1 exonuclease reaction, the 3′-terminal base of the primer is removed extremely slowly (data not shown). In addition, similarly limited exonuclease activity is observed using the 95-bp AP substrate (Figures 1A and 8 and data not shown). Although it is conceivable that the active Rrp1 protein possesses a binding site larger than a 37-bp oligonucleotide, it is unlikely that the Rrp1 binding site is larger than a 95-bp oligonucleotide. This diminishes the possibility that poor Rrp1 exonuclease activity results from ineffective substrate binding due to substrate size. However, the DNA sequence may influence the Rrp1 exonuclease rate with oligonucleotide substrates, since polyadenine sequences are degraded more rapidly than other substrates that have been tested (data not shown). In other studies, we observe that Rrp1 removes 20–50 nucleotides per end from an 800-bp dsDNA fragment with blunt ends; in contrast, exonuclease III removes up to 400 nucleotides per end (Sander et al., 1991b, 1993; Lowenhaupt et al., 1989; and data not shown).

Class II AP endonucleases are involved in the repair of DNA strand breaks carrying 3′-blocking lesions that inhibit polymerase function. The weak 3′-exonuclease activity of Rrp1 observed at nicked AP sites (Figures 5 and 6) suggests that this function may not be conserved. However, this suggestion is not consistent with recent studies demonstrating that Rrp1 repairs both alkylation- and oxidation-induced DNA damage in *E. coli* repair deficient cells (Gu et al., 1993). The possibility that Rrp1 acts as a 3′-phosphodiesterase against blocking lesions more efficiently than as a 3′-exonuclease is currently being tested. It is also possible that the Rrp1 exonuclease is more active *in vivo* than *in vitro*.

At least five AP endonucleases are known in *E. coli*, including two well-characterized class II enzymes: exonuclease III and endonuclease IV. While exonuclease III is constitutive in *E. coli*, Rrp1 is expressed in a developmentally regulated manner in *Drosophila* (data not shown), suggesting that other AP endonucleases could be important in DNA repair in *Drosophila*. In addition to Rrp1, several distinct activities have been identified (Spiering & Deutsch, 1981; Spiering &

Deutsch, 1986; Grabowski et al., 1991). The precise roles of the Rrp1 AP endonuclease and 3′-exonuclease activities *in vivo* are not yet known. Unlike exonuclease III and the other protein homologues, Rrp1 can also carry out homologous recombination reactions *in vitro*, including DNA strand transfer and ssDNA renaturation (Sander et al., 1991b, 1993). It is possible that the DNA repair and homologous recombination activities of Rrp1 function in a coordinate manner *in vivo*; alternatively, Rrp1 may play distinct roles in DNA repair and homologous recombination. It is hoped that future studies will distinguish among these possibilities.

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