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Action of *Escherichia coli* and human 5' → 3' exonuclease functions at incised apurinic/apyrimidinic sites in DNA

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The 5' → 3' exonuclease activity of *E. coli* DNA polymerase I and a related enzyme activity in mammalian cell nuclei, DNase IV, are unable to catalyse the excision of free deoxyribose-phosphate from apurinic/apyrimidinic (AP) sites incised by an AP endonuclease. Instead, the sugar phosphate residue is slowly released as part of a short oligonucleotide. These products have been characterised as dimers and trimers by comparison of their retention time on reverse-phase HPLC with reference compounds prepared by acid depurination of a dinucleotide, trinucleotide and tetranucleotide containing a 5'-terminal dAMP residue. The similar mode of action of these enzymes at 5'-incised AP sites provides an explanation for the minority of repair patches larger than one nucleotide observed when AP sites are repaired by *E. coli* and mammalian cell extracts in vitro and strengthens the functional analogy between the two activities.

Apurinic/apyrimidinic sites; DNase IV; DNA polymerase I

1. INTRODUCTION

Apurinic/apyrimidinic (AP) sites are common lesions of DNA which may arise spontaneously [1] or following the excision of altered bases by DNA glycosylases [2,3]. The correct nucleotide is restored in prokaryotes and eukaryotes by a highly conserved pathway of excision-repair. The process is initiated by an AP endonuclease [4] which incises the phosphodiester bond 5' to the AP site. This produces a 3'-hydroxyl terminus which can be used as a primer by a DNA polymerase, and a 5'-terminal deoxyribose-phosphate (dRp) residue which must be removed before ligation of the DNA strand. The major activity catalysing the excision of the baseless sugar-phosphate moiety in extracts of *E. coli* and mammalian cell nuclei is a DNA deoxyribophosphodiesterase (dRpase). This enzyme has no exonuclease activity and does not extend the gap in DNA beyond one nucleotide [5–7].

The major human nuclear 5' → 3' exonuclease, DNase IV, was unable to release a 5'-terminal dRp residue in free form but could apparently catalyse the slow excision of short oligonucleotides containing dRp from an incised AP site [6]. A small proportion (5–20%) of the repair patches filled-in at AP sites by *E. coli* and mammalian cell extracts comprised more than one nucleotide [7]. Moreover, it has been observed that *E. coli* DNA polymerase I, which possesses a 5' → 3' exonuclease activity, is able to participate in the complete repair of AP sites in cell-free assays in the absence of other functions [8,9]. Thus, this enzyme and the analogous DNase IV from mammalian nuclei may represent a back-up function to dRpase, in addition to their more usual activity of catalysing the excision of ribonucleotide primers prior to joining of Okazaki fragments [10–12].

To clarify the role of these 5' → 3' exonucleases in the excision-repair of AP sites, reverse-phase high performance liquid chromatography (HPLC) was used to identify the products released from AP sites during incubation with the two enzymes. It is shown here that both activities only release dimers and trimers containing a 5'-terminal sugar phosphate from incised AP sites. This confirms that neither enzyme catalyses the excision of free dRp.

2. MATERIALS AND METHODS

2.1. *Oligonucleotide substrates*

Poly(dA-dT) containing incised ^{32}P-labeled 5'-AP sites was prepared as described [6]. Briefly, poly(dA-dT) containing occasional ^{32}P-labeled dUMP residues was synthesized by incubation of poly(dA-dT), [α-^{32}P]dUTP, dATP and TTP with the Klenow fragment of *E. coli* DNA polymerase I. After heat inactivation of the polymerase, the polynucleotide was incubated with *E. coli* uracil-DNA glycosylase and with *E. coli* exonuclease III in 0.1 M NaCl, 10 mM sodium citrate (pH 7.0), under which conditions the latter enzyme functions only as an AP endonuclease [13]. The incised polynucleotide was precipitated with ethanol and stored in 10 mM citrate buffer (pH 6.2) at −20°C.

5'-[^{32}P]oligo(dT)$_{15}$poly(dA) was prepared as described [14]. dT$_{15}$, produced on a commercial DNA synthesiser, was end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (Boehringer Mannheim) and annealed to poly (dA).

To obtain HPLC reference compounds for comparison with the excision products released by exonucleases, the di-, tri-, and tetranu-

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cleotides AT, ATT, and ATTT were depurinated to produce ST, STT and STTT, where S represents dRp. The oligonucleotides were prepared on a commercial DNA synthesizer and desalted by application to a Brownlee C-18 reverse phase (4.6 mm × 3 cm) cartridge column and elution in methanol. After drying under vacuum, the oligonucleotides (0.2–0.25 nmol) were depurinated by incubation in 0.1 M HCl (90 μl) for 90 min at 37°C. Following depurination, 500 μl 50 mM NaH_2PO_4 (pH 4.4) was added to the reaction mixture and the oligonucleotides stored at −20°C.

2.2. *Reagent enzymes*

The holoenzyme and the Klenow fragment of *E. coli* DNA polymerase I, and *E. coli* exonuclease III were purchased from Boehringer Mannheim. Uracil-DNA glycosylase [15], human DNase IV [6,16] and human DNA dRpase [6] were purified as described.

2.3. *Repair assays*

Repair assays were performed as described [6]. Reaction mixtures (100 μl) containing poly(dA-dT) with incised ^{32}P-labeled 5'-AP sites in 50 mM HEPES-NaOH (pH 8.0), 3 or 10 mM $MgCl_2$, 1 mM DTT were incubated at 37°C for 20 min with either *E. coli* DNA polymerase I (1 U), DNase IV (0.025 U) or human DNA dRpase (0.005 U). The reactions were terminated by freezing to −20°C and the material kept at this temperature until product analysis was performed. After thawing, the whole reaction mixture was applied to an HPLC column as described below.

2.4. *Product analysis*

The reaction products were separated by reverse-phase HPLC using a Varian Micropak MCH-10 column (4 mm × 30 cm). A gradient of 2% methanol/0.1 M ammonium formate (pH 5.0) to 100% methanol was applied over 20 min at 1 ml/min, followed by 5 min isocratic elution with 100% methanol at 1 ml/min. Fractions (0.5 min) were collected and the radioactive material eluting in each fraction determined by liquid scintillation counting. Deoxyribose phosphate (Sigma), as determined by colorimetric estimation using 2,4-dinitrophenylhydrazine, and P_i eluted at 3–4 min, and dUMP (Sigma) at 6–7 min as determined by ultraviolet-light absorption.

3. RESULTS AND DISCUSSION

3.1. *Kinetics of the exonuclease functions of* E. coli *DNA polymerase I at 5'-termini*

The 5' → 3' exonuclease activities under investigation have been shown previously to be unable to catalyse the release of free dRp from 5'-incised AP sites [5,6,8], but appeared to release slowly short oligonucleotides containing dRp. In order to demonstrate that the 5' → 3' exonuclease function of *E. coli* DNA polymerase I, like DNase IV, acted less efficiently at 5'-terminal sugar phosphate residues than nucleotides at 5'-termini, poly(dA-dT) containing ^{32}P-labelled incised AP sites and [5'-^{32}P]oligo(dT).poly(dA) were incubated with 0.01–0.02 U of DNA polymerase I (Fig. 1). In a series of experiments the initial rate of excision of the 5'-terminal nucleotide residue was approximately 6-fold greater from the latter polynucleotide than the rate of liberation of dRp from the polymer containing a nicked abasic site. This result indicates that the major 5' → 3' exonuclease activities from both bacterial and mammalian cells act preferentially at normal rather than baseless end groups.

In order to exclude the possibility that the appearance

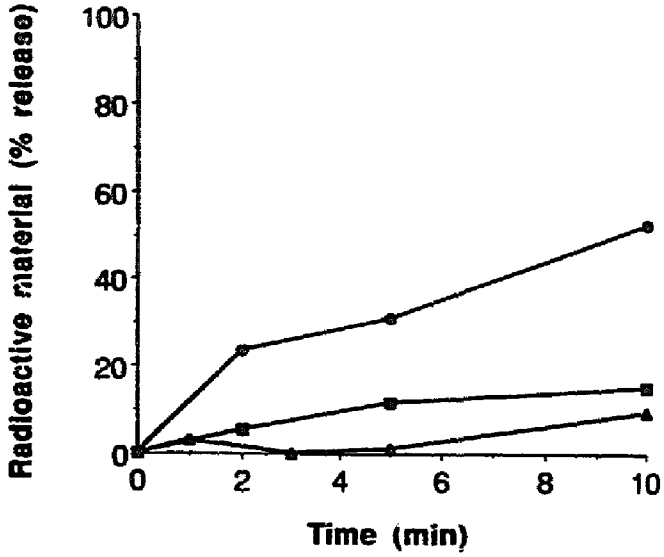

Fig. 1. Time course of release of 5'-terminal residues from polydeoxynucleotides by exonuclease functions of *E. coli* DNA polymerase I. Reaction mixtures containing 50 mM HEPES/NaOH (pH 8.0), 10 mM $MgCl_2$, 1 mM DTT, polynucleotide substrate (approximately 1–2 fmol 5'-termini) and either the holoenzyme or Klenow fragment of *E. coli* DNA polymerase I were incubated at 37°C. Data shown represent ● release of TCA-soluble radioactive material from [5'-^{32}P]oligo(dT).poly(dA) on incubation with 0.01 U DNA polymerase I; ■ release of TCA-soluble radioactive material from poly(dA-dT) containing ^{32}P-labeled incised AP sites by 0.01 U DNA polymerase I; ▲ release of TCA-soluble radioactive material from poly(dA-dT) containing ^{32}P-labeled incised AP sites in presence of 0.05 U Klenow fragment.

of TCA-soluble radioactive material from the former substrate was due to the 3' → 5' exonuclease domain of *E. coli* DNA polymerase I, a control experiment was performed in which the polymer with radioactively-labeled apyrimidinic sites was incubated with a higher concentration (0.05) U of the Klenow fragment. This protein lacks a 5' → 3' exonuclease function. Excision of the 5'-terminal dRp residue in acid-soluble form was approximately 10–20 times less efficient than with the holoenzyme, and was not detectable during the first part of the reaction (Fig. 1).

3.2. *Elution times of depurinated standards*

To characterise the short oligonucleotide released from 5'-terminal AP sites by the exonuclease activities, compounds containing a 5'-terminal baseless sugar phosphate as part of a dimer, trimer or tetramer were prepared by acid depurination of a di-, tri- or tetranucleotide, and used as references for the elution of the enzymatic products on reverse phase HPLC. Since the radioactively-labeled enzyme substrate contained a terminal apyrimidinic site rather than an apurinic site, the expected products of the exonucleases (SA, SAT and SATA) were slightly different from those of the depurinated standards (ST, STT and STTT). The elution profiles of the markers are shown in Fig. 2. The acid-

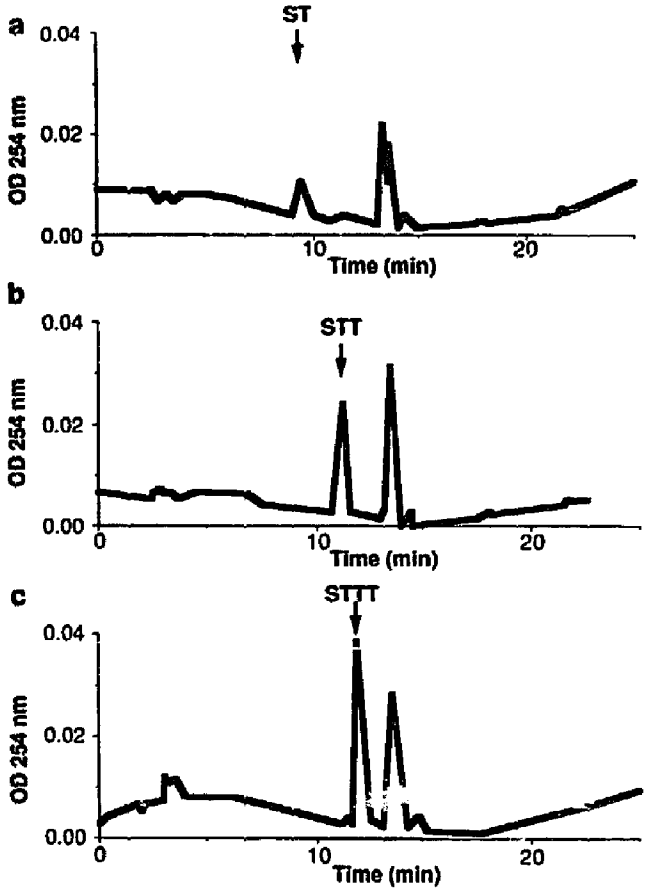

Fig. 2. Elution profiles of depurinated reference oligonucleotides. The oligonucleotides AT, ATT and ATTT were depurinated with 0.1 M HCl as described in Section 2. After mixing with 500 μl 50 mM NaH_2PO_4 (pH 4.4), 100 μl of the standard was applied to a a Varian Micropak MCH-10 column (4 mm × 30 cm) and a gradient of 2% methanol/0.1 M ammonium formate (pH 5.0) to 100% methanol was developed over 20 min at 1 ml/min, followed by 5 min isocratic elution with 100% methanol at 1 ml/min. The quantity of oligonucleotide was determined by absorption at 254 nm. The profiles shown indicate the elution times of ST (a), STT (b) and STTT (c). All oligonucleotides were eluted as a mixture of depurinated and non-depurinated compounds, since depurination was incomplete under the conditions used.

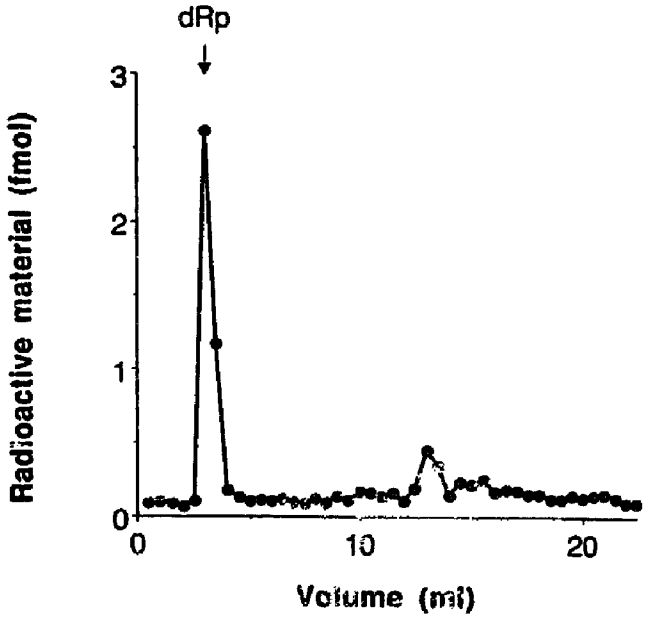

Fig. 3. HPLC analysis of excision products released from 5'-incised AP sites by human DNA dRpase. Poly(dA-dT) containing incised ^{32}P-labeled 5'-AP sites (approximately 12 fmol AP sites) was incubated with 0.005 U human DNA dRpase under the conditions described in Section 2. The whole reaction mixture was applied to a a Varian Micropak MCH-10 column (4 mm × 30 cm) and a gradient of 2% methanol/0.1 M ammonium formate (pH 5.0) to 100% methanol was developed over 20 min at 1 ml/min, followed by 5 min isocratic elution with 100% methanol at 1 ml/min. Unretarded material appeared after approximately 2.5 min. Fractions (0.5 min) were collected and the radioactive material (●) present in each determined by liquid scintillation counting. The arrow indicates the elution volume of dRp as determined by colorimetric analysis of eluted material by 2,4-dinitrophenylhydrazine following application of 100 μg deoxyribose phosphate to the HPLC column.

catalysed release of adenine was incomplete under the depurination conditions employed [17]. In each case, two main peaks of UV-absorbing material were observed, the earlier due to the oligomer containing a 5'-terminal sugar phosphate and the later to that with a 5'-terminal nucleotide. ST eluted at 9–10 min (Fig. 2a), STT at 11–11.5 min (Fig. 2b) and STTT at 11.5–12.5 min (Fig. 2c).

3.3. *Products released from 5'-incised AP site by 5' → 3' exonucleases*

In order to identify the form in which the 5'-terminal dRp residue was excised, the polynucleotide containing incised ^{32}P-labeled 5'-AP sites was incubated with a 5' → 3' exonuclease function, and the entire reaction mixture injected on to a reverse-phase HPLC column. The elution volumes of the radioactively-labeled eluants were compared with those of the acid-depurinated markers. Following incubation of the polynucleotide with human DNA dRpase, [^{32}P]dRp was released, eluting at 3–4 min (Fig. 3), as previously described [6].

After incubation of the polynucleotide with *E. coli* DNA polymerase I (Fig. 4a), ^{32}P-labeled material appeared after 10–12 min indicating that the dRp residue was excised as part of a mixture of small oligonucleotides, predominantly comprising dinucleotides. No detectable material (< 0.02 fmol) was found at the position of free dRp. The small difference between the retention time of the radioactively-labeled excision product (10 min) and the apurinic dinucleotide standard (9.5 min) was probably due to the presence of a pyrimidine rather than a purine in the latter.

When the polynucleotide was incubated with DNase IV (Fig. 4b), two separate peaks of radioactive material were observed after 10–10.5 and 11.5–12 min respectively. In this instance, where a 5' → 3' exonuclease activity without an associated 3' → 5' exonuclease function was used, the ^{32}P-labeled incised 5'-AP sites coe-

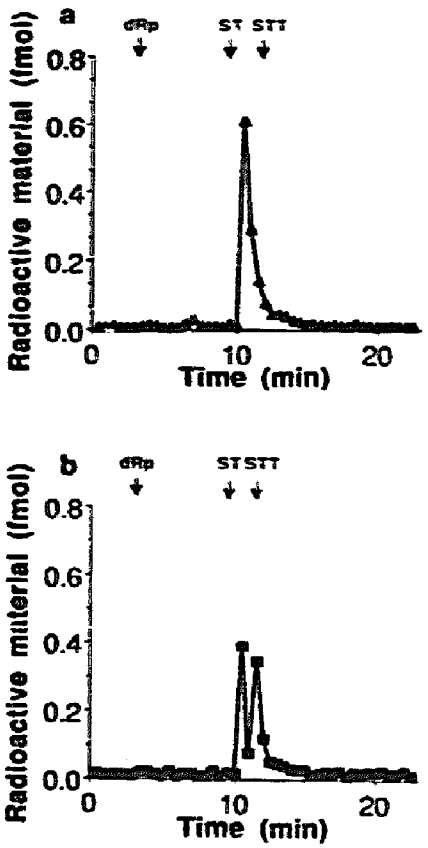

Fig. 4. HPLC analysis of excision products released from a 5'-incised AP site by *E. coli* and human 5' → 3' exonucleases. Poly(dA-dT) containing incised ^{32}P-labeled 5'-AP sites (approximately 1–2 fmol AP sites) was incubated with either *E. coli* DNA polymerase I (1 U) (a; ▲) or HeLa DNase IV (0.025 U) (b; ■) under the conditions described in Section 2. The whole reaction mixture was applied to a 3 Varian Micropak MCH-10 column (4 mm × 30 cm) and a gradient of 2% methanol/0.1 M ammonium formate (pH 5.0) to 100% methanol was developed over 20 min at 1 ml/min, followed by 5 min isocratic elution with 100% methanol at 1 ml/min. Fractions (0.5 min) were collected and the radioactive material present in each determined by liquid scintillation counting. Arrows show the elution time of the indicated reference compounds.

luted with the depurinated di- and trinucleotide reference compounds after enzymatic excision. Again, the radioactively-labeled apyrimidinic dinucleotide eluted slightly later than the acid-depurinated dinucleotide, and no free dRp was detected.

3.4. *Potential role of 5' → 3' exonucleases in excision-repair*

Gossard and Verly [8] observed that, after incision of a reduced AP site by *E. coli* exonuclease III, the 5' → 3' exonuclease of *E. coli* DNA polymerase I was able to catalyse the release of di- and trinucleotides containing the 5'-terminal reduced sugar phosphate residue. They proposed that this activity was involved in the repair of AP sites produced by depurination and the activity of DNA glycosylases in vivo. Although we have recently shown that repair replication by both *E. coli* and human cell extracts in vitro results in the insertion of a single nucleotide in most cases [7], a small number of larger repair patches, which could not be explained by the action of a dRpase, were observed. It seems probable that the exonucleases investigated here are responsible for the excision of the dRp residue in repair reactions which produce these larger replication patches in vitro. Moreover, these activities are likely to participate in the repair of AP sites in systems where a dRpase is not present.

The capacity of both the 5' → 3' exonuclease activity of *E. coli* DNA polymerase I and DNase IV to catalyse the excision of dRp as part of a di- or trinucleotide strengthens the functional analogy between these two enzymes and supports the idea that they may function as a secondary back-up mechanism in the excision-repair of AP sites in vivo.

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