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Generation of Single-Nucleotide Repair Patches Following Excision of Uracil Residues from DNA

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The extent and location of DNA repair synthesis in a double-stranded oligonucleotide containing a single dUMP residue have been determined. Gently prepared *Escherichia coli* and mammalian cell extracts were employed for excision repair in vitro. The size of the resynthesized patch was estimated by restriction enzyme analysis of the repaired oligonucleotide. Following enzymatic digestion and denaturing gel electrophoresis, the extent of incorporation of radioactively labeled nucleotides in the vicinity of the lesion was determined by autoradiography. Cell extracts of *E. coli* and of human cell lines were shown to carry out repair mainly by replacing a single nucleotide. No significant repair replication on the 5′ side of the lesion was observed. The data indicate that, after cleavage of the dUMP residue by uracil-DNA glycosylase and incision of the resultant apurinic-apyrimidinic site by an apurinic-apyrimidinic endonuclease activity, the excision step is catalyzed usually by a DNA deoxyribophosphodiesterase rather than by an exonuclease. Gap-filling and ligation complete the repair reaction. Experiments with enzyme inhibitors in mammalian cell extracts suggest that the repair replication step is catalyzed by DNA polymerase β.

DNA bases altered by hydrolytic deamination, oxygen radical-induced damage, and alkylation are often excised by DNA glycosylases (44, 54). Certain mismatched residues, such as adenine or thymine opposite guanine, may also be removed by specific DNA glycosylases (1, 56, 57). Thus, a common intermediate in the repair of several different types of DNA damage is an apurinic-apyrimidinic (AP) site. Such sites are also generated by nonenzymatic hydrolysis of base-sugar bonds, in particular by depurination (26), and as a consequence of exposure to ionizing radiation (12, 27, 34). AP endonucleases, for example, *Escherichia coli* exonuclease III and endonuclease IV, rapidly incise DNA at the 5′ side of an AP site (9). Subsequently, an excision step occurs to generate a small gap, which is filled in by repair replication. A DNA ligase completes the repair process. These consecutive events take place rapidly and efficiently in vivo. It has been shown that AP sites occur transiently in DNA after X-irradiation of mammalian cells but disappear in 2 to 4 min without any detectable accumulation of DNA containing single-strand interruptions (34).

The excision of a depurinated-depyrimidinated site has remained the least understood of these events in the base excision repair process. Early work by Painter and Young (37) and Regan and Setlow (40) showed that repair replication of X-irradiated and alkylated DNA in vivo involved the filling-in of much smaller gaps than those observed after UV light-induced DNA damage. Subsequent studies have also indicated that patches only 1 to 4 nucleotides long may be generated during repair of AP sites (8, 32). The excision of a 5′-terminal base-free sugar-phosphate residue, possibly accompanied by the removal of one or a few adjacent residues, has been ascribed to exonuclease action. However, recent investigations of the properties of enzymes that may remove deoxyribose phosphate (dRp) residues from incised AP sites permit a more detailed description of the process. Excision of dRp by 5′→3′ exonucleases, such as the 5′→3′ exonucleolytic function of *E. coli* DNA polymerase I or the apparently analogous DNase IV of mammalian cell nuclei (17, 22, 39), is inefficient. An important aspect of the action pattern of these enzymes is that they are unable to catalyze the liberation of free dRp from incised AP sites. Instead, they release dRp as part of a small oligonucleotide (15, 16, 39). Moreover, they do so at a reduced rate compared with excision of undamaged 5′ nucleotide residues at nicks in DNA (35, 39). *E. coli* endonuclease III, which is a pyrimidine hydrate-DNA glycosylase with an associated AP lyase activity, promotes chain cleavage by β-elimination at the 3′ side of an AP site (2), but such cleavage does not occur at sites already hydrolytically incised on the 5′ side by an AP endonuclease (15, 24). In a search for alternative excision functions, the major activity detected in cell extracts of *E. coli* (15) and human cells (39) was a protein of approximately 50 kDa which could hydrolytically excise free dRp from AP sites preincised by an AP endonuclease. This enzyme, DNA deoxyribophosphodiesterase (dRpase), does not have detectable exonuclease activity.

In the present work, we have investigated the fine structure of the repaired region after excision repair of DNA uracil residues. For this purpose, we have employed synthetic double-stranded oligonucleotides with sequences suitable for restriction enzyme analysis. The experiments have been carried out with mammalian cell extracts similar to those utilized for studies of transcription (30), splicing (36), and excision repair of pyrimidine dimers (59) and with *E. coli* extracts similar to those used to observe the generation and resolution of recombination intermediates (6). Specifically, we have questioned whether a repair patch size of 2 or more nucleotides occurs, which would be expected as a consequence of 5′→3′ exonuclease action, or whether single-nucleotide patches can be detected, indicative of the action of a dRpase (Fig. 1). In addition, we have used inhibitors of different mammalian DNA polymerases to assess which of these enzymes catalyzes DNA repair synthesis at an AP site.

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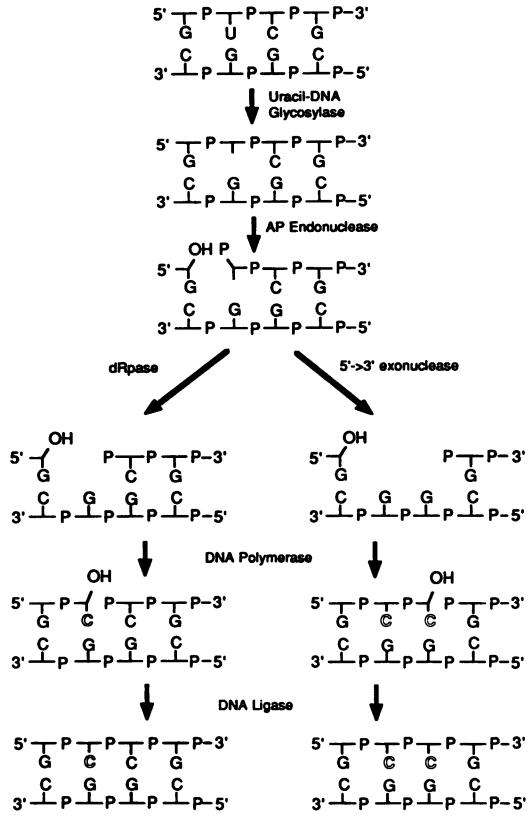

FIG. 1. Pathways for repair of a dUMP residue in DNA. After cleavage of the base-sugar bond of the dUMP residue by uracil-DNA glycosylase and incision of the phosphodiester bond 5′ to the AP site by an AP endonuclease, the baseless sugar-phosphate residue could be excised by a dRpase or a 5′→3′ exonuclease. In the former case, a single-nucleotide repair patch would result, while in the latter instance, at least two nucleotides would be replaced. The polymerase step could occur either after (as shown) or before the excision step.

MATERIALS AND METHODS

Cell extracts. *E. coli* BD10 (*ung thyA deoC*) and its *ung*$^+$ parent strain (W3110) (11) were obtained from B. Weiss. *E. coli* NH5033 (*recB sbcB endA*) was provided by S. C. West. Bacteria were grown in Luria broth, and extracts were prepared by lysozyme-EDTA treatment as described by Connolly and West (6). The human lymphoblastoid cell lines GM1310B and GM1953A, from healthy individuals, and GM1526B, from an individual with ataxia telangiectasia, were obtained from the Human Genetic Mutant Cell Repository (Camden, N.J.). The human cells were grown in suspension culture at 37°C in RPMI 1640 medium supplemented with 10% fetal calf serum. The Chinese hamster ovary (CHO) cell line AA8 and a cell line (EU2) derived from it with mutations in both the *ERCC-1* and the *XRCC-1* genes were obtained from the collection of D. Busch (5). The CHO lines were grown in roller bottles in Dulbecco's minimal essential medium supplemented with 10% fetal calf serum. Mammalian cell extracts were prepared by the method of Manley et al. (31).

Oligonucleotide substrates. Single-stranded oligonucleotides for substrates a through e (see Fig. 2) and dT_{30} were prepared on a commercial DNA synthesizer. For annealing of complementary strands, equimolar quantities of single-stranded oligonucleotides were incubated for 2 min at 65°C (a through c) or 3 min at 85°C (d and e) in a solution containing 0.25 M NaCl, 10 mM Tris-HCl (pH 8.0), and 1 mM EDTA and allowed to cool slowly to 25°C. The oligonucleotides were precipitated with 3 volumes of ethanol, resuspended in 10 mM Tris-HCl (pH 8.0)–1 mM EDTA, and stored at −20°C. The duplex DNA substrates were electrophoresed in nondenaturing 20% polyacrylamide gels to confirm that annealing had occurred.

Poly(dA · dT) containing incised ^{32}P-labeled 5′-terminal AP sites was prepared as previously described (39). Briefly, poly(dA · dT) containing ^{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. The polynucleotide was incubated with *E. coli* uracil-DNA glycosylase and then with *E. coli* exonuclease III in 0.1 M NaCl–10 mM sodium citrate (pH 7.0). Under these conditions, the latter enzyme functions as an AP endonuclease but not as an exonuclease (28). The incised polynucleotide was precipitated with ethanol and stored at −20°C either in ethanol or in 10 mM citrate buffer (pH 6.2). A polynucleotide containing ^{32}P-labeled reduced 5′-terminal AP sites was obtained by treating the incised polymer with sodium borohydride (19). The polynucleotide was resuspended in 100 μl of 0.5 M phosphate buffer (pH 6.8), and sodium borohydride was added in aliquots to a final concentration of 0.25 M. After incubation at room temperature for 90 min, the polynucleotide was dialyzed overnight against 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)–NaOH (pH 8.0)–1 mM EDTA and stored at −20°C. Approximately 93% of the 5′-terminal dRp residues were converted to an alkali-stable form by the sodium borohydride treatment. ^{32}P-labeled oligo(dT) · poly(dA) was prepared by end labeling dT_{30} with T4 polynucleotide kinase (29) and annealing to poly(dA) (52).

Reagent enzymes. *E. coli* uracil-DNA glycosylase (25), *E. coli* DNA dRpase (15), bovine DNA ligase I (52), human DNase IV (18, 39), and partially purified human DNA dRpase (39) were prepared as previously described. *E. coli* exonuclease III was obtained from Boehringer Mannheim. T4 polynucleotide kinase and the restriction endonucleases *Hae*III, *Hpa*II, and *Sac*I were purchased from New England Biolabs, Inc. dRpase and 5′→3′ exonuclease activities were measured as previously described (39).

Base excision repair and product analysis. For repair by *E. coli* or human cell extracts, standard reaction mixtures (50 μl) containing 100 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM EDTA, 2 mM ATP, 0.5 mM NAD, 20 μM (each) three deoxynucleoside triphosphates (dNTPs), 5 mM diTris-phosphocreatine, 10 U of creatine phosphokinase, 2 pmol of duplex oligonucleotide, 20 pmol of carrier single-stranded noncomplementary oligonucleotide, 2 to 20 μM the fourth dNTP (including 2.5 to 20 μCi of [α-^{32}P]dNTP), and 50 μg of protein extract were incubated for 10 min at 37°C. The reactions were stopped by the addition of EDTA and NaCl to final concentrations of 50 mM and 0.3 M, respectively, and the DNA was extracted with phenol-chloroform and precipitated with 3 volumes of nonchilled ethanol. The precipitates were collected immediately by centrifugation to minimize coprecipitation of dNTPs, dried under a vacuum, and resuspended in 100 μl of 10 mM Tris-HCl (pH 8.0)–1 mM EDTA. The duplex oligonucleotide (0.1 to 0.2 pmol) was incubated for 1 h at 37°C with 20 to 50 U of restriction endonuclease under the conditions recommended by the manufacturer. After incubation at 95°C for 2 min in 5 μl of 80% formamide–0.1% xylene cyanol–0.1%

bromophenol blue, the DNA was electrophoresed on a 20% polyacrylamide gel containing 7 M urea in 89 mM Tris–89 mM boric acid–2 mM EDTA (pH 8.8). The band intensities on the autoradiogram were quantified by using an LKB Ultrascan XL scanning laser densitometer.

For repair reactions which were analyzed by precipitation of oligonucleotides on glass filters to quantitate the incorporation of radioactive material, reaction mixtures (100 μl) containing 50 mM HEPES-NaOH (pH 8.0), 10 mM $MgCl_2$, 60 mM KCl, 2 mM dithiothreitol, 2 mM ATP, 20 μM each dNTP, 0.5 mM EDTA, 50 mM phosphocreatine, 10 U of creatine phosphokinase, 25 μg of bovine serum albumin, 0.5 μg (25 pmol) of duplex DNA substrate, 0.5 to 1 pmol of an [α-^{32}P]dNTP (3,000 Ci/mmol; Amersham), and 50 μg of protein were incubated for 5 min at 37°C. The entire reaction mixture was applied to a 1-ml Sephadex G-50 column and centrifuged at 700 × *g* for 4 min (29). A 20-μl volume of 0.5% calf thymus DNA and 13 μl of 50% trichloroacetic acid were added to the eluate, and the mixture was incubated at 0°C for 10 min. The material was applied to a GF/C glass filter (Whatman) prewashed in 0.2 M sodium phosphate–5% trichloroacetic acid. The filter was washed twice with 5% trichloroacetic acid and twice with ethanol and dried, and the amount of radioactive material retained on the filter was determined by scintillation counting. Reactions with inhibitors of DNA polymerases were performed as described above in the presence of ddTTP (Sigma), aphidicolin (Sigma), or dimethyl sulfoxide, as described in Results.

RESULTS

Stability of double-stranded oligonucleotides in cell extracts. Preliminary experiments established that blunt-ended, double-stranded oligonucleotides were not appreciably degraded in gently prepared cell extracts of *E. coli* NH5033 and human lymphoblastoid cell lines under the conditions employed in this study. In order to suppress oligonucleotide degradation, this *E. coli* strain lacked functional RecBCD nuclease, exonuclease I, and endonuclease I, which are unlikely to participate in the repair of AP sites. Wild-type *E. coli* extracts were also used, and they yielded results similar to those of the experiments described below but gave appreciable background degradation, which allowed incubation times of only 2 rather than 10 min. The 3′-terminal nucleotide residues in each strand showed limited turnover both in *E. coli* and in human cell extracts, presumably because of nonprocessive 3′→5′ exonuclease action and repolymerization. Thus, 30-mer oligonucleotides were usually reisolated after incubation with cell extracts as a mixture of 29-mers and 30-mers (see Fig. 3), and 3′-terminal restriction enzyme fragments appear as double bands after gel electrophoresis (see Fig. 4 and 5). For this reason, all oligonucleotides were constructed to contain at least two base residues at the 3′ end which did not occur in the immediate vicinity of the centrally located uracil residue under investigation to reduce the replacement of the excised 3′-terminal nucleotides by radioactive material (Fig. 2). The relative stability of double-stranded oligonucleotides incubated for extended periods with gently prepared mammalian cell extracts has also been noted by others (4, 56).

To assess the susceptibility of the 5′ termini of a double-stranded oligonucleotide to exonuclease digestion, the rates of release of 5′-terminal nucleotide residues from a substrate containing single-strand interruptions, ^{32}P-labeled oligo (dT) · poly(dA), and from the end-labeled blunt-ended substrate d (Fig. 2) were compared. The substrate containing

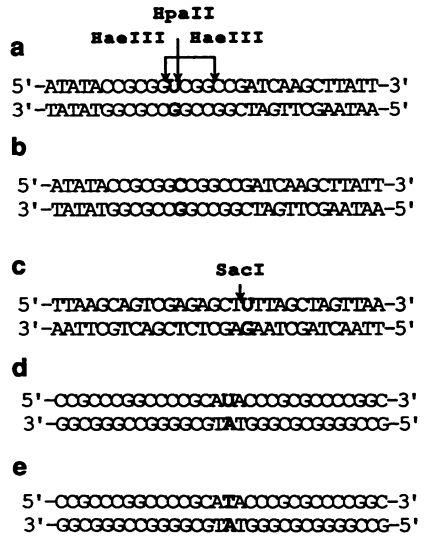

FIG. 2. Sequences of duplex oligonucleotide substrates. Oligonucleotides a, c, and d contained a dUMP residue, while b and e were control oligonucleotides identical to a and d, respectively, but containing a dCMP (b) or a TMP (e) residue in place of the dUMP residue. Restriction endonuclease sites in substrates a and c are indicated.

internal strand breaks was approximately 10 times more susceptible to exonuclease digestion than the blunt-ended substrate. Thus, with 0.00125 U of DNase IV under standard assay conditions, approximately 30% of the radioactive material was released in acid-soluble form in 5 min from the substrate containing single-strand interruptions and less than 3% was released from the blunt-ended substrate, whereas 0.00625 U of DNase IV liberated approximately 5% of the radioactive material from the latter substrate. In conclusion, the 5′ termini of the blunt-ended oligonucleotide substrates used in these experiments were relatively resistant to digestion by the major 5′→3′ exonuclease of mammalian cell nuclei.

DNA repair replication following excision of a uracil residue by *E. coli* cell extracts. Substrate a (Fig. 2), a double-stranded oligonucleotide containing a G · U mispair, and substrate b, which was identical except for the presence of the correct G · C pair, were used to determine the extent to which dCMP incorporation by an *E. coli* cell extract depended on the excision of uracil by uracil-DNA glycosylase. Figure 3 shows that turnover of dAMP and TMP residues occurred in substrates a (lanes 2 and 3) and b (lanes 5 and 6) to a similar extent but that the incorporation of dCMP was 20-fold greater in substrate a (lane 1) than in substrate b (lane 4). Moreover, separate experiments (results not shown) with extracts from isogenic *ung*$^+$ and *ung* strains established that uracil excision by uracil-DNA glycosylase was required for excision repair. The results (Fig. 3) indicate that excision of the uracil residue occurred in substrate a and was followed by replacement of the resultant AP site with a dCMP residue. The amount of substitution was close to 100% in these experiments. Turnover of nucleotide residues at 3′ termini was not dependent on the presence of a damaged residue in the central part of the oligonucleotide.

The design of the two substrates was such that if dCMP but not dGMP was utilized in repair replication after uracil excision, the repair patch could be defined as having a size of not more than 2 nucleotides. Incubation of substrate a with

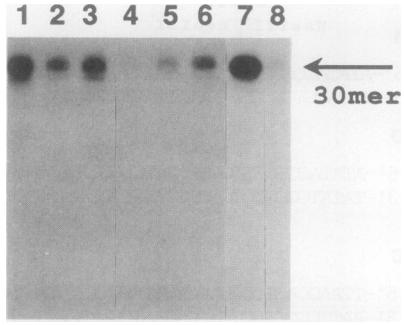

FIG. 3. Incorporation of deoxynucleoside monophosphate into oligonucleotides by an *E. coli* cell extract. The autoradiograms show incorporation of radioactive residues into an oligonucleotide containing a single dUMP residue by an *E. coli* cell extract. Substrate a containing a G · U base pair and substrate b containing the correct G · C base pair were incubated with an extract of *E. coli* NH5033 in the presence of an [α-^{32}P]dNTP as described in Materials and Methods, purified by phenol-chloroform extraction and ethanol precipitation, and analyzed by electrophoresis on 20% polyacrylamide gels. Data from two separate experiments (lanes 1 through 6 and lanes 7 and 8) are shown. Oligonucleotide a was incubated with [α-^{32}P]dCTP (lanes 1 and 7), [α-^{32}P]dATP (lane 2), [α-^{32}P]TTP (lane 3), and [α-^{32}P]dGTP (lane 8); substrate b was incubated with [α-^{32}P]dCTP (lane 4), [α-^{32}P]dATP (lane 5), and [α-^{32}P]TTP (lane 6).

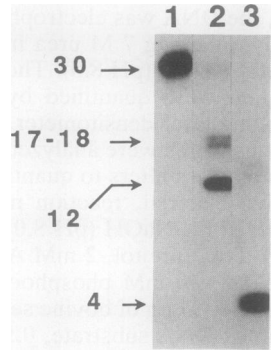

FIG. 4. Restriction enzyme analysis of substrate a after excision repair by an *E. coli* cell extract. The autoradiogram shows oligonucleotide a digested with *Hae*III or *Hpa*II after repair of a DNA uracil residue by an *E. coli* cell extract. After incubation of substrate a with an extract of *E. coli* NH5033 in the presence of [α-^{32}P]dCTP under the conditions and buffer described in Materials and Methods, the DNA was purified by phenol-chloroform extraction and ethanol precipitation. The redissolved oligonucleotide was incubated with no restriction enzyme (lane 1), 50 U of *Hpa*II (lane 2), or 50 U of *Hae*III (lane 3). The products of restriction enzyme digestion were separated by gel electrophoresis on a 20% polyacrylamide gel. Fragment sizes (-mer) are indicated.

a cell extract in the presence of either [α-^{32}P]dCTP or [α-^{32}P]dGTP showed that approximately 30 times as much dCMP (Fig. 3, lane 7) as dGMP (Fig. 3, lane 8) was incorporated at the repair patch. As a control, standard nick translation of DNA (41) with *E. coli* DNA polymerase I showed similar incorporation with either the [α-^{32}P]dCTP or the [α-^{32}P]dGTP used in this study. Thus, following the excision of a uracil residue, greater than 95% of the DNA repair replication events in the oligonucleotide involved no more than the excised nucleotide residue and one residue 3′ to the AP site.

Analysis of DNA repair replication in *E. coli* cell extracts by restriction enzyme digestion of oligonucleotides. In order to further define the size of the resynthesized patch, after excision repair of the dUMP residue substrate a was subjected to restriction endonuclease digestion, gel electrophoresis, and autoradiography. The oligonucleotide was designed so that if only one nucleotide was substituted during repair of the dUMP residue, *Hae*III digestion would result in [^{32}P]dCMP being present in a 4-nucleotide fragment and *Hpa*II digestion would result in its being present only in a 12-nucleotide fragment (Fig. 2). Any extension of the resynthesized segment 3′ to the AP site would be observed as incorporation of [^{32}P]dCMP in the 18-nucleotide fragment obtained after *Hpa*II digestion. Scanning of the autoradiograph shown in Fig. 4 indicated that after excision repair by an *E. coli* extract, more than 90% of the radioactive material was found within the 4-nucleotide fragment after digestion by *Hae*III and 70 to 75% of the radioactive material was found in the 12-nucleotide fragment after digestion by *Hpa*II. The data indicate that more than 70% of the repair events occurring in the cell-free system involved the incorporation of only a single nucleotide, whereas approximately 20 to 25% of the repair events involved incorporation of two or more nucleotides 3′ to the AP site.

In order to confirm the size of the resynthesized segment and to determine whether nucleotide incorporation 5′ to the uracil residue would occur in an oligonucleotide of a different sequence, substrate c was constructed. This oligonucleotide was A · T rich in the region of the uracil residue (Fig. 2). Consequently, extension of the resynthesized segment 5′ to the AP site would result in the presence of [^{32}P]TMP and possibly [^{32}P]dCMP in the 17-nucleotide fragment after *Sac*I digestion, and extension 3′ to the AP site would result in the occurrence of [^{32}P]TMP in the 13-nucleotide fragment after *Sac*I digestion. In Fig. 5, results are shown for DNA repair synthesis by an *E. coli* extract with [α-^{32}P]dCTP (lanes 1 and 2) or [α-^{32}P]TTP (lanes 3 and 4). Incorporation of radioactively labeled nucleotides was confined almost entirely to the

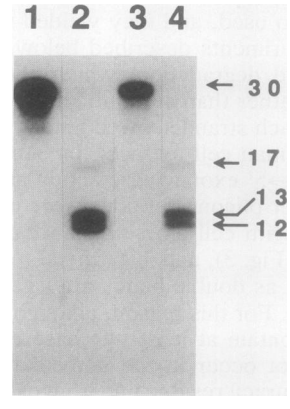

FIG. 5. Restriction enzyme analysis of substrate c after excision repair by an *E. coli* cell extract. After incubation of substrate c with an *E. coli* NH5033 extract in the presence of [α-^{32}P]dCTP or [α-^{32}P]TTP under the conditions described in Materials and Methods, the DNA was purified by phenol-chloroform extraction and ethanol precipitated. The oligonucleotide was redissolved and incubated either with no restriction enzyme or with 50 U of *Sac*I, and the nucleotide fragments were separated on a 20% polyacrylamide gel. Lane 1, [α-^{32}P]dCTP with no restriction enzyme digestion; lane 2, [α-^{32}P]dCTP with 50 U of *Sac*I; lane 3, [α-^{32}P]TTP with no restriction enzyme; lane 4, [α-^{32}P]TTP with 50 U of *Sac*I. Fragment sizes (-mer) are indicated.

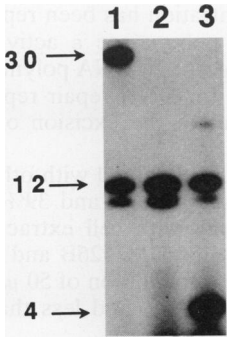

FIG. 6. Restriction enzyme analysis of substrate a following repair of a DNA uracil residue by a human cell extract. Substrate a was incubated with 50 μg of protein from the human lymphoblastoid cell line GM1953A in the presence of [α-^{32}P]dCTP under the conditions described in Materials and Methods. The DNA was extracted with phenol-chloroform, ethanol precipitated, redissolved, and incubated with no restriction enzyme (lane 1), 50 U of *Hpa*II (lane 2), or 50 U of *Hae*III (lane 3). The nucleotide fragments were separated on 20% polyacrylamide gels. The minor band immediately below the 12-mer represents this fragment in its 5′-phosphorylated form, because of the presence of polynucleotide kinase activity in the human cell extract. The radioactive material at the lower edge is unincorporated [α-^{32}P]dCTP.

13-nucleotide restriction fragment, with approximately three times as much [^{32}P]dCMP as [^{32}P]TMP being utilized. Little incorporation of [^{32}P]TMP (<2% of total) was found in the 17-nucleotide restriction fragment, indicating that extension of the repair patch beyond one nucleotide occurred only 3′ to the excised uracil residue. These data demonstrate that the majority of DNA repair replication events in the *E. coli* cell extract involved the replacement of only one nucleotide.

DNA repair replication in human cell extracts following excision of a uracil residue. Oligonucleotide d (Fig. 2) was designed to allow quantitation of the number of single-nucleotide repair patches relative to the number of longer patches by measurement in parallel experiments of the excess employment of [^{32}P]TMP over [^{32}P]dAMP. In several experiments with cell extracts from human lymphoblastoid cell lines, the utilization of TMP in repair replication was 3 to 5.5 times that of dAMP, indicating that 70 to 80% of the newly synthesized patches in the cell-free system after uracil excision contained only one nucleotide. The incorporation of TMP in substrate e, which contained the correct A · T base pair, was only 7% of that observed in substrate d with an A · U base pair after incubation with extracts, confirming that [^{32}P]TMP occurred in the oligonucleotides largely as a consequence of repair of the dUMP residue.

Extent of DNA repair replication by human cell extracts. In order to further analyze the amount of DNA repair synthesis carried out by human cell extracts, a restriction enzyme digest of oligonucleotide a was performed after excision repair by an extract from the human lymphoblastoid cell line GM1953A (Fig. 6). As with *E. coli* cell extracts, excision and repolymerization of the 3′-terminal nucleotide were observed with human cell extracts. The presence of both a 30- and a 12-nucleotide fragment prior to restriction enzyme digestion (Fig. 6, lane 1) suggested that ligation of the two fragments was incomplete after excision and gap-filling by the cell extract. Addition of purified bovine DNA ligase I (30 ng) to the reaction mixture did not reduce the number of unligated fragments, indicating a partial block to ligation following DNA polymerization, possibly by a DNA-binding protein, rather than inadequate DNA ligase activity in the extract. *Hae*III digestion (Fig. 6, lane 3) demonstrated that dCMP incorporation was confined largely to the 4-nucleotide fragment containing the site of the excised uracil residue and the residues 3′ to it. *Hpa*II digestion (Fig. 6, lane 2) of the repaired oligonucleotide indicated that the dCMP incorporation occurred within the 5′-terminal 12-nucleotide fragment containing the gap formed by the excision of the uracil residue. More than 95% of the radioactive material in the oligonucleotide was present in the *Hpa*II fragments containing the replaced dCMP moiety and the residues 5′ to it. Taken together, these results show that DNA repair replication catalyzed by activities in the cell extract was limited to a single nucleotide replacing the AP site, with little extension of the repair patch 3′ or 5′ to the dUMP residue.

Chemical reduction of AP sites prevents dRpase activity after incision. AP sites in DNA are alkali labile but can be stabilized by reduction of the free aldehyde group with sodium borohydride. Alternatively, a 3-hydroxy-2-hydroxymethyltetrahydrofuran residue can be synthesized as a structural analog of a stable reduced AP site and incorporated into oligonucleotides by automated DNA synthesis (49). AP endonucleases can incise DNA containing such residues but only at a greatly reduced rate (32, 45, 49). In order to assess whether such stable reduced forms of AP sites are useful analogs in excision repair reactions, we have assessed the ability of dRpases from *E. coli* and human cells to catalyze the release of a reduced 5′-terminal dRp residue. Poly(dA · dT) containing ^{32}P-labeled incised AP sites (15, 39) was reduced with sodium borohydride (19). On incubation with 0.002 U of 300-fold-purified *E. coli* dRpase under standard assay conditions (15), very little detectable release (less than 5%) of reduced dRp residues occurred, although the enzyme effectively liberated dRp residues from an analogous unreduced substrate. The difference between the activities of dRpase on nonreduced and reduced substrates was greater than 10-fold, a more distinct difference than that noted in a preliminary study with a crude enzyme preparation (14). In experiments with partially purified dRpase from a human lymphoblastoid cell line (39), only small amounts of radioactive material (less than 5%) were liberated from a poly(dA · dT) substrate containing reduced 5′-incised AP sites by 0.003 U of dRpase, apparently corresponding to the small proportion of dRp residues which had not been reduced during the sodium borohydride treatment. We conclude that the use of reduced analogs of AP sites in excision repair experiments with cell extracts or purified enzymes will register alternative repair pathways not involving dRpase action.

Cofactor requirements. The presence of either 2 or 5 mM ATP in reaction mixtures caused an apparent threefold stimulation of site-specific excision repair in both *E. coli* and mammalian cell extracts compared with reaction mixtures without ATP. A similar effect of ATP on DNA repair replication in bleomycin-treated permeabilized mouse sarcoma cells has been observed (47). The stimulation observed here was in part due to stabilization of the dNTP pools, since it was clearly smaller when additional dNTP (100 μM each) was added. In contrast to the absolute dependence on ATP in the excision repair of pyrimidine dimers in extracts of *E. coli* (46) and human cells (59), it is presently unclear whether ATP is required for excision repair of dUMP residues except in its role as a cofactor of a mammalian DNA ligase. When ATP was absent from the *E. coli* repair reactions, an increased amount of nonspecific nucleotide incorporation was seen in addition to specific DNA repair replication. A

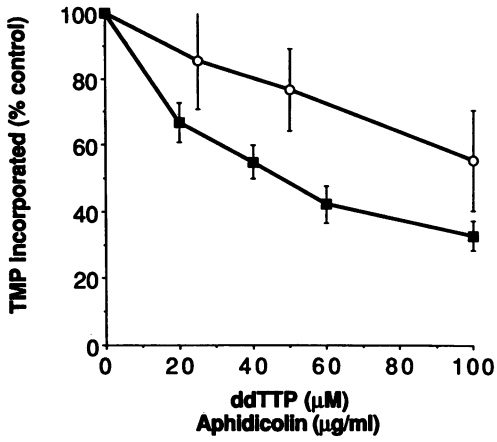

FIG. 7. Effect of DNA polymerase inhibitors on TMP incorporation during repair of a uracil residue in DNA by a human cell extract. Reaction mixtures (100 μl) containing 0.5 μg of substrate d, 50 μg of protein from a GM1310B human lymphoblastoid cell extract, 1.5 to 3 μCi of [α-^{32}P]TTP, and the concentrations of ddTTP (■) or aphidicolin (○) shown were incubated under the conditions described in Materials and Methods. Reactions were stopped by the addition of 100 μg of calf thymus DNA and trichloroacetic acid to a final concentration of 5%. The oligonucleotide was collected on a glass filter and washed with 5% trichloroacetic acid, and the amount of radioactive material retained on the filter was determined. Error bars indicate the standard errors for five experiments.

slight stimulatory effect of NAD^+ addition on uracil excision repair was also observed with human cell extracts (data not shown).

Effects of DNA polymerase inhibitors on repair replication by mammalian cell extracts. In order to ascertain which DNA polymerase might be involved in DNA repair replication at an AP site, the incorporation of [^{32}P]TMP during repair of the uracil residue in oligonucleotide d in the presence of various DNA polymerase inhibitors was measured. DNA polymerases α (48), δ (38), and ε (55) are inhibited by aphidicolin at concentrations which do not affect DNA polymerase β. In contrast, DNA polymerase β is inhibited by ddTTP at concentrations which have only minor effects on the other nuclear DNA polymerases (38). In the latter case, ddTTP does not function primarily by causing chain termination but by acting as a competitive inhibitor for TTP (13). Dimethyl sulfoxide (10% [vol/vol]) suppresses the activity of DNA polymerase ε but has much less effect on DNA polymerases α and δ (55).

The results of experiments examining the effects of aphidicolin and ddTTP on DNA repair replication by a human lymphoblastoid cell extract (from line GM1310B) are shown in Fig. 7. Aphidicolin had only a relatively minor effect in experiments with substrate d (approximately 14% inhibition at 25 μg/ml and 23% inhibition at 50 μg/ml) at levels at which greater than 95% inhibition of DNA replication (21) or long-patch DNA repair (20, 51) has been observed. Similar results were obtained in experiments with substrate a in which dCTP utilization was assessed. Furthermore, addition of 100 μM ddTTP to the reaction mixture in place of aphidicolin caused 67% inhibition of repair replication at uracil residues in substrate d. This ddTTP concentration has been reported to inhibit purified bovine DNA polymerases α, δ, and ε by only approximately 25% (55). In separate experiments, the presence of 10% dimethyl sulfoxide resulted in a 17% stimulation of DNA repair synthesis, whereas this concentration has been reported to cause 70% inhibition of DNA polymerase ε activity (55). Taken together, the data suggest that DNA polymerase β is the major activity responsible for DNA repair replication at AP sites produced as a result of the excision of uracil residues in human cell extracts.

Similar results were obtained with other cell lines. Thus, 40 μM ddTTP caused 48, 44, and 39% inhibition, respectively, in experiments with cell extracts from the human lymphoblastoid cell line GM1526B and the CHO cell lines AA8 and EU2, whereas addition of 50 μg of aphidicolin per ml resulted in only 11, 10, and less than 5% inhibition of repair replication, respectively.

DISCUSSION

Repair of an AP site is initiated by an AP endonuclease which catalyzes chain breakage at the 5′ side of the base-free site. Consequently, a separate excision function is required prior to gap-filling and ligation. It has been generally assumed that a 5′→3′ exonuclease accounts for this process. However, such enzymes would liberate the dRp residue as part of a small oligonucleotide, since they are unable to catalyze the release of free dRp from a DNA 5′ terminus (39). The main finding in the present work, which employed cell extracts of *E. coli* and human cells, is that the prevalent repair reaction involves the filling-in of a gap of only a single nucleotide. These results strongly indicate that an enzyme different from the 5′→3′ exonuclease function of *E. coli* DNA polymerase I or its mammalian equivalent, DNase IV, accounts for the excision step in the repair of AP sites.

Direct attempts to identify such an excision function led to the detection of DNA dRpase in *E. coli* (15) and human cell nuclei (39). This enzyme, which has no associated exonuclease or DNA phosphatase activity, hydrolytically releases dRp residues in free form from enzymatically incised AP sites. To date, no genetic evidence that dRpase accounts for the excision step in vivo during excision repair of AP sites is available. However, the approximately 50-kDa dRpase is the dominant activity of this type in crude cell extracts of *E. coli* and mammalian cells (15, 39). The molecular mass of dRpase is larger than that of AP endonucleases or DNA glycosylases-AP lyases, which are typically 20- to 40-kDa proteins, indicating that the main excision function is a distinct enzyme. A number of other cellular proteins might serve as backup functions, however, contributing to the release of free dRp from incised AP sites by β-elimination. The intrinsic property of dRpase of being unable to liberate mononucleotides from termini in DNA provides a satisfactory explanation for the very small patch size observed in the present work: the dRpase can release the base-free sugar-phosphate from an incised AP site but is then incapable of extending the gap.

A different route of excision repair might occur during the removal of oxidative DNA lesions, such as pyrimidine hydrates and 8-hydroxyguanine, as well as thymine residues at G · T mismatches. The DNA glycosylases catalyzing these reactions also have the ability to promote β-elimination of the phosphodiester bond on the 3′ side of the lesion (2, 3, 50, 56). Thus, a nonincised AP site in DNA may never be a reaction intermediate in these cases, since the repair enzymes both release a free base by hydrolysis and cleave a phosphodiester bond. The 3′-terminal sugar-phosphate residue occurring in these cases can be excised by an AP endonuclease, since such enzymes can cleave at the 5′ side of the lesion independent of the existence of a strand break

on the 3′ side (24, 43). The Fpg protein, a DNA glycosylase-AP lyase which liberates free 8-hydroxyguanine and formamidopyrimidine residues from DNA, and the mammalian enzyme removing ring-saturated pyrimidine residues have an intrinsic ability to cleave first on the 3′ side and then on the 5′ side of the lesion (3, 10). Such repair would not be significant, however, at AP sites generated by nonenzymatic hydrolysis, i.e., depurination and depyrimidination, nor would it occur as a consequence of the action of abundant repair enzymes such as uracil-DNA glycosylase or 3-methyladenine-DNA glycosylase, which lack the ability to promote β-elimination at AP sites in DNA (7, 9).

A detailed study of the excision repair of a tetrahydrofuran analog of an AP site by *Xenopus laevis* cell extracts has been performed by Matsumoto and Bogenhagen (32, 33). This residue is repaired by the extracts in an ATP-dependent reaction with the generation of patches of about 3 nucleotides. After incision at damaged sites, nicked intermediates accumulate in the system, indicating that the excision of the tetrahydrofuran residue is relatively inefficient and is the rate-limiting step in the reaction. As shown in the present work, dRpase does not seem to act at reduced 5′-terminal sugar-phosphates, so the reaction investigated by Matsumoto and Bogenhagen apparently reflects an alternative pathway, probably involving slow excision by a 5′→3′ exonuclease which generates a patch of 2 to 3 nucleotides. Recently, Withka and coworkers (58) demonstrated that the conformational properties of duplex DNA containing the naturally occurring aldehyde abasic site are different from those of duplex DNA containing a tetrahydrofuran analog of the abasic site.

Experiments on very short patch repair usually have not addressed directly the critical question of whether the patch is just 1 or at least 2 nucleotides in size (Fig. 1). However, an early observation by Kataoka and Sekiguchi (23) on the repair of randomly depurinated sites in DNA by *E. coli* cell extracts indicated that TTP is employed in repair replication much less frequently than dGTP or dATP, consistent with a patch size of only 1 nucleotide in most cases. Subsequent gap-filling is most likely performed by DNA polymerase I in *E. coli* and, as suggested by the aphidicolin resistance observed in the present work, by DNA polymerase β in mammalian cells. Wiebauer and Jiricny (57) have reported that antibodies against DNA polymerase β interfere with gap-filling in a cell-free system during very short patch repair of G · T mismatches in DNA.

A major goal in studies on DNA replication, excision repair of bulky residues such as pyrimidine dimers, and mismatch correction by human cell-free systems is to define the various enzymes and protein factors required. This approach involves the relatively laborious identification and purification of relevant proteins by in vitro complementation assays (42, 53). In contrast, the biochemistry of short-patch excision repair involving AP sites as the reaction intermediates seems better understood, and enzymes which are good candidates to carry out each step in the pathway have already been isolated. Consequently, it should now be possible to perform the reactions described above with purified enzymes, in order to determine whether additional proteins are required for efficient repair or whether the entire excision repair pathway only reflects the consecutive actions of the five enzymes uracil-DNA glycosylase, AP endonuclease, dRpase, DNA polymerase, and DNA ligase.

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