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Major oxidative products of cytosine, 5-hydroxycytosine and 5-hydroxyuracil, exhibit sequence context-dependent mispairing *in vitro*

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ABSTRACT

Two major stable oxidation products of 2′-deoxycytidine are 2′-deoxy-5-hydroxycytidine (5-OHdC) and 2′-deoxy-5-hydroxyuridine (5-OHdU). In order to study the *in vitro* incorporation of 5-OHdC and 5-OHdU into DNA by DNA polymerase, and to check the base pairing specificity of these modified bases, 5-OHdCTP and 5-OHdUTP were synthesized. Incorporation studies showed that 5-OHdCTP can replace dCTP, and to a much lesser extent dTTP, as a substrate for *Escherichia coli* DNA polymerase I Klenow fragment (exonuclease free). However, 5-OHdUTP can only be incorporated into DNA in place of dTTP. To study the specificity of nucleotide incorporation opposite 5-hydroxypyrimidines in template DNA, 18- and 45-member oligodeoxyribonucleotides, containing an internal 5-OHdC or 5-OHdU in two different sequence contexts, were used. Translesion synthesis past 5-OHdC and 5-OHdU in both oligonucleotides occurred, but pauses both opposite, and one nucleotide prior to, the modified base in the template were observed. The specificity of nucleotide incorporation opposite 5-OHdC and 5-OHdU in the template was sequence context dependent. In one sequence context, dG was the predominant nucleotide incorporated opposite 5-OHdC with dA incorporation also observed; in this sequence context, dA was the principal nucleotide incorporated opposite 5-OHdU. However in a second sequence context, dC was the predominant base incorporated opposite 5-OHdC. In that same sequence context, dC was also the predominant nucleotide incorporated opposite 5-OHdU. These data suggest that the 5-hydroxypyrimidines have the potential to be premutagenic lesions leading to C → T transitions and C → G transversions.

INTRODUCTION

Oxidative damage to DNA produced during normal aerobic metabolism and oxidative stress has been implicated in a variety of diseases (1–3) as well as in ageing (4,5). In the cell, oxidative damage to DNA is mediated by hydroxyl radicals formed in its vicinity. Hydroxyl radicals interact with cytosine residues primarily by addition to the 5–6 double bond of the heterocycle resulting in the formation of a number of stable oxidation products via hydroperoxy radicals and hydroperoxides. These products are similar to those derived from hydroxyl radical attack on thymine. However, a major cytosine product, cytosine glycol, is not stable in aqueous solution and deaminates to form uracil glycol or dehydrates to form 5-hydroxycytosine (6,7). The 5-hydroxypyrimidines, 2′-deoxy-5-hydroxycytidine (5-OHdC), and 2′-deoxy-5-hydroxyuridine (5-OHdU), have been reported to be major stable oxidation products of 2′-deoxycytidine (6,7,8–10). Recent studies have shown that the steady state levels of 5-hydroxypyrimidines in DNA are high and increase substantially when DNA is exposed to ionizing radiation or oxidizing agents (7). In fact, the steady state level of 5-OHdC in DNA extracted from normal tissue is similar to that of 8-oxoguanine (7), a product recently shown to have important biological consequences (11–14). Because the 5-hydroxypyrimidines are stable products, and have not yet been demonstrated to be substrates for any repair enzymes, it is important to determine their mutagenic potential.

Here we report the chemical synthesis of 5-OHdCTP and 5-OHdUTP and their utilization as substrates for *E.coli* DNA polymerase I Klenow fragment. We further demonstrate unusual base-pairing properties of the 5-hydroxypyrimidines during translesion synthesis past the lesions in oligodeoxyribonucleotide templates.

MATERIALS AND METHODS

Chemicals, enzymes and DNA

2,4,6-Collidine and pyridine were obtained from Aldrich; bromine and all organic solvents used were obtained from J.T.Baker; γ-^{32}P-ATP (>5000 Ci/mmole, 10 mCi/ml) was obtained from Amersham; 2′-deoxynucleoside triphosphates were purchased from Pharmacia; 2′,3′-dideoxynucleoside triphosphates were obtained from US Biochemicals; DEAE sephadex A-25 was obtained from Sigma. All enzymes and M13 mp18 DNA were purchased from US Biochemicals.

Oligodeoxyribonucleotides

The oligonucleotides used were synthesized by the standard phosphoramidite method on an ABI 380A DNA synthesizer

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(Department of Microbiology and Molecular Genetics, University of Vermont). The phosphorylating agent (5' Phosphate-ON) was purchased from Clontech (Palo Alto, CA).

The oligonucleotides were purified by Mono Q (Pharmacia) anion-exchange chromatography on a Milton Roy HPLC system. The Mono Q column (HR 5/5) was equilibrated with buffer A (0.18 M NaCl, 0.01 M NaOH) at 1 ml/min. Crude deprotected oligonucleotides were loaded on the column and then eluted with a linear gradient of 100% buffer A to 50% buffer A/ 50% buffer B (0.9 M NaCl, 0.01 M NaOH) over 60 minutes. After purification, oligonucleotides were concentrated on a Speed Vac (Savant) and desalted by gel-filtration on a NEP-5 column (Pharmacia) using water as an eluent.

The oligonucleotides were 5'-^{32}P-labeled with γ-^{32}P-ATP using T4 polynucleotide kinase following standard procedures. Labeled oligonucleotides were further purified using a NENSORB 20 Nucleic Acids Purification Cartridge (DuPont). To obtain the desired final specific radioactivity, labeled oligonucleotides were combined with the appropriate cold oligonucleotides.

The oligonucleotides to be used in the ligation reaction were synthesized with 5'-phosphate. To control the ligation, and to detect the ligation product, 5'-phosphate was removed from a small amount of the particular oligonucleotide (5 nmoles) with 0.01 units of shrimp alkaline phosphatase in a buffer containing 20 mM Tris−HCl, pH 8.0 and 10 mM $MgCl_2$. Dephosphorylated oligonucleotides were purified using a NENSORB 20 cartridge and 5'-^{32}P-labeled with γ-^{32}P-ATP using standard procedures. Labeled oligonucleotides were further purified using a NENSORB 20 cartridge and combined with the same cold 5'-phosphorylated oligonucleotide to obtain the desired final specific radioactivity.

Synthesis of 5-OHdCTP

5-Hydroxy-2'-deoxycytidine 5'-triphosphate was synthesized using a procedure similar to that described for the synthesis of 5-hydroxycytidine 5'-diphosphate (15). 25 mg (45 μmole) of dCTP (sodium salt) was dissolved in 200 μl of water and then cooled to 4°C. Bromine was slowly added with vigorous mixing to the dCTP solution until the yellow color persisted. To remove excess bromine, 7 μl of cyclohexene was added with shaking, followed by 0.1 ml of 2,4,6-collidine. The emulsion was incubated for about 2 hours at 37°C and then extracted with ether (4×0.5 ml). The aqueous layer was evaporated under vacuum, redissolved in water, and loaded on a DEAE-Sephadex A-25 column (about 80 ml, HCO_3^- form). The fraction containing triphosphates was eluted from the column with a linear gradient of triethylammonium bicarbonate (TEAB), pH 7.5−8 (5 mM to 0.8 M). Fractions containing a mixture of nucleoside triphosphates were pooled and evaporated several times with 50% ethanol to remove TEAB. 5-OHdCTP was further purified twice on a Mono Q column using first a linear gradient of NaCl from 5 mM to 0.7 M in 20 mM Tris−HCl buffer, pH 7.5, and finally a linear gradient from 5 mM to 0.7 M sodium phosphate buffer, pH 3.5. The peak of 5-OHdCTP was collected, diluted with water, and reloaded on a DEAE sephadex A-25 column (2.5 ml, HCO_3^- form). The column was washed with 0.1 M ammonium bicarbonate to remove salt followed by the elution of 5-OHdCTP with 0.6 M ammonium bicarbonate. The ammonium bicarbonate was removed by repeated evaporation with 50% ethanol. The yield of 5-OHdCTP was about 25−30%. The molar absorptivity [ϵ = 7700 (λ_{max}=292 nm)] (15) was used to calculate the amount of 5-OHdCTP.

Synthesis of 5-OHdUTP

5-Hydroxy-2'-deoxyuridine 5'-triphosphate was synthesized by bromination of dUTP in aqueous medium followed by pyridine treatment as described for the synthesis of 5-hydroxyuridine 5'-triphosphate (16). 5-OHdUTP was purified first on a DEAE sephadex A-25 column as described above for 5-OHdCTP (to separate the fraction containing nucleoside triphosphates) and finally on a Mono Q column using a linear gradient of NaCl from 5 mM to 0.7 M in 20 mM Tris−HCl buffer, pH 7.5. The 5-OHdUTP collected was desalted by ion-exchange chromatography on a DEAE sephadex A-25 column as described above for 5-OHdCTP. The yield of 5-OHdUTP was about 20−25%. Molar absorptivity [ϵ=7335 (λ_{max}=278 nm)] (7) used to calculate the molar amount of 5-OHdUTP.

Synthesis of oligonucleotides containing an internal 5-OHdC or 5-OHdU

Oligonucleotides containing a single, internal 5-OHdC or 5-OHdU (templates 1 and 2, Fig. 3) were prepared by a modification of the method previously described (18). 1−2.5 nmoles of GCAGCCAAAACGTCC or CCTTCG were incubated for 30 min. at 30°C in 65 μl of buffer containing 100 mM sodium cacodylate pH 7.0, 1 mM $CoCl_2$, 0.1 mM EDTA, 50 μg/ml of BSA, 0.1 mM DTT, 10 μM 5-OHdCTP or 5-OHdUTP, and 100 units of terminal deoxynucleotidyl transferase. The oligonucleotides, extended from the 3'-end with a single 5-OHdCMP or 5-OHdUMP, were then HPLC purified on a Partisphere SAX column (0.4×12.5 cm, Whatman) using a linear gradient of sodium phosphate buffer, pH 6.3 (from 5 mM to 0.5 M over 60 min), containing 25% acetonitrile. The purified extended oligonucleotides, GCAGCCAAAACGTCCX or CCTTCGX (X = 5-OHdC or 5-OHdU), were desalted using a NEP-5 column (Pharmacia) and ligated using T4 DNA ligase with ^{32}pGGATGGTCTGTCCCTTGAATCGATAGGGG or ^{32}pTACTTTCCTCT (both oligonucleotides contained a small amount of 5'-^{32}P label for detection of ligation products), respectively, using the appropriate templates, GACAGACCATCCGGGACGTTTTGGCTGC or AGAGGAAAGTAGCGAAGG. Ligation reactions were performed for 10−15 hours at 15°C in 50 mM Tris−HCl, pH 7.6, 10 mM $MgCl_2$, 0.1 mM EDTA, 10 mM DTT, 0.3 mM ATP, 10 μM of nicked duplex oligonucleotide and 0.5 unit/μl of reaction mixture of T4 DNA ligase. After ligation, the strands containing the lesion were separated from the templates by electrophoresis on 20% PAG under denaturing conditions, eluted from the gel, and ethanol precipitated.

DNA polymerase reaction with 5-OHdCTP and 5-OHdUTP

The primer ^{32}pGTAAAACGACGGCCAGT, annealed to M13 mp18 DNA, was extended using 0.2 unit of *E.coli* DNA polymerase I Klenow fragment lacking proofreading activity (Kf Exo$^-$). The reaction mixture (6 μl) contained 15 mM Tris−HCl, pH 7.5, 7.5 mM $MgCl_2$, 30 mM NaCl, 4 mM DTT (buffer 'P'), 20 nM of primed DNA and all four combinations (dA,dT,dC; dG,dT,dC; dG,dA,dC and dG,dA,dT, 50 μM each) of the three normal dNTPs. DNA polymerase reactions were incubated at 37°C for 15 min. in the absence or presence of 50 μM 5-OHdCTP (or 5-OHdUTP).

DNA polymerase reactions

A 5'-^{32}P-labeled primer (20 nM), annealed to a template with a single 5-OHdC or 5-OHdU, was incubated at 37°C for 15 min in 6 μl of buffer 'P' containing 0.01 unit of Kf and four dNTPs (50 μM each).

'One nucleotide extension' reactions were performed similarly, using 50 nM primer-template and 5 μM of only one of the four dNTPs. The reaction mixture was incubated for 15 min at 15°C.

Dideoxy sequencing reactions, using the exonuclease free version of phage T7 DNA polymerase (Sequenase v.2), were performed following the recommendations of the supplier (US Biochemicals).

Electrophoresis

All enzymatic reactions were terminated by the addition of an equal volume of loading buffer (95% formamide, 0.05% bromphenol blue, 0.05% xylene cyanol and 20 mM EDTA). Reaction products were analyzed by electrophoresis on 0.4 mm thick 13% polyacrylamide gels containing 8 M urea. The gels were electrophoresed in 50 mM Tris–borate, 2 mM EDTA buffer, pH 8.3 for 2–4 h at 3000V, dried under vacuum, and exposed to X-ray film.

RESULTS AND DISCUSSION

Synthesis of 5-OHdCTP and 5-OHdUTP

In order to study the incorporation of the 5-hydroxypyrimidines into DNA by DNA polymerase *in vitro*, and to examine the base-pairing properties of these products, we synthesized both 5-OHdCTP and 5-OHdUTP. 5-OHdCTP was prepared by the bromination of dCTP in aqueous medium, followed by 2,4,6-collidine catalyzed hydrolysis (15). The purification of 5-OHdCTP included two separations on a Mono Q column. To remove the remainder of unreacted dCTP, the first column was eluted with a linear gradient of NaCl from 5 mM to 0.5 M in 20 mM Tris–HCl buffer, pH 7.5 (Fig. 1, panel A). For the second separation, a linear gradient from 5 mM to 0.7 M sodium phosphate buffer, pH 3.5, was used to remove the small amount (3–6%) of 5-OHdUTP formed at the stage of 2,4,6-collidine-catalyzed hydrolysis as a result of deamination of 5-OHdCTP (Fig. 1, panel B).

5-OHdUTP was synthesized similarly except pyridine was used in the hydrolysis step (11). 5-OHdUTP was purified on a Mono Q column using a gradient elution at pH 7.5 (similar to the buffer conditions described for the purification of 5-OHdCTP) to separate unreacted dUTP (about 5%) and 5-BrdUTP (25–30%) (Fig.1, panel C).

The final purity of 5-OHdCTP and 5-OHdUTP was confirmed by anion-exchange and reverse phase (after treatment with alkaline phosphatase) HPLC with UV- and electrochemical detection. 5-OHdCTP and 5-OHdUTP so prepared contained less then 5% of 5-OHdCDP and 5-OHdUDP, correspondingly.

The specificity of incorporation of 5-OHdCTP and 5-OHdUTP during DNA synthesis

To examine the specificity of incorporation of 5-OHdCTP and 5-OHdUTP into DNA in place of normal dNTPs by Kf Exo polymerase, we used a traditional 'three dNTP' assay (17). In

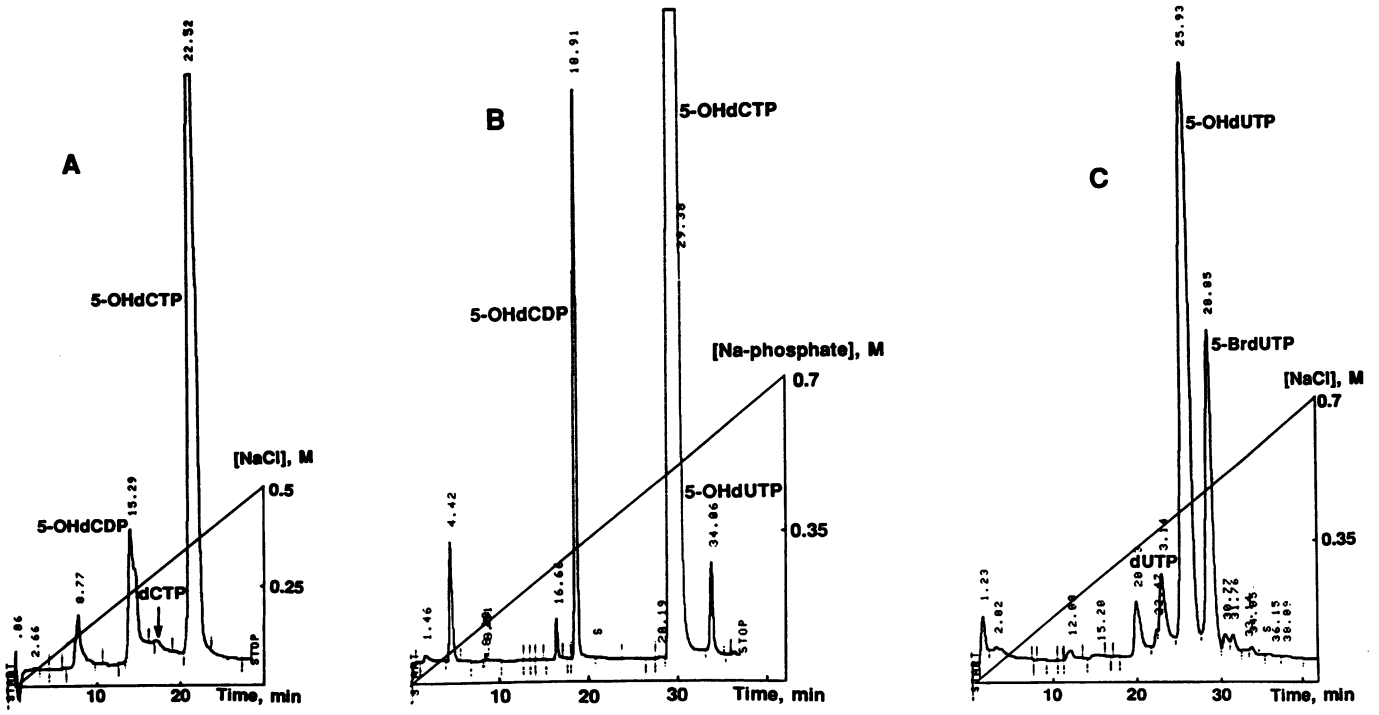

Figure 1. The elution profiles of Mono Q ion-exchange chromatography of reaction mixtures containing 5-OHdCTP (panels A and B) or 5-OHdUTP (panel C). Panel A: Elution of 5-OHdCTP with a linear gradient of NaCl from 5 mM to 0.5 M in 20 mM Tris–HCl buffer, pH 7.5; UV detection at λ = 292 nm; Panel B: Elution of 5-OHdCTP with a linear gradient from 5 mM to 0.7 M of sodium phosphate buffer, pH 3.5; UV detection at λ = 292 nm; Panel C: Elution of 5-OHdUTP with a linear gradient of NaCl from 5 mM to 0.7 M in 20 mM Tris–HCl buffer, pH 7.5; UV detection at λ = 278 nm.

this experiment, the primer-template complex was incubated with DNA polymerase in the presence of only three of the four dNTPs ('minus' reaction). DNA polymerase catalyzed primer elongation pauses opposite each template position complementary to the 'missing' dNTP. If, in the 'plus' reaction (same three dNTPs plus a modified dNTP), the added modified dNTP is incorporated in place of the missing dNTP, stimulation of the primer elongation reaction is observed.

Figure 2 shows the specificity of incorporation of 5-OHdCTP into DNA. Primer ^{32}pGTAAAACGACGGCCAGT was annealed to M13 mp18 DNA and extended using Kf Exo$^-$. Reaction mixtures contained all four combinations of three normal dNTPs and DNA polymerase reactions were conducted in the absence or presence of 5-OHdCTP. The extension products were analyzed by high resolution sequencing gels. A comparison of these products showed that 5-OHdCTP replaced both dCTP (compare lanes 11 and 12) and dTTP (compare lanes 8 and 9). However, the efficiency of incorporation of 5-OHdCTP in place of dCTP was much greater than in place of dTTP (compare lanes 9–12). It is clear that the presence of 5-OHdCTP does not inhibit the polymerase activity of Kf Exo$^-$ (compare lanes 13 and 14).

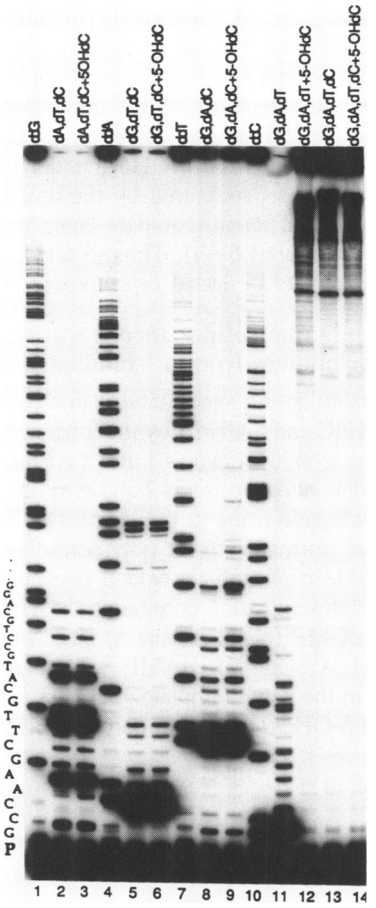

Figure 2. Specificity of incorporation of 5-OHdCTP catalyzed by *E.coli* DNA polymerase I Klenow fragment (exonuclease free). The extension of ^{32}pGTAAAACGACGGCCAGT, annealed to M13mp18 DNA, using *E.coli* Kf(Exo$^-$). Lanes 2 and 3 represent extension in the absence of dGTP ('-dG' reaction) without and with the addition of 50 μM 5-OHdCTP, respectively. Lanes 5 and 6 represent the '-dA' reaction without and with the addition of 50 μM 5-OHdCTP, respectively. Lanes 8 and 9 represent the '-dT' reaction without and with the addition of 50 μM 5-OHdCTP, respectively. Lanes 11 and 12 represent the '-dC' reaction without and with the addition of 50 μM 5-OHdCTP, respectively. Lane 13 shows extension of the primer in the presence of all four dNTPs (50 μM each); lane 14, same as 13, but containing, in addition, 50 μM 5-OHdCTP. Lanes 1, 4, 7 and 10, standard dideoxy sequencing reactions using the same primer and Sequenase.

The unusual incorporation of 5-OHdCMP in place of dTMP in the newly synthesized strand was also confirmed by the extension of ^{32}P-labeled oligo[dT]$_{12-18}$ annealed to poly[dA] using 5-OHdCTP and Kf Exo$^-$. In the control experiment, using similar conditions and dCTP, the extension of oligo[dT]$_{12-18}$ was not observed. To prove the presence of 5-OHdC in the extension product, the DNA fraction was separated from the components of the reaction mixture on a NENSORB 20 column and enzymatically digested to nucleosides with P1 nuclease and alkaline phosphatase. Analysis of the hydrolysate by reverse phase HPLC with UV- and electrochemical detection confirmed the presence of 5-OHdC in DNA (data not shown).

Using the similar approach (data not shown), we found that 5-OHdUTP can be incorporated into DNA but only in place of dTTP. Incorporation occurred with high efficiency.

Preparation of oligonucleotides containing a single internal 5-OHdC or 5-OHdU

In order to elucidate the interaction between DNA polymerases and 5-hydroxypyrimidines in template DNA, it was necessary to construct oligonucleotides containing each of these lesions in a specific position in the nucleotide chain. Two oligonucleotides, of different sequence and length, containing an internal 5-OHdC or 5-OHdU, were prepared. One of them, a 45-mer (template 1, Fig.3), was used for dideoxy sequencing and lesion bypass experiments. Another one, an 18-mer (template 2, Fig. 3), was used to study the specificity of nucleotide incorporation opposite the lesion in DNA polymerase reactions using the approach described for 8-oxodG (7). To compare the specificity of nucleotide incorporation in different sequence contexts, both the 45- and 18-mers were used in single nucleotide extension experiments. To prepare these two templates, oligonucleotides GCAGCCAAAACGTCC and CCTTCG were 3′ extended with either a single 5-OHdCMP or 5-OHdUMP using terminal deoxynucleotidyl transferase as previously described (18). To internalize the lesion, the purified oligonucleotides containing

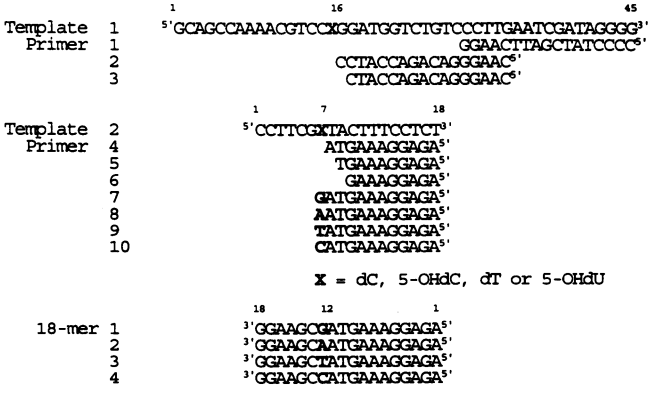

Figure 3. Sequence of templates and primers.

5-OHdC or 5-OHdU on the 3′-end, were ligated using T4 DNA ligase with a second oligonucleotide in the presence of the appropriate complementary templates (see Materials and Methods). The efficiency of ligation in the case of modified oligonucleotides was lower (about 50%) than for the similar oligonucleotides containing dC and dT on the 3′-end instead of 5-OHdC and 5-OHdU. The oligonucleotides used are depicted in Figure 3.

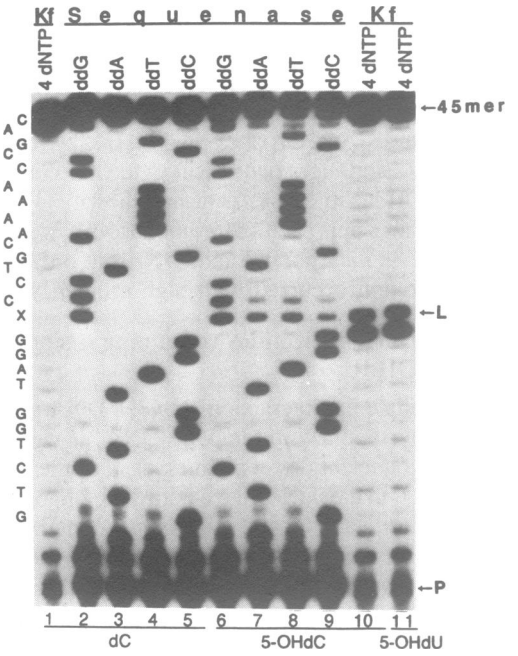

Figure 4. The effect of 5-OHdC and 5-OHdU in the template on DNA synthesis catalyzed by Kf and Sequenase. The 5′-^{32}P-labeled primer 1, annealed to a 5-OHdC- (lane 10) or a 5-OHdU (lane 11) containing template 1, was extended using Kf in the presence of four dNTPs. Lane 1, same experiment with undamaged template 1 containing dC at position 16; lanes 2–9, ddNTP sequencing reactions using Sequenase and primer 1 annealed to undamaged template 1 (lanes 2–5) and a 5-OHdC-containing template 1 (lanes 6–9). 'ddG' sequencing reaction, lanes 2 and 6; 'ddA', lanes 3 and 7; 'ddT', lanes 4 and 8; 'ddC', lanes 5 and 9. X = dC, 5-OHdC or 5-OHdU (P denotes the primer; L, the position of the lesion.)

Translesion DNA synthesis

To examine whether DNA polymerases can bypass 5-hydroxypyrymidines in the template, we studied the DNA polymerase catalyzed extension of primer 1 (see Fig. 3) annealed to template 1 containing either dC, 5-OHdC or 5-OHdU at position 16. As can be seen in Figure 4, 5-OHdC and 5-OHdU were efficiently bypassed by Kf with sites of pausing occurring both at and one nucleotide prior to the lesion (compare lanes 10 and 11).

Using Sequenase and the template containing 5-OHdC (lanes 2–9), termination bands were observed opposite the lesion in every lane, albeit with slightly different frequencies (the band in the ddG lane dominated). Termination bands were also present, although less pronounced, one nucleotide beyond the lesion (compare lanes 2–5 with 6–9). Similar results were obtained with template 1 containing 5-OHdU at the same position (data not shown), however the band in the ddA lane predominated. Dideoxy sequencing analysis does not give a clear picture of the base pairing specificity of 5-hydroxypyrimidines since the results can be explained either by misincorporation of all four ddNTPs opposite the lesion and some misincorporation opposite the next nucleotide in the template, or by the pausing of T7 DNA polymerase at the lesion site. However, even if Sequenase (or Kf) pauses at 5-OHdC and 5-OHdU under these steady state conditions, bypass, as measured by the amount of full length product, was very efficient (compare lanes 10 and 11 to lane 1 and lanes 2–5 to lanes 6–9). Thus, 5-OHdC and 5-OHdU in DNA are unlikely to be lethal or cytotoxic lesions.

Specificity of nucleotide incorporation opposite the 5-hydroxypyrimidines from a 'standing start' position

In order to examine the specificity of nucleotide incorporation opposite 5-OHdC and 5-OHdU by the 'one nucleotide extension' assay, oligonucleotide templates 1 and 2 containing these lesions were annealed to labeled primers 2 and 4, respectively, extending up to one nucleotide prior to the lesion (see Fig 3). A typical 'standing start' format with Kf polymerase was used with the deoxynucleoside triphosphates added one at a time. Figure 5 shows that, with template 1 containing 5-OHdC, primer 2 was extended by dGMP (panel A lane 5) and to a lesser extent by dAMP (panel A, lane 6). dAMP was also misincorporated opposite dC in the same template, but to a much lesser extent than opposite 5-OHdC (panel A, compare lanes 2 and 6). With

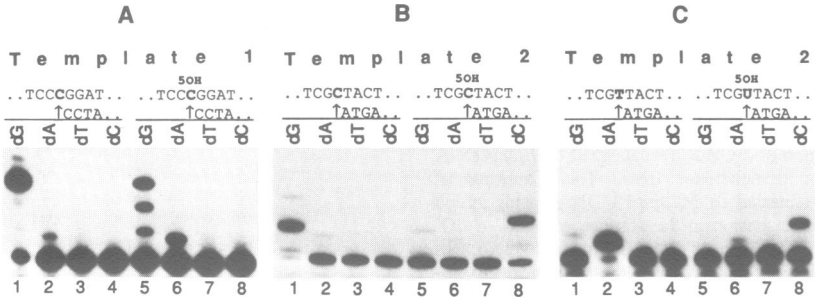

Figure 5. The specificity of nucleotide incorporation opposite 5-OHdC and 5-OHdU. The extension of 5′-^{32}P-labeled primer 2, annealed to template 1 containing dC (panel A, lanes 1–4), or 5-OHdC (panel A, lanes 5–8) at position 16; or 5′-^{32}P-labeled primer 4 annealed to template 2 containing dC (panel B, lanes 1–4), 5-OHdC (panel B, lanes 5–8), dT (panel C, lanes 1–4) or 5-OHdU (panel C, lanes 5–8) at position 7. Each extension experiment was performed in the presence of only one of four dNTPs.

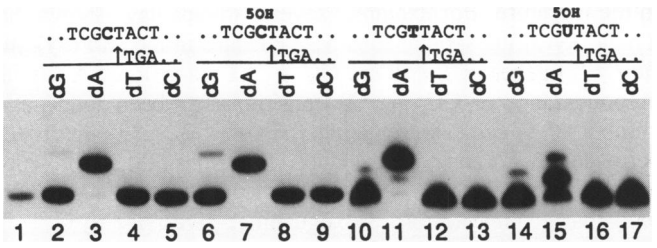

Figure 6. The specificity of nucleotide incorporation at the position one nucleotide prior to the lesion. 5′-^{32}P-labeled primer 5 (lane 1) was annealed to template 2 containing dC (lanes 2–5), 5-OHdC (lanes 6–9), dT (lanes 10–13), and 5-OHdU (lanes 14–17), and incubated with Kf in the presence of only one of four dNTPs. The reaction mixtures were analyzed by electrophoresis in 13% PEG containing 8 M urea.

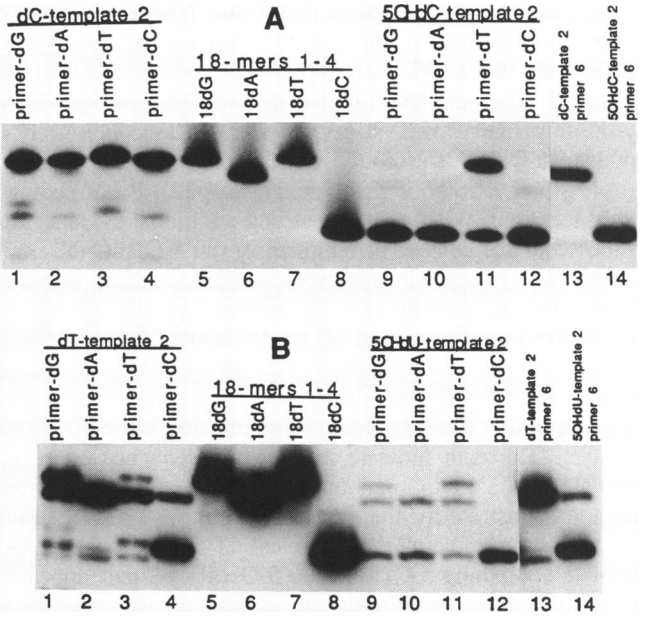

Figure 7. The specificity of nucleotide incorporation opposite 5-OHdC and 5-OHdU from a 'running start', and proofreading of the 3′ terminus by Kf. Primer 6 was annealed to template 2 containing dC or 5-OHdC (lanes 13 and 14, panel A), or dT or 5-OHdU (lanes 13 and 14, panel B), and extended using Kf and all four dNTPs (50 μM each). The mobility in 20% polyacrylamide gels of full length extension products (18-mers) was compared with the mobility of control 18-mers 1–4 (see Fig. 2) containing dG, dA, dT and dC at position 12 (lanes 5–8, both panels). To measure proofreading by Kf of the 3′-end nucleotide opposite 5-OHdC or 5-OHdU, primers 7–10 (see Fig. 2) were annealed to template 2. Each primer was extended in the presence of four dNTPs using Kf and loaded on to a 20% polyacrylamide gel to compare the mobility of full length extension products with the mobility of control 18-mers 1–4. Panel **A**: Lanes 1–4, extension products of primers 7–10 annealed to template 2 containing dC at position 7; lanes 5–8, control 18-mers 1–4; lanes 9–12, extension products of primers 7–10 annealed to template 2 containing 5-OHdC at position 7. Panel **B**: Lanes 1–4, extension products of primers 7–10 annealed to template 2 containing dT at position 7; lanes 5–8, control 18-mers 1–4; lanes 9–12, extension products of primers 7–10 annealed to template 2 containing 5-OHdU at position 7.

the same template containing 5-OHdU, extension was only observed with dATP (data not shown). This result is in good agreement with the specificity of incorporation of 5-OHdCTP and 5-OHdUTP into DNA.

Quite a different pattern of incorporation was observed with template 2 which contained the lesions in a different sequence context. Here, surprisingly, the analogous primer 4 was extended predominantly by dCMP opposite 5-OHdC (panel B, lane 8). Only a small amount of extension was observed with dGTP and dATP (panel B, lanes 5 and 6). With the same template containing 5-OHdU, again, predominantly dCMP was incorporated opposite the lesion (panel C, lane 8) with only trace amount of dAMP (panel C, lane 6). In the case of the control template containing dC or dT at the same position, dGMP and dAMP were incorporated opposite them respectively (panel B, lane 1; panel C, lane 2).

Specificity of nucleotide incorporation one nucleotide prior to 5-hydroxypyrimidines

Since Kf was found to pause one nucleotide prior to the lesion, we also examined the specificity of nucleotide incorporation at this position (Figure 6). Misincorporation was not observed one base prior to the lesion with the possible exception of template 2 containing 5-OHdC where dGMP was incorporated opposite dT in excess over the control (compare lane 2 and 6). In the same template containing 5-OHdU, extension of the normal dA incorporated opposite dT 3′ to 5-OHdU was less efficient than if the template contained dT instead of 5-OHdU (compare lanes 11 and 15). This observation is in agreement with the data described above where we found the dA·5-OHdU pair in the sequence context of template 2 to be less stable than the dC·5-OHdU pair. No differences were observed in either incorporation or extension of nucleotides incorporated one base prior to the lesion when template 1 was used (data not shown). These results suggest that 5-OHdC and 5-OHdU do not introduce significant distortion into the DNA molecule.

Specificity of nucleotide incorporation from a 'running start' position and proofreading of nucleotides inserted opposite the 5-hydroxypyrimidines

The unusual specificity of nucleotide incorporation opposite 5-OHdC and 5-OHdU in template 2 was also examined in a 'running start' reaction. Here, the annealed labeled primer 6 terminated three nucleotides prior to the lesion in the template strand. The full length products (18-mers) of Kf-catalyzed extension of primer 6 were analyzed by comparing their mobility in 20% PAG (Fig. 7) to the mobility of four control 18-mer oligonucleotides 1–4 (12) with dG, dA, dT and dC at position 12 (Fig. 7, panel A, lanes 5–8). These data show that, in agreement with the results obtained with 'standing start' reactions (Fig. 5), almost 100% of the full length extension products contained dC at position 12 opposite 5-OHdC in template 2 (Fig. 7, compare lane 14 with lanes 5 and 8, panel A). With the control template 2, only dGMP was incorporated opposite dC in the template strand (compare lane 13 with lanes 5 and 8, panel A). With the same template containing 5-OHdU, about 85% of the full length products contained dC opposite 5-OHdU and about 15% contained dA (compare lane 14 with lanes 6 and 8, panel B). Almost 100% of dA was placed opposite dT at the same position (lane 13, panel B).

The data presented in Figure 7 suggest that the 5-OHdC-dC and 5-OHdU-dC pairs in the sequence context of template 2 are relatively stable. To further support these observations, in four different experiments, primers 7–10 containing each of four bases opposite the lesion were annealed to template 2 containing either 5-OHdC or 5-OHdU, and used as substrates for Kf with 3′ → 5′ proofreading activity in the presence of all four dNTPs.

Comparison of the mobilities of the full length proofreading-extension products showed that dG and dA in primers 7 and 8, respectively, were efficiently replaced opposite 5-OHdC by dC. Less efficient (about 45%) replacement of dT opposite 5-OHdC by dC in primer 9 was observed (panel A, lane 11). Only dC opposite 5-OHdC was found to be totally stable towards the proofreading activity of Kf (panel A, lane 12). Proofreading of the 3′ terminus by Kf provides strong additional evidence that, in the sequence context of template 2, the 5-OHdC-dC pair is the most stable pair. Unexpectedly, the proofreading activity of Kf on template 2 containing dT at position 7 provided just partial (about 40%) replacement of dG by dA, about 95% replacement of dT by dA, and left dC (80% dC and 20% dA) opposite dT almost intact. At the same time, incorporation of dC opposite dT using primer 6 in a 'running start' reaction was not observed (Fig. 7). The data indicate that, in the sequence context of template 2, the dT-dC pair once formed is surprisingly stable and not a substrate for the proofreading activity of Kf. In fact, it is almost as stable as the canonical dT-dA pair. In the same template containing 5-OHdU, the 5-OHdU-dC pair appears to be the most stable, both under the conditions of 'running start' synthesis and proofreading. We found partial replacement of dG by dA and dC; dA by dC; dT by dA and dC. Only dC opposite 5-OHdU formed a stable pair in the proofreading experiment. These data suggest that, in the sequence context of template 2, 5-OHdU can pair with dA but it predominantly pairs with dC.

Taken together, the data demonstrate that 5-OHdCTP can replace dCTP, and to a much lesser extent dTTP as a substrate for DNA polymerase; however, 5-OHdUTP can only be incorporated into DNA in place of dTTP. Translesion synthesis past 5-OHdC and 5-OHdU in oligodeoxyribonucleotide templates was efficient, but the DNA polymerase paused both opposite and one base prior to the position of the damage in the template. Finally, the specificity of nucleotide incorporation opposite 5-OHdC and 5-OHdU was sequence context dependent. In one sequence context, dGMP was the predominant nucleotide incorporated opposite 5-OHdC with dAMP incorporation also observed. In this sequence context, dAMP was the principal nucleotide incorporated opposite 5-OHdU. However, in another sequence context, dCMP was the predominant nucleotide incorporated opposite 5-OHdC. In the latter sequence context, dCMP was also the predominant nucleotide incorporated opposite 5-OHdU.

NMR and UV absorption studies of 5-hydroxycytosine and 5-hydroxyuracil suggest that these structures retain the enol rather than the keto configuration (19, 20). Thus, it might be expected that in DNA, the base pairing characteristics of the 5-hydroxy-pyrimidines would be correspondingly normal. This is the case for 5-OHdU which, like uracil, pairs as thymine with A. However, since 5-OHdU is derived from dC, like dU it is likely to be a potent premutagenic lesion leading to C → T transitions. 5-OHdC, in addition to its expected interaction with dG, is capable of interacting with dA both in the template and in place of dTTP during incorporation. In both cases, these mispairings with dA would lead to C → T transitions. Of particular interest from a structural point of view, is the sequence context CCTT-CGXTACTTTCCTCT where X is either 5-OHdC or 5-OHdU. Here, dC is inserted opposite the lesion. For both 5-OHdC and 5-OHdU, this misinsertion would give rise to C → G transversions.

The pairing characteristics of the 5-hydroxypyrimidines described above can explain a number of unresolved observations in the literature. For example, several studies have shown that a majority of the spontaneous base changes in the *lacI* (21) and *supF* (22) genes of *E.coli* are GC → AT transitions. A similar preponderance of GC → AT transitions has been found in a number of systems for ionizing radiation (22–25), hydrogen peroxide (26,27) and copper-induced mutations (28), all of which interact with DNA via hydroxyl radicals. Since the principal oxidative guanine lesion, 8-oxoguanine, gives rise to G → T transversions (11, 13, 14), it cannot be responsible for the above described mutations. Also, 8-oxoquanine is processed by multiple repair systems (14, 29). In contrast, 5-OHdC, uracil glycol and 5-OHdU in DNA, are present in significant steady state levels (7), and after oxidation (6, 7–10), and are not processed by any known DNA repair activities. Also, 5-OHdC and 5-OHdU have the potential, based on the studies described here, to produce GC → AT transitions. Thus, the observed GC → AT transitions observed in spontaneous mutants and mutants derived from radiation and chemical oxidation may be due to oxidation of dC.

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