Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 1642–1646, March 1994
Biochemistry

Efficient removal of uracil from G·U mispairs by the mismatch-specific thymine DNA glycosylase from HeLa cells

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Communicated by Maurice S. Fox, September 30, 1993

ABSTRACT **The uracil DNA glycosylases (EC 3.2.2.3) characterized to date remove uracil from DNA irrespective of whether it is base paired with adenine or mispaired with guanine in double-stranded substrates or whether it is found in single-stranded DNA. We report here the characterization of uracil glycosylase activity that can remove the base solely from a mispair with guanine. It does not recognize uracil either in A·U pairs or in single-stranded substrates. The enzyme, a 55-kDa polypeptide, was previously characterized as a mismatch-specific thymine DNA glycosylase and was thought to be responsible solely for the correction (to G·C) of G·T mispairs that arise as a result of spontaneous hydrolytic deamination of 5-methylcytosine to thymine. Given the broader substrate specificity of the enzyme (in addition to uracil and thymine, the protein can also remove 5-bromouracil from mispairs with guanine), we propose that its biological role *in vivo* may also include the correction of a subset of G·U mispairs inefficiently removed by the more abundant ubiquitous uracil glycosylases, such as those arising from cytosine deamination in G+C-rich regions of the genome.**

Uracil residues arise in DNA either by cytosine deamination (1) or by incorporation into the nascent strand during replication as dUMP (2). The former process gives rise to mutagenic G·U mispairs, whereas the latter yields A·U "pairs." Uracil is removed from both these lesions and from single-stranded DNA by ubiquitous uracil DNA glycosylases (EC 3.2.2.3) (2, 3). A recent study addressing the removal of uracil from a large number of different sequence contexts suggested that the major uracil glycosylases of both mammals and bacteria may require local strand separation for efficient removal of the base from DNA (4). Correspondingly, G·U mispairs in a G+C-rich sequence context were found to be poor substrates for these enzymes *in vitro*, although this may not be so for all uracil glycosylases (5). Interestingly, analysis of mutational spectra of several mammalian genes published to date revealed no evidence for an increased rate of C → T transition mutations that could be associated with G+C-rich regions (6, 7). This would imply either that the ubiquitous enzyme is so abundant that it manages to repair even the less accessible substrates or that a second enzyme may be able to compensate for the inefficiency of the ubiquitous activity. We describe here another glycosylase activity that, due to its different substrate recognition requirements, efficiently removes uracil from G·U mispairs in double-stranded DNA and may thus be a candidate for such a back-up role.

MATERIALS AND METHODS

All the reagents and solvents used in this study were of analytical purity. The restriction endonucleases were from Boehringer Mannheim. T4 polynucleotide kinase was from Pharmacia. [γ-^{32}P]ATP was from Amersham International. The oligonucleotides were made on an Applied Biosystems model 380B synthesizer and purified by denaturing PAGE. All the standard manipulations (5′-labeling of the oligonucleotides, gel electrophoresis, DNA precipitations, etc.) were carried out as described by Sambrook *et al.* (8). SDS/PAGE molecular mass standards were from Bio-Rad.

Preparation of Whole Cell Extracts. These extracts were prepared from 180 g of frozen HeLa cells in three 60-g batches as described (9).

Fractionation of the Cell Extracts. The extract was diluted 1:4 with HE buffer [25 mM Hepes, pH 7.8/1 mM EDTA/1 mM dithiothreitol/10% (vol/vol) glycerol] and incubated batchwise for 1 h with phosphocellulose P11 (Whatman) (20 mg of protein per ml of matrix) that had been pretreated as directed by the manufacturer and equilibrated with HE buffer containing 0.1 M NaCl. The phosphocellulose was washed stepwise in a sintered glass funnel with three matrix volumes of HE buffer containing 0.1, 0.3, 0.5, and 1 M NaCl. The active fractions (0.5 M NaCl) were pooled and dialyzed overnight against two 10-liter changes of HE buffer containing 0.1 M NaCl. The dialyzed fractions were loaded onto a 30-ml heparin-Sepharose column (Pharmacia), and the proteins were eluted using a 260-ml gradient from 0.1 to 1.0 M NaCl in HE buffer. Active fractions (0.61–0.87 M NaCl) were pooled, dialyzed against two 2-liter changes of HE buffer containing 0.1 M NaCl. Ten percent of the protein pool was loaded onto a 1-ml Mono Q FPLC column (Pharmacia). Proteins were eluted with a 12.5-ml gradient from 0.1 to 0.5 M NaCl in HE buffer. To concentrate the protein fractions, active fractions (0.23–0.31 M NaCl) from all 10 Mono Q columns were loaded onto the same Mono Q column and material was eluted with the same gradient. Active fractions of this Mono Q column were diluted 1:1.4 with HE buffer and loaded onto a 0.5-ml Blue-Sepharose column (Pharmacia) equilibrated with HE buffer containing 0.25 M NaCl. Material was eluted stepwise with three matrix volumes (collected in three aliquots) of HE buffer containing 0.25, 0.4, 0.7, and 1 M NaCl. Active fractions (the third 0.4 M NaCl fraction and all three 0.7 M NaCl fractions) were pooled, diluted 1:3 with HE buffer, and loaded directly onto a 1-ml Mono S FPLC column (Pharmacia). Elution of proteins was carried out with a 10-ml NaCl gradient from 0.25 to 0.5 M NaCl in HE buffer. The activity was recovered in the fractions eluting with 0.4 M NaCl (0.37–0.44 M NaCl). These fractions still contained detectable amounts of uracil glycosylase and could, therefore, be used solely in the band-shift and cross-linking assays described below.

Purification of the G·T Glycosylase by Affinity Chromatography. This purification scheme is described in detail in ref. 9. The glycosylase fraction obtained after the first pass through the affinity column had a specific activity of 103,000 units/mg (where 1 unit is defined as 1 fmol of thymine liberated after a 10-min incubation at 37°C). This fraction was used in the SDS/PAGE–renaturation experiment described

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Abbreviation: UGI, uracil glycosylase inhibitor.
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below and shown in Fig. 2. The activity obtained after the second affinity chromatography step was nearly homogeneous and free of uracil glycosylase, as judged by its lack of activity on a single-stranded uracil-containing oligonucleotide (see Fig. 4). This G·T glycosylase fraction also had no AP-endonuclease activity (9); consequently, after incubation with the enzyme, all DNA substrates had to be treated with NaOH prior to loading on gels to ensure quantitative cleavage of the sugar-phosphate backbone at the AP sites (see below).

Enzymatic Activity Assays. The purification of the enzymatic activity was followed by using a nicking assay (9).

Band Shift and UV-Cross-Linking Assays. The experiment shown in Fig. 1 was carried out as follows: the duplexes G·T, G·U, and G·C (40 fmol), formed by annealing the 16-mer oligonucleotide 5′-GATCCGTCGACCBGCA-3′ with the 34-mer 5′-AGCTTGGCTGCAGGTYGACGGATCCCCGGGAATT-3′, labeled at its 5′ end with ^{32}P (where B is 5-bromouracil and Y is T, U, or C), were incubated with the active Mono S FPLC fraction (containing 1 or 2 μg of protein) for 30 min at 37°C in the presence or absence of 50 ng of poly[d(I-C)]·poly[d(I-C)] (Pharmacia) as described (10). In parallel, 1 μg of active or inactivated MutS protein was incubated with the G·T duplex under similar conditions (20 mM Tris·HCl, pH 7.6/5 mM $MgCl_2$/1 mM dithiothreitol/0.1 mM EDTA) (11), but without competitor DNA. The total volume of the assays was 25 μl. To a 5-μl aliquot of the reaction mixture was added 1 μl of 20% (wt/vol) Ficoll and the sample was loaded onto a 6% nondenaturing polyacrylamide gel electrophoresed at room temperature in TEA buffer (10). The autoradiograph is shown in Fig. 1*A*.

The remaining 20 μl of the above mixture was UV-irradiated as described (10), mixed with 5 μl of SDS-loading dye (8), and loaded onto a SDS/15% polyacrylamide gel. The low molecular mass protein standards (Bio-Rad; 5 μl), diluted 1:20, were loaded onto the same SDS gel. After electrophoresis, the protein standards were visualized by staining with Coomassie blue. The gel was then dried and autoradiographed at −80°C (see Fig. 1*B*).

Renaturation of the Glycosylase Activities After SDS/PAGE. This experiment was carried out as described (9). The first and second lanes of the SDS/PAGE gel were loaded with 10 μl (1 μg) of the active fraction eluted from the first affinity column with 0.4 M NaCl (9); the third lane was loaded with 10 μl (0.1 μg) of low molecular mass protein marker (Bio-Rad). After the electrophoresis, the first lane was cut into 12 equal slices, and lanes two and three were silver-stained.

HeLa Uracil Glycosylase Substrate Specificity Assays. Single-stranded or double-stranded 34-mer oligonucleotides (40 fmol) were incubated with 5 μl of the phosphocellulose column flow-through fraction containing the HeLa uracil glycosylase(s) (10 μg of total protein, see above) for 30 min at 37°C. The inhibition experiments were carried out by adding increasing amounts of uracil glycosylase inhibitor (UGI) (12) or of a solution of uracil in dimethyl sulfoxide to the reaction mixture. The inhibitor concentrations are given in Fig. 3. Electrophoresis was carried out as described above.

Thymine Glycosylase Substrate Specificity Assays. The twice-affinity-purified protein fraction (1 μl; ≈20 ng) eluted from the column with 0.4 M NaCl (9), was incubated with 40 fmol of the 34-mer substrates G·T, U, B, A·U, G·U, G·B, and A·B for 30 min at 37°C as described above. For the G·T duplex, the assay was carried out in the presence or absence of UGI (2.5 units per assay) or uracil (10 mM).

RESULTS

The Enzyme Forms Stable Complexes with Oligonucleotide Duplexes Containing a G·T or a G·U Mispair. The purification scheme described above was intended to yield the pure G·T mismatch-specific thymine DNA glycosylase, as identified (13). Unfortunately, despite the numerous chromatographic steps, the protein mixture remained highly complex, such that we were unable to associate the desired enzymatic activity with a specific protein band. We therefore decided to purify the protein by affinity chromatography. To this end, we performed a series of band-shift experiments to determine the best affinity matrix. As shown in Fig. 1*A*, the active Mono S fractions contain a protein that forms a complex with a G·T-mismatch-containing oligonucleotide duplex. [The band shifts had to be carried out with the Mono S fractions, because the early activity-containing fractions contain the G·T mismatch binding protein (10) that obliterates any gel shift due to the thymine glycosylase.] We assumed that the protein component of this complex was the HeLa G·T-specific thymine glycosylase. Interestingly, however, a protein–DNA complex with a similar electrophoretic mobility was also observed upon incubation of the same protein fraction with an oligonucleotide duplex containing a G·U mispair (Fig. 1*A*). As protein–DNA cross-linking experiments confirmed that the two complexes were due to the same polypeptide (see below), prior to embarking on another purification attempt, we decided to characterize this protein

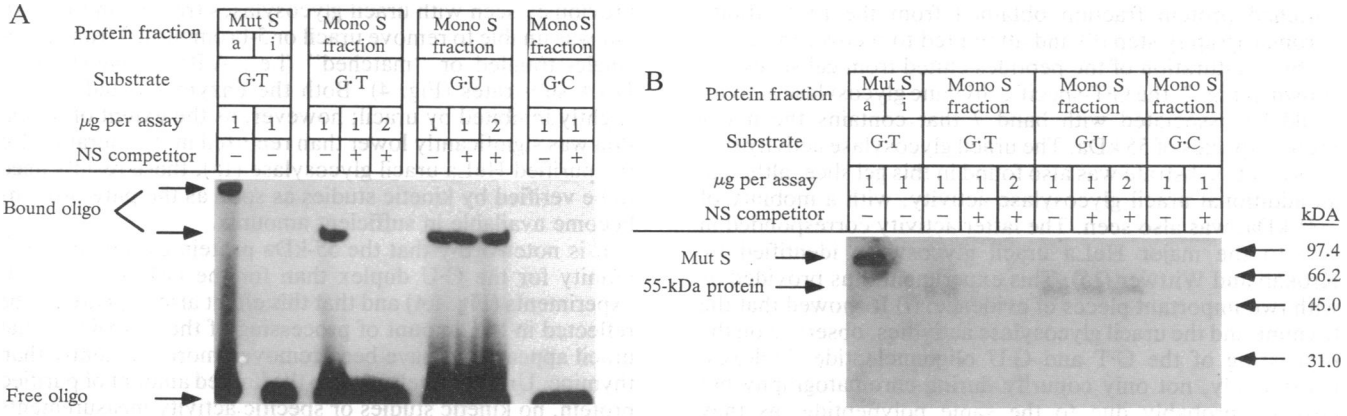

FIG. 1. Binding of a HeLa protein to G·T and G·U duplexes. (*A*) Protein–DNA complexes formed upon incubation of a Mono S FPLC fraction of HeLa extracts with the ^{32}P-labeled oligonucleotide probes G·T, G·U, and G·C, as visualized by a band-shift assay. The active (a) or heat-inactivated (i) bacterial mismatch-binding protein MutS (a kind gift of P. Modrich, ref. 11) was used as a reference. The assays were carried out in the presence (+) or absence (−) of nonspecific competitor DNA [poly[d(I-C)]·poly[d(I-C)]. The figure shows an autoradiogram of a native 6% polyacrylamide gel. (*B*) UV-cross-linking of the bound proteins to the oligonucleotide probes G·T and G·U. The position of the covalently bound protein–DNA complexes is indicated on the left; the positions of molecular mass standards are shown on the right. An autoradiogram of a denaturing SDS/15% polyacrylamide gel is shown. NS, nonspecific.

better to ensure that we were not attempting to isolate a known uracil glycosylase. Indeed, although all uracil glycosylases characterized to date are highly specific for uracil, at least one could be inhibited also by thymine (14).

As shown in Fig. 1*A*, the protein–DNA complex containing the G·T and G·U duplexes migrated in native polyacrylamide gels considerably faster than the G·T duplex bound by the *Escherichia coli* mismatch-binding protein MutS, a 97-kDa polypeptide (11), suggesting that the protein bound to the DNA in the former case was smaller than MutS. UV-cross-linking of the protein to DNA duplexes (Fig. 1*B*) yielded in both cases a band of ≈60 kDa on a denaturing SDS/polyacrylamide gel that corresponds to the single-stranded 16-mer oligonucleotide (≈5 kDa) covalently linked to a protein of 55 kDa. From the size of the protein, it appeared unlikely that it might correspond to a known uracil glycosylase, as most uracil glycosylases characterized to date were polypeptides of ≈30 kDa. We therefore decided to proceed with the purification of this protein by affinity chromatography, using a DNA matrix containing the G·U mispair, due to the fact that the protein appeared to bind to this substrate with an affinity higher than to G·T (Fig. 1*A*).

The purification of the enzymatic activity, described in detail elsewhere (9), was followed by incubation of the protein fractions with a ^{32}P-labeled G·T-mispair-containing oligonucleotide duplex (13), whereby the removal of the mispaired thymine resulted in a cleavage of the labeled strand at the abasic site under the conditions of the assay. However, as the affinity matrix contained a G·U mispair, we needed to ensure that the pure fractions were free of contaminating uracil glycosylase activity. We therefore tested them also with three additional substrates: G·U, A·U, and single-stranded U. Although all these substrates were cleaved upon incubation with the thymine glycosylase-containing protein fractions from the early stages of purification (data not shown), only the G·T and the G·U duplexes were processed by the pure protein (see below). These results suggested that the mismatch-specific thymine and uracil glycosylase activities copurify or are the functions of the same protein.

The Thymine and Uracil Glycosylase Activities Are Functions of the Same Polypeptide. The evidence presented so far that suggests that G·T and the G·U are substrates for the same protein cannot be considered conclusive. We therefore attempted to establish whether the two activities that appear to copurify are mediated by the same polypeptide. To this end, we carried out denaturing SDS/PAGE of the substantially enriched protein fraction obtained from the first affinity chromatography step (9) and attempted to recover the activity by renaturation of the peptides eluted from gel slices. As shown in Fig. 2, the G·T-specific thymine glycosylase activity could be associated with band 7 that contains the major protein species of 55 kDa. The uracil glycosylase activity that uses a G·U substrate was also found in this gel slice, although an additional uracil glycosylase activity, with a mobility of ≈31 kDa, was also seen. The latter activity corresponded in size to the major HeLa uracil glycosylase identified by Krokan and Wittwer (15). This experiment thus provided us with two important pieces of evidence. (*i*) It showed that the thymine and the uracil glycosylase activities, observed on the processing of the G·T and G·U oligonucleotide duplexes, respectively, not only copurify during chromatography but also are probably due to the same polypeptide, as they comigrate through a polyacrylamide gel under denaturing conditions. (*ii*) The experiment confirmed our findings that the first affinity fraction is still contaminated with the major HeLa uracil glycosylase and that the substrate specificity studies using uracil-containing DNA had to be carried out with the second affinity fraction that is essentially homogeneous and free of this latter enzyme, as observed by its lack

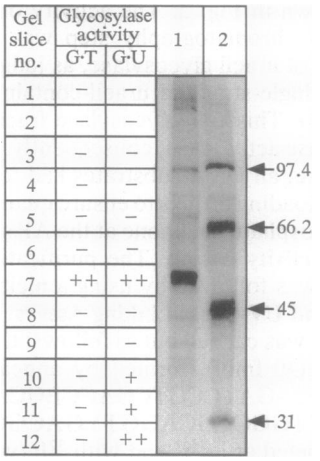

FIG. 2. G·T and G·U processing activities comigrate in a polyacrylamide gel under denaturing conditions. A photograph of a silver-stained SDS/polyacrylamide gel is shown. Lanes: 1, affinity-purified glycosylase—10 μl (1 μg total protein) of the protein fraction eluted from the first affinity column (9) with 0.4 M NaCl; 2, low molecular mass marker proteins (Bio-Rad, the sizes are indicated on the right in kDa). Positions of the gel slices into which the duplicate non-silver-stained lane was cut are indicated and the G·T and G·U columns indicate the presence (+) or absence (−) of thymine or uracil glycosylase activity, respectively, in the appropriate slices after recovery of the protein and renaturation. Note the presence of uracil glycosylase activity also in the fractions flanking the 31-kDa marker.

of activity on a single-stranded uracil-containing oligonucleotide (see Fig. 4).

The Enzyme Removes Thymine and Uracil from Mispairs with Guanine. Figs. 3 and 4 show a comparison of the substrate specificities of the purified mismatch-specific thymine glycosylase and the uracil glycosylase activities present in the HeLa extract fractions that contained no detectable G·T glycosylase activity (the phosphocellulose flow-through). The major HeLa uracil glycosylase(s) catalyzed the removal of uracil from A·U, G·U, and U but not of 5-bromouracil from G·B oligonucleotide substrates. The activity could also be inhibited by UGI, an allosteric uracil glycosylase inhibitor (12) (Fig. 3). In contrast, the mismatch-specific thymine DNA glycosylase clearly processed solely the G·U and G·T duplexes and was not inhibited to any significant extent by UGI (Fig. 4). In addition, the enzyme also excised 5-bromouracil from a mispair with guanine, an activity not previously seen with uracil glycosylases (ref. 16 and Fig. 3), but was unable to remove uracil or 5-bromouracil from either single-stranded or "matched" (i.e., A·B) double-stranded DNA substrates (Fig. 4). Both the enzymes could also be slightly inhibited by uracil; however, as the extent of inhibition was significantly lower than reported in the literature for the purified HeLa uracil glycosylase (15), these results need to be verified by kinetic studies as soon as the pure proteins become available in sufficient amounts.

It is noteworthy that the 55-kDa protein exhibited higher affinity for the G·U duplex than for the G·T in band-shift experiments (Fig. 1*A*) and that this effect also appeared to be reflected in the amount of processing of the mispair, in that uracil appeared to have been removed more efficiently than thymine. Unfortunately, due to the limited amount of purified protein, no kinetic studies or specific activity measurements on the G·T and the G·U substrates could be carried out that would confirm these findings.

DISCUSSION

The substrate specificity exhibited by the thymine DNA glycosylase would appear to be unique. As reported else-

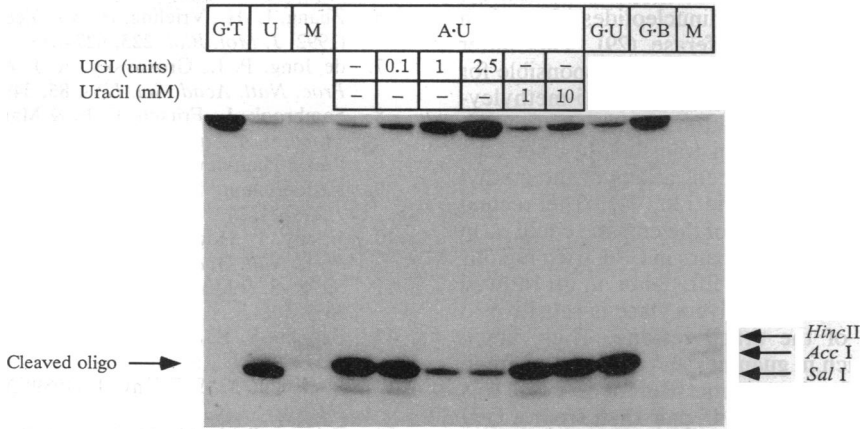

FIG. 3. HeLa uracil glycosylase does not process G·T or G·B mispairs and can be inhibited by UGI and uracil. The 34-mer oligonucleotide substrates G·T, U, A·U, G·U, and G·B were incubated with HeLa uracil glycosylase(s) in a phosphocellulose fraction of the whole cell extract that had no demonstrable thymine DNA glycosylase activity, and an autoradiogram of a denaturing 20% polyacrylamide gel containing reaction products is shown. Lane M contains marker 34-mer oligonucleotide duplex G·C, digested with *Hin*cII, *Acc* I, and *Sal* I (9). Processing of the apyrimidinic site by the double-strand-specific 5′-AP-endonuclease present in the fraction generated a fragment comigrating with the *Acc* I marker that corresponds to the 5′-terminal 15 nucleotides of the labeled thymidine oligonucleotide cleaved 5′ from the mispaired thymine. It should be noted that the corresponding labeled 15-mer fragment of the single-stranded substrate uracil migrates faster, due to the fact that the protein fraction contains no single-strand-specific AP endonuclease. The apyrimidinic site within this substrate was thus chemically cleaved by treatment with hot alkali and contains a 3′-terminal phosphate, rather than a 3′-hydroxyl group as in the enzymatically cleaved products.

where (9), the enzyme can remove thymine not only from the G·T mispair but also from any duplex where the thymine is not in a Watson–Crick base pair with adenine, at least *in vitro*. We show here that the enzyme can also recognize and remove uracil and 5-bromouracil from mispairs with guanine. To our knowledge, such a range of substrates is unprecedented for a glycosylase. The bacterial mismatch-specific enzyme, product of the *E. coli mutY* gene, apparently recognizes G·A and hoG·A mispairs (where hoG is 8-hydroxyguanine) and, to a lesser extent, also C·A and oxA·A mispairs (where oxA is 8-oxoadenine) but only catalyzes the removal of the mispaired adenine (ref. 17 and references therein). The product of the *E. coli mutM* gene, the formamidopyrimidine glycosylase, also exhibits a very broad substrate specificity range (18, 19). However, it can remove damaged bases also from single-stranded DNA (3), which implies that its mode of substrate recognition is different from the mismatch-specific enzymes.

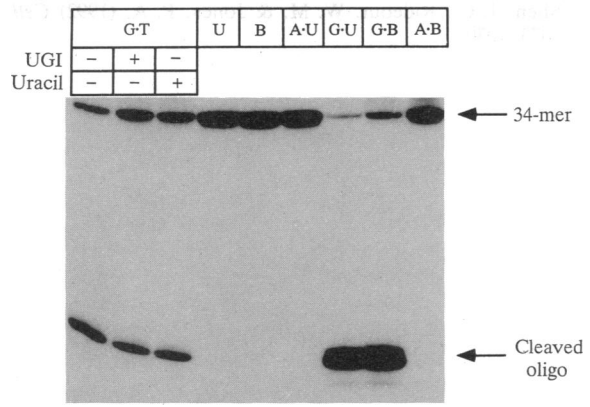

FIG. 4. The 55-kDa glycosylase does not remove uracil or 5-bromouracil from single-stranded DNA or from base pairs with adenine and is not significantly inhibited by UGI peptide or uracil. The experimental conditions employed in this experiment were identical to those shown in Fig. 3, except that the oligonucleotides were incubated with the purified thymine glycosylase. The UGI concentration was 2.5 units per assay and the uracil concentration was 10 mM. An autoradiogram of a denaturing 20% polyacrylamide gel is shown.

Thus, unlike typical glycosylases that recognize their respective substrates in any DNA, the thymine glycosylase and the MutY protein do so solely within the context of a mispair in a duplex.

Despite the fact that the removal of uracil from DNA was the first base-excision DNA repair process to be characterized (20), the mismatch-specific thymine glycosylase escaped detection, probably because the enzymatic activity was always monitored by the release of ^{3}H-labeled uracil from uniformly labeled DNA, where most uracil residues were present in A·U pairs. As shown above (Fig. 4), such DNA is not a substrate for the mismatch-specific thymine glycosylase. Moreover, the existence of a G·U-specific uracil glycosylase activity was not anticipated, because bacterial and yeast uracil glycosylases address all uracil-containing substrates. This is substantiated by the finding that these enzymes are encoded by a single gene that evolutionarily appears to be highly conserved. Accordingly, inactivation of this master gene yields organisms with a high frequency of spontaneous C → T mutations (21–23).

Although sufficient for the removal of uracil from the genomes of small organisms such as bacteria and yeast, a single protein may not suffice in vertebrates. Indeed, three genes encoding uracil glycosylases have been identified to date in human cells (24–26). Although the major part of uracil is probably removed, as in the lower organisms, by the product of a single gene [as judged by the fact that its inactivation leads to a loss of >80% of the total uracil glycosylase activity (27) and by its sequence similarity with the bacterial gene (24)], it is likely that the other enzymes carry out more limited, but perhaps also more specialized, functions.

What could, therefore, be the biological role of the mismatch-specific thymine glycosylase in mammalian cells? Judging by its low abundance and slow action (9, 13), it is unlikely to play a major part in the removal of uracil from DNA. We originally proposed that this enzyme counteracts the effects of the spontaneous hydrolytic deamination of 5-methylcytosine to thymine by initiating the correction of G·T mispairs to G·C pairs (13, 28). However, in view of the discovery of its uracil glycosylase activity, it is conceivable that the enzyme could also play a role in the correction of a subset of G·U mispairs, such as those arising by the deami-

nation of cytosines within CpG dinucleotides through a "malfunction" of the methyltransferase (29). This latter enzyme, which is, under normal conditions, responsible for converting cytosines in CpG sequences to 5-methylcytosines, brings about the deamination of cytosines under conditions where the methyl group transfer does not take place—e.g., at low or limiting concentrations of the methyl group donor, *S*-adenosylmethionine (30, 31). The second and, possibly, more likely function of the enzyme could be in the removal of uracil from G·U mispairs in G+C-rich regions of the genome. A recent report by Eftedal *et al.* (4) showed that a purified mammalian uracil glycosylase is sensitive to the sequence context of the uracil residue. Thus, uracil residues in sequences rich in guanines and cytosines were in general poor substrates for the enzyme. Moreover, uracil was removed slightly faster from an A·U pair than from a G·U mispair in the same sequence context (4), which is contrary to expectations. Indeed, the uracil glycosylase of *E. coli*, a "real" antimutator enzyme (24), could be shown in our preliminary experiments to catalyze the removal of uracil from the G·U mispair with an efficiency greater than from an A·U pair (data not shown).

Both the above hypotheses are testable by expressing the enzyme in *ung*$^-$ cells lacking the wild-type uracil glycosylase that exhibit an elevated rate of C → T transition mutations. The mismatch-specific thymine glycosylase would be expected to compensate for the deficiency in the repair of the mutagenic G·U mispairs and thus rescue the mutator phenotype of such cells. These and other experiments must await the identification of a cDNA clone encoding this activity and the purification of the enzyme in large amounts.

We express our gratitude to Karin Wiebauer for many helpful suggestions and discussions, to Raffaele De Francesco and Paola Gallinari for critical reading of the manuscript, to Dale Mosbaugh for the UGI inhibitor, to Tomas Lindahl for the gift of the bacterial uracil glycosylase, to Paul Modrich for the purified MutS fraction, to Yves Cully for photographic assistance, and to Philippe Neuner for the synthetic oligonucleotides.

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