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Uracil-DNA glycosylases preferentially excise mispaired uracil

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We have investigated the substrate specificity of human, viral and bacterial uracil-DNA glycosylases employing as substrate double-stranded oligonucleotides containing in the same position of the 5'-^{32}P-labelled strand an uracil residue facing, on the complementary strand, guanine (mimicking cytosine deamination) or adenine (mimicking dUTP misincorporation). The enyzmic removal of uracil was monitored and quantified by the generation of alkali-sensitive apyrimidinic sites. All three uracil-DNA glycosylases excise uracil from mispaired oligonucleotides (U/G) more efficiently than from paired oligonucleotides (U/A). The enzymes also remove uracil from single-stranded oligonucleotide with an efficiency similar to that observed with U/A paired oligonucleotide. The efficient recognition of U/G mispair by uracil-DNA glycosylase is important in minimizing miscoding transcripts and C/G → T/A transitions in proliferating cells.

INTRODUCTION

The variety of DNA damage is mirrored by a variety of DNA-repair systems which correct the error and thus restoring the normal nucleotide sequence. One of them is the base excision repair, in which the first step of the repair mechanism is the excision of the incorrect or modified base by the action of DNA glycosylases. These enzymes specifically hydrolyse the *N*-glycosylic bond linking damaged or incorrect bases to the sugar-phosphate backbone of DNA (Friedberg, 1985). Several DNA glycosylases have been described in viruses, bacteria and eukaryotic cells; one of them is the uracil-DNA glycosylase, which is ubiquitously distributed in nature (Lindahl, 1974, 1977; Friedberg *et al.*, 1975; Borle *et al.*, 1979, 1982; Caradonna & Cheng, 1980; Krokan & Wittwer, 1981; Blaisdell & Warner, 1983; Friedberg, 1985; Worrad & Caradonna, 1988; Sirover, 1979; Mullaney *et al.*, 1989). It catalyses the specific removal of uracil from DNA. This base, normally confined to RNA, can occur in DNA as a consequence of cytosine deamination, which is estimated to occur approximately at a rate of 100/day per bacterial genome (Friedberg, 1985) or by dUTP misincorporation, instead of dTTP, by the action of DNA polymerases (Tye *et al.*, 1978; Focher *et al.*, 1990). The deriving uracil, facing guanine or adenine respectively, is removed by the action of uracil-DNA glycosylase, both in prokaryotes and in eukaryotes.

In double-stranded DNA, uracil derived from cytosine deamination can be easily distinguished from uracil deriving from dUTP misincorporation because, in the first case, uracil pairs with guanine, leading to a mismatched base-pair (U/G), whereas the misincorporated uracil, sterically similar to thymine, perfectly matches with adenine on the complementary strand (U/A).

In the absence of DNA repair, deamination of cytosine would be highly mutagenic in proliferating cells, as it induces the C/G → T/A transition, although the correction of U/G and U/A base-pairs is also necessary in resting cells. In fact, as we have recently demonstrated, the uracil present in DNA either derived from cytosine deamination or dUTP misincorporation alters the DNA–protein interactions (Verri *et al.*, 1990). Specifically in the cyclic AMP-responsive element (CRE) sequence of the rat somatostatin gene promoter, the uracil derived from dUTP misincorporation decreases the binding capability of CRE-binding protein (CREBP) to about 60%, whereas when the uracil is derived from cytosine deamination, it increases the specific binding of CREBP to CRE sequences up to 10 times (Verri *et al.*, 1990). Thus the overall lower tolerance of U/G mispairing than for U/A pairing implies that uracil-DNA glycosylase should more efficiently remove uracil arising from cytosine deamination.

In the present study we have tested this hypothesis by challenging, *in vitro*, three different uracil-DNA glycosylases, coded for by human, Herpes simplex 1 (HSV1) and *Escherichia coli* genomes, with several oligonucleotides corresponding to a fragment of the rat somatostatin gene containing the CRE sequence (TGACGTCA) in which point mutations were introduced in order to mimic uracil arising either from misincorporation or cytosine deamination.

METHODS

Chemicals and enzymes

All chemicals used to prepare buffers were of analytical reagent grade and were purchased from Merck (Darmstadt, Germany) or Fluka Chemie AG (Buchs, Switzerland). Acrylamide, bisacrylamide, ammonium persulphate and *NNN'N'*-tetramethylethylenediamine were obtained from Bio-Rad Laboratories (Richmond, CA, U.S.A.). Sephadex G-50 (fine grade) was purchased from LKB–Pharmacia (Uppsala, Sweden), [γ-^{32}P]ATP (3000 Ci/mmol) from Amersham Corp. (Arlington Heights, IL, U.S.A.). T4 polynucleotide kinase and *E. coli* uracil-DNA glycosylase were purchased from Boehringer (Mannheim, Germany).

Purification of human and HSV1 uracil DNA-glycosylases

Human uracil DNA-glycosylase was purified from HeLa cells as described by Krokan & Wittwer (1981) up to the phosphocellulose step and further purified on heparin–Sepharose and poly(U)–Sepharose. The final preparation (1400 units/mg) was nuclease-free under the assay conditions used.

The viral enzyme was purified essentially as the human enzyme

Abbreviations used: CRE, cyclic AMP-responsive element; CREBP, CRE-binding protein; HSV1, Herpes simplex (virus) 1; AP, apyrimidinic; oligo, oligonucleotide.

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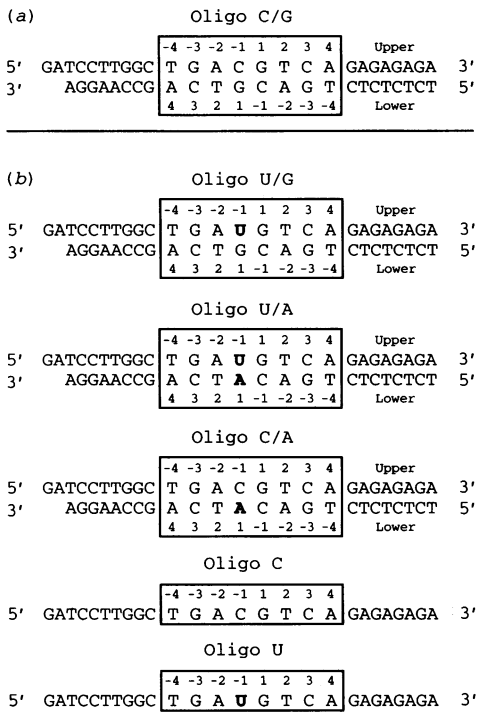

Fig. 1. (*a*) Synthetic oligonucleotide (C/G) corresponding to CRE sequence in rat somatostatin gene and (*b*) synthetic oligonucleotides derived from CRE sequence and employed in the present study

Oligo U/G contains an uracil derived from cytosine deamination in position −1 in the upper strand (U = G mismatch); oligo(U/A) contains an uracil derived from dUTP misincorporation in the same position (U = A match); oligo C/A contains a mismatch in the same position (C = A mismatch); oligonucleotides C and U are normal and uracil-containing single-stranded oligonucleotides.

from HSV1-infected HeLa cells, collected at 10 h after infection, when the induction of viral enzyme is maximal. The final preparation (11000 units/mg) was nuclease-free under the assay conditions employed.

One unit of uracil-DNA glycosylase is defined as 1 nmol of uracil removed from the [^{3}H]uracil-labelled DNA in 1 h at 37 °C (Focher *et al.*, 1990).

Oligonucleotides

All oligonucleotides were synthesized using a Beckman System 1 plus DNA Synthesizer and purified as described by Verri *et al.* (1990). Oligonucleotides were always ^{32}P-end-labelled separately with T4 polynucleotide kinase to a specific radioactivity of 0.3 μCi/pmol. Purified complementary strands were mixed in 10 mM-Tris/HCl (pH 7.5)/1 mM-EDTA to a final concentration of 100 nM double-stranded oligonucleotide, heated to 95 °C for 5 min and allowed to cool to room temperature.

Uracil-DNA glycosylase assay

A final volume of 25 μl contained 100 mM-Tris/HCl, pH 8, 5 mM-dithiothreitol, 10 mM-EDTA, 0.5 pmol of [^{32}P]oligonucleotide (660000 c.p.m./pmol) and 0.2 unit of enzyme to be tested. After incubation at 37 °C for 30 min, 25 μl of 0.2 M-NaOH was added and the tubes were incubated for 30 min at 90 °C in order to cleave the sugar–phosphate bond at the apyrimidinic site. Samples were then freeze-dried and resuspended in 50 μl of a buffer containing 6% (w/v) sucrose, 2 mM-Tris/HCl, pH 8, 0.05% Bromophenol Blue and 0.05% xylene cyanole. A 2 μl portion of this solution was applied on a 16% (w/v) polyacrylamide gel containing 7.5 M-urea. After electrophoresis, gels were dried and autoradiographed with Amersham Hyperfilm-MP. Quantitative determination of uracil-DNA glycosylase activity was performed by cutting the radioactive spots from dried gels and counting the radioactivity in a β-radiation counter.

RESULTS

The double-stranded synthetic oligonucleotide C/G shown in Fig. 1(*a*), corresponding to a fragment of the rat somatostatin gene containing the CRE sequence (TGACGTCA), was modified in position −1 in the upper strand (C → U) and in position 1 in the lower strand (G → A). The normal and uracil-containing oligonucleotides were 5′-^{32}P-labelled with polynucleotide kinase before annealing with a complementary oligonucleotide. The annealing produced the variety of double-stranded oligonucleotides shown in Fig. 1(*b*), which were used as substrates for human, HSV1 and *E. coli* uracil-DNA glycosylases. Oligo U/G contains a uracil, mimicking a deaminated cytosine, in position −1; oligo U/A contains a uracil, mimicking a misincorporated dUTP, in the same position. As control we also prepared oligo C/A, where a mispair without uracil is present.

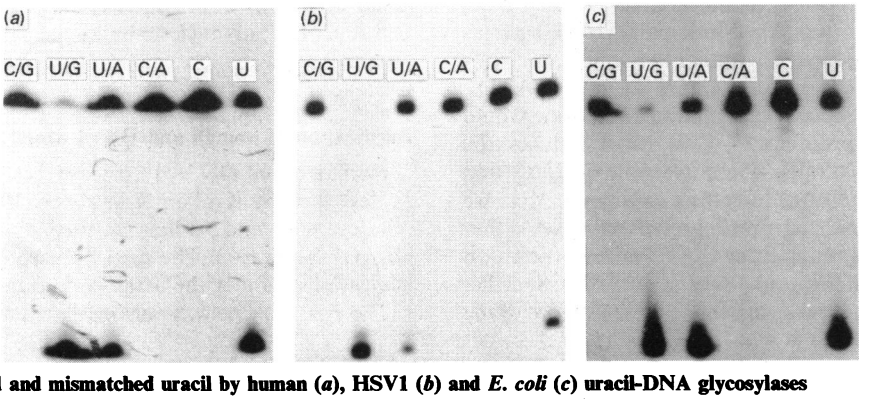

Fig. 2. Removal of matched and mismatched uracil by human (*a*), HSV1 (*b*) and *E. coli* (*c*) uracil-DNA glycosylases

Uracil-DNA glycosylase assay and further processing of oligonucleotides is described in the Methods section. The upper bands correspond to the 26-mer intact oligonucleotide; lower bands correspond to the alkali-cleaved AP-oligonucleotide 12-mer derived from the 26-mer substrate of the enzymes. Letters over the lanes correspond to the oligonucleotides described in Fig. 1.

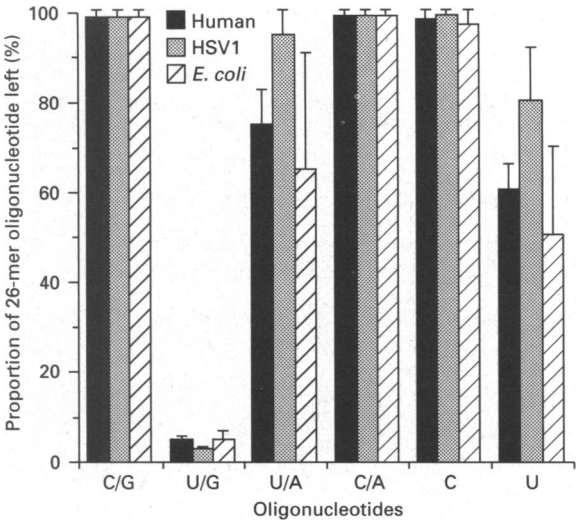

Fig. 3. Quantitative determination of the efficiency of uracil-DNA glycosylases in the removal of uracil from the oligonucleotides described in Fig. 1

Bars correspond to the 26-mer substrate left after 30 min of incubation with human, viral and bacterial uracil-DNA glycosylases.

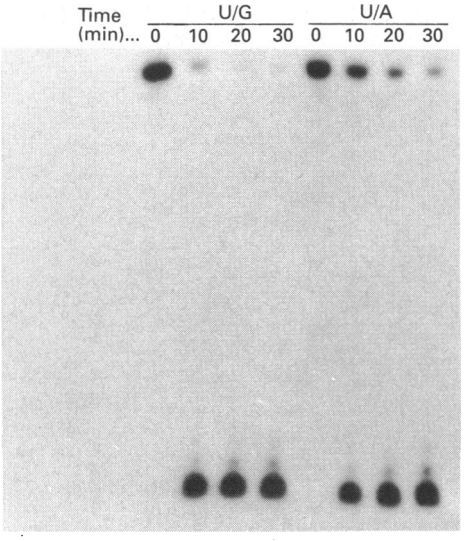

Fig. 4. Enzyme kinetics with human uracil-DNA glycosylase

A 0.25 pmol portion of [^{32}P]oligonucleotides were incubated with the enzyme in 25 μl of reaction mixture as described in the Methods section. Upper bands correspond to the 26-mer intact oligonucleotide; lower bands correspond to the alkali-cleaved AP-oligonucleotide 12-mer.

After incubation with uracil-DNA glycosylases to produce apyrimidinic (AP) sites, and alkali treatment to generate the complete cleavage of the sugar–phosphate bond at the 3′ side of the AP site, the oligonucleotides were freeze-dried, resuspended and applied on a 16% denaturating polyacrylamide gel as described in the Methods section. Fig. 2 shows the specific cleavage of uracil-containing oligonucleotides by human, HSV1 and *E. coli* uracil-DNA glycosylases (Figs. 2*a*, 2*b* and 2*c* respectively) monitored as production of ^{32}P-labelled 12-mer. The radioactive spots were then cut from the gel and counted for radioactivity to quantify the percentage 26- and 12-mer present in the gel. Fig. 3 shows that all three enzymes remove uracil from

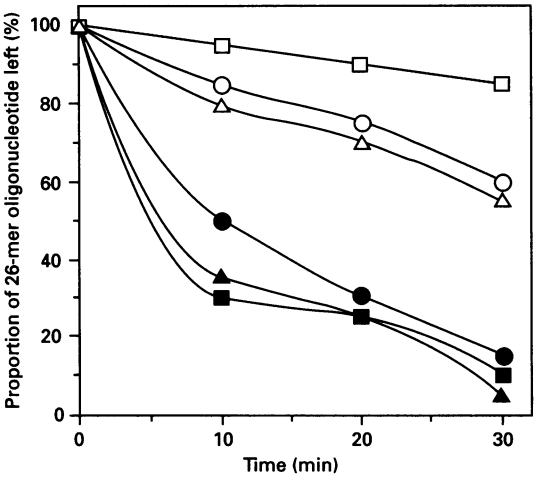

Fig. 5. Enzyme kinetics with human (●/○), HSV1 (■/□) and *E. coli* (▲/△) uracil-DNA glycosylases

Closed symbols: oligonucleotide U/G; open symbols: oligonucleotide U/A; 0.5 pmol of [^{32}P]oligonucleotides was incubated with the enzyme in 25 μl of reaction mixture as described in the Methods section. Quantitative determination of uracil-DNA glycosylase activity was performed by cleavage of the radioactive spots from dried gels and counting the radioactivity in a β-radiation counter. Each point is an average for three experiments.

both single-stranded and double-stranded uracil-containing DNAs. However, they act preferentially on double-stranded DNA, where uracil replaces a cytosine residue. The decreased amount of 26-mer of oligo U/G is then dependent on the simultaneous presence of both uracil and a mismatch, whereas a mismatch alone has no effect on the action of uracil-DNA glycosylases (Figs. 2 and 3; compare oligo U/G and C/A).

To study the kinetics of removal of uracil from U/G and U/A oligonucleotides, we performed preliminary kinetic experiments at different concentrations of substrate. Fig. 4 shows one of these experiments with human uracil-DNA glycosylase on 10 nM-U/G and U/A oligonucleotides. At this substrate concentration more than 90% of mismatched uracil was removed by the human enzyme in 10 min. Similar results were obtained with the viral and bacterial uracil-DNA glycosylases (results not shown). Therefore for 30 min kinetic experiments we used the substrate oligonucleotides at 20 nM. The rate of uracil release by human, viral and bacterial uracil-DNA glycosylases are reported in Fig. 5. All three uracil-DNA glycosylases remove uracil faster from U/G than from U/A oligonucleotides.

DISCUSSION

By exploiting the recent availability of dUTP for the programmed chemical synthesis of oligonucleotides we have synthesized oligonucleotides with U/G or U/A in the same position and used them to reveal a preferential recognition of U/G mismatch over U/A by human, viral and bacterial uracil-DNA glycosylases. This behaviour clearly resembles the one of hypoxanthine-DNA glycosylase that removes more efficiently hypoxanthine base residues derived from hydrolytic adenine deamination than from occasional dITP misincorporation during DNA synthesis (Dianov & Lindahl, 1991).

In both cases this is selectively advantageous because of the higher mutagenic potentiality of the deaminated bases compared with the misincorporated bases. Occasional misincorporation of dUTP and dITP residues into DNA could only affect DNA–protein interaction (Verri *et al.*, 1990) in both proliferating and

resting cells. On the other hand, the generation of uracil and hypoxanthine residues by spontaneous hydrolytic deamination, leading to U/G and I/T mispairs in coding sequences, would not only alter DNA–protein recognition, but would codify for incorrect messengers, with an adenine or a cytosine present in place of a guanine or thymine respectively. Moreover, in proliferating cells, U/G and I/T mispairs would also lead to the mutagenic C/G → T/A or T/A → C/G transitions. The biological significance of the formation of uracil in DNA by the deamination of cytosine is demonstrated by the findings that *E. coli* (Duncan *et al.*, 1978; Duncan & Weiss, 1982) or *Saccharomyces cerevisiae* (Impellizzeri *et al.*, 1991) defective in the removal of uracil from DNA have an elevated rate of spontaneous mutations, specifically of C/G → T/A transitions.

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