**Human RAD2 Homolog 1 5’- to 3’-Exo/Endonuclease Can Efficiently Excise a Displaced DNA Fragment Containing a 5’-Terminal Abasic Lesion by Endonuclease Activity**

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Repair of abasic lesions, one of the most common types of damage found in DNA, is crucial to an organism’s well-being. Studies in vitro indicate that after apurinic-apyrimidinic endonuclease cleaves immediately upstream of a baseless site, removal of the 5’-terminal sugar-phosphate residue is achieved by deoxyribophosphodiesterase activity, an enzyme-mediated β-elimination reaction, or by endonucleolytic cleavage downstream of the baseless sugar. Synthesis and ligase complete repair.

Eukaryotic RAD2 homolog 1 (RTH1) nuclease, by genetic and biochemical evidence, is involved in repair of modified DNA. Efficient endonucleolytic cleavage by RTH1 nuclease has been demonstrated for annealed primers that have unannealed 5’-tails. In vivo, such substrate structures could result from repair-related strand displacement synthesis. Using 5’-tailed substrates, we examined the ability of human RTH1 nuclease to efficiently remove 5’-terminal abasic residues. A series of upstream primers were used to increasingly displace an otherwise annealed downstream primer containing a 5’-terminal deoxyribose-5-phosphate. Until displacement of the first annealed nucleotide, substrates resisted cleavage. With further displacement, efficient cleavage occurred at the 3’-end of the tail. Therefore, in combination with strand displacement activity, RTH1 nucleases may serve as an important alternative to other pathways in repair of abasic sites in DNA.

Abasic lesions occur in DNA for several reasons, including spontaneous depurination (1), release of the base from a damaged sugar residue, or enzymatic removal of an inappropriate (e.g., uracil) or damaged (e.g., alkylated, deaminated, or oxidized) base by specialized glycosylases (2). Timely repair of abasic lesions is necessary, since during replication the lesion could lead to potentially lethal or mutagenic substitutions (3).

Repair of an abasic site is predominantly initiated by an apurinic-apyrimidinic (AP) endonuclease that cleaves immediately upstream of the baseless sugar, creating a 3’-hydroxyl terminus and a 5’-deoxyribose-5-phosphate terminus (4, 5). The next step, removal of the baseless sugar from the downstream strand, likely occurs by one of three distinct mechanisms.

One mechanism involves removal of 5’-terminal sugar-phosphate residues by a Mg²⁺-dependent hydrolytic reaction, which releases 2-deoxyribose-5-phosphate. The activity responsible for this reaction, deoxyribophosphodiesterase (dRpase), was discovered in *Escherichia coli* (6). It was later shown to result from cleavage by either exonuclease I (7, 8) or the RecJ protein (9). The product of the *RecJ* gene was previously identified as a single strand-specific 5′- to 3′-exonuclease (10). Nonetheless, enzymes with dRpase activity have no associated double strand-specific 5′- to 3′-exonuclease function, so only a 1-nucleotide gap is produced after removal of the baseless sugar. A DNA polymerase fills in the single gap, and then a DNA ligase fuses the nick. There is support for this pathway, known as base excision repair. It has been shown in vitro that the majority of uracil residues in DNA are repaired by replacing a single nucleotide (11), and this pathway has been reconstituted with purified enzymes, one of which was the RecJ protein (12). There is also evidence of dRpase activity in *Deinococcus radiodurans* (13) and in humans (14), but these activities have been only partially purified and, therefore, have not yet been linked with a specific gene.

Another mechanism entails removal of a 5′-terminal baseless sugar-phosphate residue by a β-elimination reaction, releasing unsaturated deoxyribose-phosphate derivatives. The C(α)-O-P bond 3′ to a 5′-terminal abasic site is extremely labile, with a half-life of about 2 h in the absence of any β-elimination catalysts (e.g., spermine or basic proteins), prompting some to propose that enzymatic removal of the sugar-phosphate residue may not be necessary (15, 16). Others have demonstrated that a β-elimination reaction can be catalyzed by the Fpg protein in *E. coli* (17) and by DNA polymerase β in a reconstituted system (18).

A third mechanism involves removal of 5′-terminal sugar-phosphate residues by endonucleolytic cleavage downstream of the baseless sugar. Previous studies indicate that the 5′- to 3′-exonuclease activity of *E. coli* DNA polymerase I (6, 19) and human DNase IV (14, 19) cannot release free 2-deoxyribose-5-phosphate from the 5′-terminus at a single strand break in

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** The abbreviations used are: AP, apurinic-apyrimidinic; dRpase, deoxyribophosphodiesterase; RTH1, RAD2 homolog 1; PEI, polyethyleneimine; PAGE, polyacrylamide gel electrophoresis; D, downstream primer; M, miscellaneous primer; T, template; U, upstream primer.
DNA. Although these enzymes can endonucleolytically remove the abasic residue as part of small oligonucleotides, cleavage occurs at a slow rate (6, 14, 19).

DNAse IV and the 5' nuclease domain of E. coli DNA polymerase I are reported to belong to a class of enzymes the members of which share structural and functional homology (20). This class includes the Saccharomyces pombe RAD2 protein (21); the product of the YKL510 open reading frame (21), called RTH1 (22); murine flap endonuclease 1 (23); human RTH1 (also called flap endonuclease 1) (24); and a calf 5'- to 3'-exonuclease and -endonuclease activities as described previously (25, 26). Active fractions were pooled and further purified by hydroxyapatite as described in Murante et al. (25) for calf RTHI. The final preparation was 95% pure according to analysis by SDS-PAGE stained with silver. Purified enzyme (0.15 mg/ml) was dialyzed into a storage buffer (20% glycerol, 50 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, and 1 mM EDTA and EGTA) and then stored at -80 °C.

3'Radiolabeled Substrates with and without an Abasic Residue at the 5'-Terminus—For control experiments using substrates without an abasic residue at the 5'-terminus, a 22-mer oligonucleotide (M22, Table I) with a deoxyuridine at the 5'-terminus was annealed to a complementary template (T22, Table I) so that a single unpaired guanine nucleotide remained at the 5'-terminus of the template. The primer M22 was radiolabeled by template-directed incorporation of [γ-32P]ATP (3000 C/mmol) at its 3'-terminus using Sequenase (version 2.0). The resulting 23-mer (D1; Table I) primer with a labeled 3'-terminus and a 5'-terminal deoxyuridine was gel purified and annealed to a 39-mer template (T39, Table I) to form one of four primary subsubstrates (D1:T39, Fig. 2). For experiments using substrates with an abasic lesion at the 5'-terminus, a 39-mer oligonucleotide (M39, Table I) with an internal deoxyuridine was 3'-radiolabeled as above, gel purified, and then annealed to the template (T39). In every instance, template T39 was allowed at least 4 residues to overhang at both 3'-ends to prevent degradation of the substrate by exonuclease III at a later step (Fig. 1, lane 1). The substrate was stored at -20 °C until use. For each experiment, a small aliquot was removed from storage and further prepared as follows. The uracil base was removed using uracil-DNA-glycosylase at 37 °C for 120 min. The substrate was then incubated at 37 °C with 2 units of exonuclease III for 35 min to specifically cleave on the 5'-side of the deoxyribose-5-phosphate lesion. Under the conditions used, exonuclease III continued to degrade the resulting upstream strand until only 3–5 nucleotides remained (Fig. 1, lane 5), at which point this short primer could be easily displaced from the template. This formed the second primary substrate (D1D2:T39, Fig. 2). Exonuclease III was removed by centrifuging the substrate through a Micropur™ EZ column (Amicon). A control experiment confirmed that exonuclease III is completely removed by the minicolumn (Fig. 1, lane 3).

TABLE I

| Downstream primers | U17(17-mer): 5'-CTTCCTCTCTTCCTCTCT-3' | U18(18-mer): 5'-CTTCCTCTCTTCCTCTCT-3' | U19(19-mer): 5'-CTTCCTCTCTTCCTCTCT-3' | U20(20-mer): 5'-CTTCCTCTCTTCCTCTCT-3' | U22(22-mer): 5'-CTTCCTCTCTTCCTCTCT-3' |
|-------------------|--------------------------------------|--------------------------------------|--------------------------------------|--------------------------------------|--------------------------------------|
| Upstream primers  | M22(22-mer): 5'-UTCTTTTTTTTTTCTTTCTT-3' | M39(39-mer): 5'-CTTCCTCTCTCTCTCTCTCT' | M44(44-mer): 5'-CTTCCTCTCTCTCTCTCTCTCT-3' |
| Templates         | T22(22-mer): 3'-CCAAAGAAGGAAGAGAAAGGGAAGG-5' |

* U within a sequence context is defined as deoxyuridine.

* S within a sequence context is defined as an abasic residue.

* U with a numerical subscript denoting length is defined as an upstream primer.

**Miscellaneous primers**

**Recombinant Human RTH1 Nuclease Removal of 5’-Terminal Abasic Lesions**

**Materials**—Unless otherwise noted, DNA modification enzymes were from Boehringer Mannheim. Oligonucleotides were from Genosys (The Woodlands, TX). Radiolabeled nucleotides were from DuPont NEN. Sequenase version 2.0 was from U. S. Biochemical Corp. Nickel nitrotriacetic acid (NTA)-agarose resin was from Qiagen. Micropure™ EZ minicolumns were from Amicon, Inc. (Beverly, MA). Polyethyleneimine (PEI) cellulose TLC plates (20 × 20 cm) were from EM Science (via WVR Scientific). All other reagents, including 2-deoxyribose-5-phosphate, were from Sigma.

**Preparation of Human RTH1 Nuclease—Recombinant human RTH1** was obtained from bacteria using the T7 RNA expression system (31) following the procedure of Shen et al. (34). Briefly, the coding sequence for human RTH1 was cloned into the expression vector pET-28b (Novagen) upstream and in frame with the coding sequence for 6 histidine residues. The resulting plasmid, pET-FCH, was transformed into E. coli strain BL21(DE3)pLysS (31). Transformants were grown at 37 °C and induced with a final concentration of 1 mM isopropyl-1-thio-β-D-galactopyranoside for 3 h. Cells were collected by centrifugation and lysed by sonication. The recombinant protein, which contains the entire amino acid sequence for human RTH1 with a histidine tag at the C-terminal end, was purified by nickel nitrotriacetic acid-agarose column chromatography as recommended by Qiagen. Fractions were collected and tested for 3'- to 3'-exonuclease and -endonuclease activities as described previously (25, 26). Active fractions were pooled and further purified by hydroxyapatite as described in Murante et al. (25) for calf RTH1. The final preparation was 95% pure according to analysis by SDS-PAGE stained with silver. Purified enzyme (0.15 mg/ml) was dialyzed into a storage buffer (20% glycerol, 50 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, and 1 mM EDTA and EGTA) and then stored at -80 °C.

**EXPERIMENTAL PROCEDURES**

**Preparation of Human RTH1 Nuclease—**Recombinant human RTH1 was obtained from bacteria using the T7 RNA expression system (31) following the procedure of Shen et al. (34). Briefly, the coding sequence for human RTH1 was cloned into the expression vector pET-28b (Novagen) upstream and in frame with the coding sequence for 6 histidine residues. The resulting plasmid, pET-FCH, was transformed into E. coli strain BL21(DE3)pLysS (31). Transformants were grown at 37 °C and induced with a final concentration of 1 mM isopropyl-1-thio-β-D-galactopyranoside for 3 h. Cells were collected by centrifugation and lysed by sonication. The recombinant protein, which contains the entire amino acid sequence for human RTH1 with a histidine tag at the C-terminal end, was purified by nickel nitrotriacetic acid-agarose column chromatography as recommended by Qiagen. Fractions were collected and tested for 3'- to 3'-exonuclease and -endonuclease activities as described previously (25, 26). Active fractions were pooled and further purified by hydroxyapatite as described in Murante et al. (25) for calf RTH1. The final preparation was 95% pure according to analysis by SDS-PAGE stained with silver. Purified enzyme (0.15 mg/ml) was dialyzed into a storage buffer (20% glycerol, 50 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, and 1 mM EDTA and EGTA) and then stored at -80 °C.
gel purified and annealed to the 39-mer template used above (T39). The substrate was stored at −20 °C until use. For each experiment, a small aliquot was removed from storage and further prepared as follows. The uracil base was removed using uracil-DNA-glycosylase at 37 °C for 120 min. The substrate was then incubated at 37 °C with 2 units of exonuclease III, quickly centrifuged through a Micro-pureTM EZ minicolumn, and then incubated for 75 min (lane 3) to confirm that the minicolumn completely removes exonuclease III. A 5- radiolabeled mononucleotide (dUMP) is shown in (lane 4). The sample from lane 2 was also incubated with exonuclease III for 45 min (lane 5) to confirm that this enzyme degrades the resulting upstream primer after initial AP endonucleolytic cleavage. A different substrate (*ApD; T39) with a 5-terminal abasic residue was subjected to gel electrophoresis (lane 6) to verify that this resolving method causes spontaneous detachment of the baseless residue. Products were analyzed by 12% PAGE-urea and autoradiography.

RESULTS

Previous work with calf RTH1 and murine flap endonuclease 1 showed that endonuclease activity was efficient on annealed primers with unannealed 5′-tails. In addition, cleavage was often enhanced when an upstream primer formed a nick structure directly adjacent to the first annealed nucleotide of the downstream primer (25, 28). Such structures would result in vivo from strand displacement synthesis activity that extends the upstream strand at a single stranded break in duplex DNA into the annealed region of the downstream strand, thereby displacing a 5′-terminal segment of the downstream strand. This prompted us to explore whether removal of 5′-terminal baseless sugars as small oligonucleotides could be enhanced by simulating strand displacement synthesis.

Substrates with an Abasic Lesion at the 5′-Terminus—Four primary substrates were first created from which all additional substrates were made. The two primary substrates (D;T39 and *D;T39; see Fig. 2) with intact 5′-terminal deoxyuridine residues and that were either 3′- or 5′-radiolabeled, respectively, were created as outlined under “Experimental Procedures.” The other two primary substrates (ApD;T39 and *ApD;T39) consisted of fully annealed downstream primers having a baseless sugar-phosphate residue at the 5′-terminus and were either 3′- or 5′-radiolabeled, respectively. Removal of a uracil base by uracil glycosylase is inefficient when the base is located near the ends of DNA (data not shown; Ref. 14). In view of this, we designed these primary substrates by either creating or using oligonucleotides containing an internal deoxyuridine residue as outlined under “Experimental Procedures.” These oligomers were annealed to a template (T39) and treated with uracil glycosylase to create an internal abasic site. The template (T39) (Table I) was designed to allow 4 residues to overhang at both 3′-ends to prevent degradation of the substrate prematurely during the following step. The substrates were then treated with exonuclease III, which has both AP endonuclease activity and 3′-to 5′-exonuclease activity on recessed

Fig. 1. Experiments to monitor substrate design procedures. *Mg2+;T39 was incubated with exonuclease III (exo III) for 60 min (lane 1) to confirm that the overhanging 3′-ends prevent this enzyme from degrading the substrate. *Mg2+;T39 was treated with uracil-DNA glycosylase (UDG) for 120 min (lane 2). The latter sample was further treated with exonuclease III, quickly centrifuged through a Micro-pureTM EZ minicolumn, and then incubated for 75 min (lane 3) to confirm that the minicolumn completely removes exonuclease III. A 5′-radiolabeled mononucleotide (dUMP) is shown in (lane 4). The sample from lane 2 was also incubated with exonuclease III for 45 min (lane 5) to confirm that this enzyme degrades the resulting upstream primer after initial AP endonucleolytic cleavage. A different substrate (*ApD; T39) with a 5′-terminal abasic residue was subjected to gel electrophoresis (lane 6) to verify that this resolving method causes spontaneous detachment of the baseless residue. Products were analyzed by 12% PAGE-urea and autoradiography.

Fig. 2. Schematic of substrates. A series of upstream primers was used to increasingly displace a fully annealed downstream primer to simulate strand displacement synthesis. Thus, the unannealed 5′-tails in this study were still complementary to the template. Each downstream primer (D, *ApD, *D, or *ApD) was separately annealed to template T39 to form four primary substrates. In turn, each upstream primer U17, U18, U19, U20 or U22 was separately annealed to each primary substrate, forming four different sets of the six structures shown above, one set for each downstream primer, pS, position of an abasic residue in those substrates that have downstream primers (ApD or *ApD); otherwise, deoxyuridine is at this position. On substrates with an abasic residue, primer U19 leaves a 1-residue gap between the annealed nucleotides of the two primers. In all other cases, the upstream primers form a nick structure between the annealed nucleotides of the two primers. Numerical subscripts, number of sugar-phosphate residues. Oligonucleotide sequences are shown in Table I.
3'-termini of double stranded DNA. This nuclease initially cleaves on the 5'-side of the internal abasic residue and then digests the resulting upstream strand. Thorough digestion of the resulting upstream primer was necessary, since we wanted to introduce several new upstream primers of increasing length. Finally, exonuclease III was removed by centrifuging the substrates through a Micropur™ EZ minicolumn (Amicon).

As shown in Fig. 1, several control experiments were conducted to monitor the substrate design procedures. Fig. 1, lane 1, depicts 5'-radiolabeled *M39:T39 (Table I) treated with exonuclease III for 60 min to verify that the overhanging 3'-ends protect the downstream primer and the template from being nuclease III for 60 min to verify that the overhanging 3'-terminus. Cleavage was monitored by the

| Substrate | Time (min) |
|-----------|------------|
| D+T39     | 0 45 10 30 45 |
| U17:D+T39 | 0 10 30 45 |
| U18:D+T39 | 0 10 30 45 |
| U19:D+T39 | 0 10 30 45 |
| U20:D+T39 | 0 10 30 45 |
| U22:D+T39 | 0 10 30 45 |

as indicated. Each substrate was incubated at 37 °C with human RTH1 nuclease for the indicated times. Length markers are shown in lanes 7, 16, 17, and 26. Products were analyzed by 12% PAGE-urea and autoradiography.

Course. Control experiments also were performed without upstream primers. Cleavage of 3'-Radiolabeled Substrates with an Intact 5'-Terminus—The first experiment was designed to determine the cleavage specificity of human RTH1 nuclease on substrates made from D+T39 with an intact 5'-terminal deoxyuridine residue (Fig. 3). Fig. 3, lanes 3–6, shows that without an upstream primer, cleavage was inefficient and somewhat nonspecific, releasing primarily 1 or 2 nucleotides. Once a nick structure was formed by primer U17 positioned immediately upstream of a fully annealed downstream primer, the 5'- to 3'-exonuclease function became predominant, releasing mostly 1 nucleotide (Fig. 3, lanes 8–11). Exonucleolytic cleavage became even more efficient (Fig. 3, lanes 12–15) when the primer (U18) was long enough to displace the 5'-terminal nucleotide on the downstream primer. Here, the majority of substrate was converted to product within the first 10 min, whereas the rest was resistant to cleavage, perhaps because some downstream primers remained completely unannealed. Cleavage activity shifted from exonucleolytic to endonucleolytic as the upstream primer became long enough to displace dimer (Fig. 3, lanes 18–21), trimer (Fig. 3, lanes 22–25), or pentamer (Fig. 3, lanes 27–30) unannealed tails on the downstream primer. The results indicate that cleavage always occurred on the 5'-side of the first annealed nucleotide at the base of the tail. Cleavage was most efficient when the unannealed 5'-tail consisted of 2 residues. The results here are consistent with an earlier finding, which showed that the nucleotide sequence of the tail near the cleavage site can influence the cleavage efficiency and position specificity of calf RTH1 nuclease (27).

Cleavage of 3'-Radiolabeled Substrates with a 5'-Terminal Abasic Lesion—Next, the cleavage specificity of human RTH1 nuclease was examined using substrates made from *A39:D+T39 that have a 3'-radiolabeled downstream primer with a baseless sugar-phosphate residue at the 5'-terminus (Fig. 4). As mentioned above, we found that gel electrophoresis of the substrates caused detachment of the baseless sugar (Fig. 1, lane 6), regardless of the conditions used. As a result, this experiment did not distinguish deoxyribose-phosphate residues that were exonucleolytically removed as monomers during the enzyme reaction from the baseless residues that were detached after the reaction by the resolving method. This problem was evident from the control with no enzyme. The downstream primer, which started as a 23-mer oligonucleotide, was resolved as a 22-mer oligonucleotide. The difference in length between the substrate and the cleavage products throughout these reactions was always 1 residue shorter than would normally be expected. Despite this limitation, as the upstream primers became long enough to displace dimer (Fig. 4, lanes 17–20), trimer (Fig. 4, lanes 21–24), and pentamer (Fig. 4, lanes 26–29) unannealed tails on the downstream primer, human RTH1 nuclease efficiently removed the 5'-terminal deoxyuridine residue on the substrate.
nuclease efficiently cleaved the tails at their 3′-end. Baseless sugar-phosphate residues were not released as monomers either prematurely or during the reaction, because results from an experiment using 5′-radiolabeled substrates made from APD:T39 and analyzed by thin layer chromatography (see below) did not show significant release of 2-deoxyribose-5-phosphate or β-elimination products. In addition, cleavage appeared appreciably more nonspecific when the upstream primer (U17) and the first annealed nucleotide of the downstream primer were separated by a single nucleotide gap (Fig. 4, lanes 8–11). This substrate (U17-APD:T39) closely resembles the structure that exists in vivo immediately after an AP endonuclease cleaves at an internal abasic site in DNA. It represents the only structure used in studies that first reported the ability of DNase IV (human RTH1) to remove 5′-terminal baseless sugar residues as part of small oligonucleotides (14, 19). The results shown in Fig. 4, lanes 8–11, appear to correlate with the earlier findings, since those studies show inefficient release of dimers and trimers.

**Cleavage of 5′-Radiolabeled Substrates with an Intact 5′-Terminus**—To confirm that the tailed polymer segments were removed endonucleolytically and not exonucleolytically, two additional substrate sets (Fig. 2) were designed with 5′-radiolabeled downstream primers. We retested the ability of human RTH1 nuclease to endonucleolytically cleave unannealed 5′-tails by monitoring the production of 5′-radiolabeled cleavage products over a time course. The first experiment was performed using substrates made from primary substrate (*D:T39) containing an intact 5′-terminal deoxyuridine residue (Fig. 5). Products were resolved on a 12% PAGE-urea sequencing gel. Fig. 5, lanes 3–6, confirms, as indicated earlier, that without an upstream primer, cleavage was inefficient, although results here suggest that release of a dinucleotide was favored. Fig. 5, lanes 7–10, confirms that once a nick structure was formed by primer U17 positioned directly adjacent to a fully annealed downstream primer, 5′-to 3′-exonuclease activity again was stimulated. As before, exonucleolytic cleavage became more efficient (Fig. 5, lanes 14–17) when the primer (U18) was long enough to displace the 5′-terminal nucleotide on the downstream primer. Cleavage activity shifted from exonucleolytic to endonucleolytic as the upstream primer became long enough to displace dimer (Fig. 5, lanes 18–21) or trimer (Fig. 5, lanes 22–25) unannealed tails. Surprisingly, the results in Fig. 5, lanes 22–25, appear to con-
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...the results from this experiment indicate that only a dinucleotide was released. A different experiment (data not shown) confirmed this discrepancy and also indicated that the first 2 residues were again released as a separate dinucleotide even when the longest primer (U22) was used. This conflict may be reconciled, however, by considering the sequence of the fully complementary downstream primers used in this study (Table I). The first five bases (5’-UTCTT-3’) suggest that a stronger G-C pairing immediately adjacent to the released dinucleotide could momentarily hinder complete tail displacement. Earlier work by Murante et al. (26) provided evidence of a model in which RTH1 nuclease slide over the 5’-end of an unannealed tail and then track along the tail before cleaving at the junction. Since the unannealed tails in this earlier work were noncomplementary with the template, displacement was always complete. In this study, incomplete tail displacement, even if momentary, could provide an alternate structure suitable for cleavage. Because the data from Fig. 3, lanes 22-25, do not show significant levels of a product that would correspond to release of only a dinucleotide, we further propose that the enzyme remained associated with the substrate so it could cleave again once tail displacement was complete. Also, cleavage was more efficient when the unannealed 5’-tail consisted of only 2 residues. Perhaps when longer primers (U20 and U22) were used, more of the substrate may have formed alternate structures unsuitable for cleavage.

Cleavage of 5’-Radiolabeled Substrates with a 5’-Terminal Abasic Lesion—This final experiment was performed using substrates made from primary substrate (\( \text{U}_{16}^{\text{A}}\text{D-T}_{39} \)) containing a baseless sugar-phosphate residue at the 5’-terminus (Fig. 6). As mentioned above, a 5’-terminal abasic lesion is extremely labile. In this experiment, since the radioactive label is attached to the lesion, the 5’-radiolabeled products had to be analyzed by a method other than gel electrophoresis. We chose PEI cellulose thin layer chromatography, because the labile substrates proved more stable using this method.

Fig. 6A, lanes 1 and 2, depicts a control reaction with no added enzyme using a substrate (\( \text{U}_{16}^{\text{A}}\text{D-T}_{39} \)) without an upstream primer. Results confirm that most substrate molecules are unaffected by TLC analysis. That is, the baseless sugar-phosphate residue remains attached to the downstream primer, with no significant increase in spontaneous detachment after a 45-min incubation at 37°C. To verify that a sizable portion of the substrate present at the TLC plate origin (Fig. 6A, lanes 1 and 2) actually had a 5’-terminal abasic lesion, we performed the same reaction in the absence and presence, respectively, of NaOH (Fig. 6A, lanes 3 and 4). Sodium hydroxide is known to induce a \( \beta \)-elimination reaction (33), so that 5’-terminal baseless sugar-phosphate residues are released as unsaturated derivatives of deoxyribose-phosphate (17). Results show (Fig. 6A, lane 4) that the released product is distinct from 2-deoxyribose-5-phosphate (Sigma), shown in Fig. 6A, lane 10. Quantitation by a PhosphorImager (Molecular Dynamics) revealed that a 20-min incubation at 37°C with 82 mM NaOH converted 34% of the substrate into a \( \beta \)-elimination product and another 18% into inorganic phosphate. Therefore, at least 52% of the primary substrate used for all the reactions depicted in Fig. 6A had a 5’-terminal abasic lesion.

To test the ability of human RTH1 nuclease to exonucleolytically remove the lesion, we performed reactions using the primary substrate (\( \text{U}_{16}^{\text{A}}\text{D-T}_{39} \)) either with no upstream primer (Fig. 6A, lanes 5 and 6), primer U17, which leaves a 1-residue gap between the annealed nucleotides of the two primers (Fig. 6A, lanes 7 and 8), or primer U18 which forms a nick between the annealed nucleotides of the two primers (Fig. 6A, lanes 9 and 13). In every case, there was no significant release of either a \( \beta \)-elimination product (Fig. 6A, lane 4) or 2-deoxyribose-5-phosphate (Fig. 6A, lane 10). We conclude that human RTH1 nuclease does not function as either a \( \beta \)-elimination catalyst or as a dRpase. The results here are consistent with earlier studies, which indicated that DNase IV (human RTH1) was inhibited by 5’-terminal baseless sugar-phosphate residues (14, 19).

Although there was no release of a monomer product, after a 30-min incubation with the enzyme, results from each reaction show evidence of migration of the radioactive label away from the origin. In keeping with the results obtained using 3’-radiolabeled substrates, this was likely due to endonucleolytic cleavage downstream of the 5’-terminal abasic site, so that small oligonucleotides were released. Another reaction was performed (Fig. 6A, lanes 14 and 15) using a substrate (\( \text{U}_{16}^{\text{A}}\text{D-T}_{39} \)) that used an upstream primer that could displace an unannealed dimer tail. In this case, the result shows increased migration of the radioactive label away from the origin, although cleavage product(s) were not identifiable. A different experiment (data not shown) used substrates made with primers U20 and U22 so that the upstream primer was further displaced. Here, the results were similar to that shown in Fig. 6A, lane 15. Unfortunately, under the conditions used for TLC analysis, oligonucleotides bearing the abasic lesion could not be resolved.

Additional control experiments were performed (Fig. 6B) to verify the integrity of the substrate and the validity of the TLC analysis. Fig. 6B, lanes 3 and 4, depict a reaction using a 5’-radiolabeled substrate (\( \text{D-T}_{39} \)) with an intact 5’-terminal deoxyuridine either without or with, respectively, 102 mM NaOH. The result confirms that an intact 5’-terminal nucleotide is resistant to detachment during the sodium hydroxide treatment used above. In the enzyme reactions shown in Fig. 6A, neither \( \beta \)-elimination products nor 2-deoxyribose-5-phosphate were detectable. An alternative explanation for this result is that the rest of the substrate in some way retarded the migration of the monomer cleavage products on the TLC plate. Three additional reactions were performed to exclude this possibility. In the first, a substrate (\( \text{U}_{16}^{\text{A}}\text{D-T}_{39} \)) with an intact 5’-terminal deoxyuridine was incubated with the enzyme for 0 and 30 min, respectively, (Fig. 6B, lanes 10 and 11). Based on results shown above (Fig. 5, lanes 14–17), the terminal nucleotide should be exonucleolytically released. As predicted, the cleavage product migrated to the same position on the TLC plate as radiolabeled dUMP in the control (Fig. 6B, lane 7). In the second reaction, a substrate (\( \text{U}_{16}^{\text{A}}\text{D-T}_{39} \)) having a 5’-terminal abasic lesion was also incubated with human RTH1 nuclease (Fig. 6B, lanes 12–14). As before, no \( \beta \)-elimination products or 2-deoxyribose-5-phosphate were detected (Fig. 6B, lane 13). In Fig. 6B, lane 14, an additional aliquot of the reaction was mixed with 2-deoxyribose-5-phosphate (Sigma) and then analyzed. The result shows that the 2-deoxyribose-5-phosphate in the mixed sample migrated to the same position on the TLC plate as 2-deoxyribose-5-phosphate in the control (Fig. 6B, lane 8). Finally, in the third reaction, two substrates (\( \text{U}_{16}^{\text{A}}\text{D-T}_{39} \) and \( \text{U}_{16}^{\text{A}}\text{D-T}_{39} \)) were mixed and then incubated with the enzyme (Fig. 6B, lanes 15 and 16). The result shows that a cleavage product migrated to the same position as the product in lane 11 and dUMP in lane 7. Taken together, these results rule out the possibility that migration of monomer cleavage products on a TLC plate is retarded by other components in the reaction. Detection of a cleavage product in the third reaction also confirms that the enzyme remains active even in the presence of a substrate that resists cleavage.
FIG. 6. Cleavage of 5'-radiolabeled substrates with a 5'-terminal abasic lesion. A, no upstream primer or U15, U16, or U17 was separately annealed to primary substrate (pAPD:T39). The noted substrates were incubated at 37 °C for the indicated times with no enzyme (lanes 1 and 2), 82 mM NaOH (lanes 3 and 4), or human RTH1 nuclease (lanes 5–8 and 12–15). A 5'-radiolabeled mononucleotide (dUMP) is shown in lane 9. The circle (lane 10) indicates the position of 2-deoxyribose-5-phosphate (Sigma) as observed by UV fluorescence. A 5'-radiolabeled dinucleotide (pUpT) is shown in lane 11. The lower bands in lanes 9 and 11 were determined to be inorganic phosphate. B, no upstream primer or U16 was annealed to primary substrates (pAPD:T39 or D:T39). The noted substrates were incubated at 37 °C for the indicated times with no enzyme (lanes 1 and 2), 102 mM NaOH (lanes 3–6), or human RTH1 nuclease (lanes 10–16). A 5'-radiolabeled mononucleotide (dUMP) is shown in lane 7. The circle in lane 8 indicates the position of 2-deoxyribose-5-phosphate (Sigma) as observed by UV fluorescence. A 5'-radiolabeled dinucleotide (pUpT) is shown in lane 9. The lower bands in lanes 7 and 9 were determined to be inorganic phosphate. In lane 14, 2-deoxyribose-5-phosphate (Sigma) was added to the reaction after 30 min, and then the mix was subjected to TLC. Products in both panels were analyzed by thin layer chromatography and autoradiography. Upper edges, solvent front.
DISCUSSION

Genetic and biochemical evidence indicates that eukaryotic RTH1 nuclease participates in removal of modified nucleotides (22, 26, 29). Previous work has shown that the exonucleolytic activity of this nuclease was ineffective at removal of 5'-terminal abasic lesions (14, 19). Here we show that this nuclease can efficiently remove such lesions by endonuclease activity. We have demonstrated that endonucleolytic cleavage downstream of a 5'-terminal abasic lesion is dependent on displacement of the downstream primer bearing the lesion. Cleavage was most efficient when an upstream primer displaced the first annealed nucleotide on the downstream primer. Further displacement of the unannealed 5'-tail continued to stimulate cleavage, although not as efficiently. Such structures are likely produced in vivo at sites of single strand breaks in DNA by a strand displacement synthesis activity. Single strand breaks are created during the initial stages of repair of an abasic DNA lesion.

Abasic DNA lesions occur for a variety of reasons, including spontaneous depurination at physiological temperature and pH (1, 34), removal of uracil from DNA by a uracil glycosylase, or removal of damaged bases by other specialized glycosylases. Some glycosylases (e.g., 2,6-diamino-4-hydroxy-5-N-formamidopyrimidine (Fapy) DNA glycosylase) cleave the N-glycosyl bond holding the base and also cleave on the 3'-side of the just formed baseless sugar (35). This creates a suitable 5'-terminus on the downstream strand for eventual ligation. In this scenario, removal of the baseless sugar from the 3'-terminus has been well characterized, occurring from cleavage by a class II AP endonuclease (4, 5). This latter class of enzyme has both 3'-phosphodiesterase and 3'-phosphatase activities. However, not every glycosylase has the ability to cleave the DNA backbone, so an internal abasic site is often produced. Repair of an internal abasic lesion is predominantly initiated by a class II AP endonuclease, which cleaves on the 5'-side of the baseless sugar, so that the 3'-terminus can support primer extension (4, 5). Subsequent removal of the abasic residue from the 5'-terminus must occur before the upstream and downstream strands are ligated.

Previous studies have identified three distinct mechanisms for this process. Two of these mechanisms entail removal of the baseless sugar as a single residue, either by a hydrolytic reaction known as dRpase activity (6, 14) or by an enzyme mediated β-elimination reaction (17, 18). In either case, the 1-nucleotide gap is filled by a DNA polymerase, and the adjacent strands are joined by a ligase. There is support for this pathway, known as base excision repair. Using both bacterial and mammalian cell extracts, the majority of uracil residues in DNA were repaired by replacing a single nucleotide (11). Base excision repair also has been reconstituted with purified enzymes from E. coli, one of which was the RecJ protein (12). In both studies, however, some of the substrates used may have been resistant to strand displacement synthesis, because the uracil residue to be repaired was incorporated in a G-C-rich region. In addition, the latter study indicates that in the absence of dRpase activity provided by the RecJ protein, longer repair patches were observed and were likely due to the strand displacement and 5'-nuclease activities of E. coli DNA polymerase I. As noted before, it has been reported that DNase IV and the 5'-nuclease domain of DNA polymerase I share structural and functional homology (20). Therefore, DNase IV (human RTH1) would likely be responsible for longer repair patches in the mammalian system. Yet another study found that E. coli Fpg/resj double mutants retain capacity to repair abasic sites in DNA, indicating the presence of a backup excision function (9).

The third mechanism by which 5'-terminal abasic lesions are repaired involves endonucleolytic cleavage downstream of the baseless residue, so that the lesion is removed as part of small oligonucleotides. Previous studies have shown that the 5'- to 3'-exonuclease activities of E. coli DNA polymerase I (6, 19) and human DNase IV (14, 19) are inhibited by a 5'-terminal deoxyribose-phosphate residue. Although the studies indicate that the enzymes can endonucleolytically remove the abasic residue as part of small oligonucleotides, they report that cleavage occurs at a slow rate. Rapid repair of an abasic DNA lesion, the investigators conclude, necessitates a dRpase activity that can rapidly remove free 2-deoxyribose-5-phosphate. The substrates used in these earlier studies, however, were limited to the structure obtained immediately after an AP endonuclease has cleaved at an internal abasic site. The results in our study are consistent with the earlier results, since we show that human RTH1 nuclease is inhibited until the first annealed nucleotide on the downstream primer is displaced.

Recent work with the calf RTH1 nuclease indicates that the enzyme can remove short RNA segments from duplex DNA when the segments are part of unannealed 5'-tails. Most initiator RNA removal from Okazaki fragments in mammals is thought to occur through the combined endonucleolytic action of RNase H and exonucleolytic action of RTH1 nuclease (30). However, calf RTH1 nuclease alone was able to remove the RNA segment endonucleolytically when the downstream primer was sufficiently displaced (27). This latter result was obtained in vitro through experiments similar to the work presented here. When downstream primers containing an RNA-DNA junction were increasingly displaced by an upstream primer to simulate strand displacement synthesis, a substrate for efficient endonucleolytic cleavage by RTH1 nuclease was created. In this way, RTH1 class nucleases can participate in the processing of Okazaki fragments through two pathways during lagging strand DNA replication. A role for RTH1 nuclease in DNA replication has gained further support by a recent finding that null mutants in the yeast RTH1 nuclease are defective in DNA replication and show elevated levels of spontaneous mitotic recombination (22). This is indicative of a suppression of Okazaki fragment processing. Interestingly, these mutants were also sensitive to methyl methane-sulfonate. This DNA-damaging agent induces lesions that are initially repaired by removal of the alkylated base by a glycosylase, thereby creating an abasic DNA lesion. Sommers et al. (22) surmised that the null mutants were incapable of removing the abasic lesion from the 5'-terminus after AP endonucleolytic cleavage.

Murante et al. (26) provided evidence that RTH1 nucleases slide over the free end of an unannealed 5'-tail and then track along the tail to its base before cleaving endonucleolytically. Other more recent work extends this model by revealing that the enzyme has a high degree of tolerance for bulky adducts along the tail, including cis-platinum, biotin, and tertiary butyl silyl adducts (29). This suggests that RTH1 nucleases can participate in repair of a variety of different lesions, when working in combination with a DNA strand displacement activity. This model implies that the structure of the 5'-terminal residue of the tail would be important for substrate recognition, tracking, or both. However, our results indicate that the base moiety on the terminal deoxyribose may not be crucial when the unannealed tail is at least 2 residues long. This is consistent with the earlier observation that calf RTH1 nuclease can also slide past a 7-methylguanine located at the 5'-terminus of the tail (26). In this case, perhaps the penultimate base also can participate in recognition and/or tracking. For the special case in which the baseless sugar forms an unannealed 1-residue 5'-tail, the base moiety might be more crucial for recognition or cleavage, since we and others (6, 14, 19) did not observe
dRpase activity by this class of enzyme.

The results presented here, together with previous work, suggest that 5'-terminal abasic lesions in mammals are repaired by at least two pathways. The first involves removal of the baseless sugar-phosphate residue by the action of either a dRpase or a β-elimination reaction. The second involves removal of the abasic lesion as part of an oligomer. Here we show that the RTH1 nuclease, implicated by both genetic and biochemical evidence in the direct removal of damaged nucleotides, has the appropriate substrate specificity to participate in the latter pathway. For the reaction to occur efficiently, the 5'-abasic residue and one or more additional nucleotides must be displaced from the template. The RTH1 nuclease can then slide over the 5'-end of the displaced tail, move to the point where the tail is annealed to the template, and then cleave endonucleolytically. Since RTH1 nuclease is also involved in both Okazaki fragment processing and the repair of adduct-damaged bases, the current results highlight the range of important functions in which this enzyme participates.

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