Enzymatic Repair of 5-Formyluracil

II. MISMATCH FORMATION BETWEEN 5-FORMYLURACIL AND GUANINE DURING DNA REPLICATION AND ITS RECOGNITION BY TWO PROTEINS INVOLVED IN BASE EXCISION REPAIR (AlkA) AND MISMATCH REPAIR (MutS)*

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5-Formyluracil (fU), a major methyl oxidation product of thymine, forms correct (fU:A) and incorrect (fU:G) base pairs during DNA replication. In the accompanying paper (Masaoka, A., Terato, H., Kobayashi, M., Honsho, A., Ohyama, Y., and Ide, H. (1999) J. Biol. Chem. 274, 25136–25143), it has been shown that fU correctly paired with A is recognized by AlkA protein (Escherichia coli 3-methyladenine DNA glycosylase II). In the present work, mispairing frequency of fU with G and cellular repair protein that specifically recognized fU:G mismatches were studied using defined oligonucleotide substrates. Mispairing frequency of fU was determined by incorporation of 2'-deoxyribonucleoside 5'-triphosphate of fU opposite template G using DNA polymerase I Klknew fragment deficient in 3'-5' exonuclease. Mispairing frequency of fU was dependent on the nearest neighbor base pair in the primer terminus and 2–12 times higher than that of thymine at pH 7.8 and 2.6–6.7 times higher at pH 9.0 with an exception of the nearest neighbor T(template):A( primer). AlkA catalyzed the excision of fU placed opposite G, as well as A, and the excision efficiencies of fU for fU:G and fU:A pairs were comparable. In addition, MutS protein involved in methyl-directed mismatch repair also recognized fU:G mismatches and bound with an efficiency comparable to T:G mismatches, but it did not recognize fU:A pairs. Prior complex formation between MutS and a heteroduplex containing an fU:G mispair inhibited the activity of AlkA to fU. These results suggest that fU present in DNA can be restored by AlkA and the methyl-directed mismatch repair pathway initiated by MutS. Biological relevance of the present results is discussed in light of DNA replication and repair in cells.

Damage to DNA base moieties alters the base pairing properties, hence generating mutation after DNA replication. Deamination of cytosine and adenine, for example, results in uracil and hypoxanthine, respectively, and changes their coding properties in an explicit manner because the deamination products can fully (uracil) or partly (hypoxanthine) adopt hydrogen bonding schemes of thymine and guanine, respectively.

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Abasic sites also result in mutation by an explicit mechanism due to the total loss of base pairing information at the sites. In contrast, some of DNA base lesions that seemingly retain intact coding regions have mutagenic potential. 7,8-Dihydro-8-oxoguanine is one of the best studied examples for this type of lesions. In this lesion, the functional groups in the pyrimidine unit responsible for canonical hydrogen bond formation remain intact, but oxidation of the imidazole unit (C-8) tends to shift the anti-syn equilibrium of the base conformation so that 7,8-dihydro-8-oxoguanine in syn conformation forms a mispair with adenine during DNA replication (1–3). Similarly, we have previously shown that 5-formyluracil (fU)1 retaining an apparently intact coding region forms a mispair with guanine, as well as a correct pair with adenine (4). The mispairing frequency of fU with guanine is significantly higher than that of parent thymine, and the decreased pKa of fU relative to thymine is responsible for the mispairing. Thus, alterations of a part of a base structure that is not directly involved in base pairing can affect anti-syn (ex. 7, 8-dihydro-8-oxoguanine) or acid-base (fU) equilibrium, thereby leading to mutation in an implicit manner.

In the accompanying paper (5), we have shown that fU paired with adenine is recognized by Escherichia coli 3-methyladenine glycosylase II (AlkA) and excised from DNA. In the present work, we quantitatively evaluated the mispairing frequency of fU based on the misincorporation of the deoxyribonucleoside triphosphate of fU (fdUTP) and then searched for cellular repair systems that recognize an fU:G mispair site-specifically introduced into oligonucleotide substrates. We report here that the mispairing frequency of fU with G is nearest neighbor base-dependent and that fU:G mispairs are specifically recognized by two repair proteins, i.e. AlkA (3-methyladenine glycosylase II) and MutS, which are involved in the base excision and methyl-directed mismatch repair pathways, respectively.

EXPERIMENTAL PROCEDURES

Chemicals—Ultra-pure dNTPs were purchased from Amersham Pharmacia Biotech. 5-Formyl-2'-deoxyuridine (fdU) and 5-formyl-2'-deoxyuridine 5'-triphosphate (fdUTP) were synthesized and purified as described (4).

Enzymes—T4 polynucleotide kinase and E. coli DNA polymerase I Klknew fragment deficient in 3'-5' exonuclease; AlkA, E. coli, 3-methyladenine DNA glycosylase II; 8oxoG, 7,8-dihydro-8-oxoguanine; BrU, 5-bromouracil; HPLC, high pressure liquid chromatography.

1 The abbreviations used are: fU, 5-formyluracil; fdU, 5-formyl-2'-deoxyuridine; fdUTP, 5-formyl-2'-deoxyuridine 5'-triphosphate; Pol I Klknew fragment deficient in 3'-5' exonuclease; AlkA, E. coli 3-methyladenine DNA glycosylase II; 8oxoG, 7,8-dihydro-8-oxoguanine; BrU, 5-bromouracil; HPLC, high pressure liquid chromatography.
Oligonucleotides—Templates and primers for DNA polymerase reactions (Table 1) were synthesized by the standard phosphoramidite method and purified by reverse phase HPLC. 25T and 25FU containing thymine and fU at the same position, respectively, were prepared by DNA polymerase reactions using template/primer 30A/P1 as described in the accompanying paper (5) and separated from the complementary strand 30A by preparative 16% polyacrylamide gel electrophoresis under denaturing conditions. 25T and 25FU were extracted from the gel and purified by a Sep-Pak cartridge (6). The amounts of the recovered oligonucleotides were calculated from the specific radioactivity of the primer P1 used in the polymerase reaction.

Enzymatic Parameters of Nucleotide Incorporation—Primers (13A, 13G, 13C, and 13T, Table I) were 5’-end labeled with γ-[32P]ATP (110 TBq/mmol, Amersham Pharmacia Biotech) and T4 polynucleotide kinase, and purified as described (6). The primers were annealed to appropriate templates, and template/primer (0.3 pmol) was incubated with Pol I Kf (exo−) (0.012 unit) and dNTP (fdUTP, dTTP or dCTP) in Buffer A (6 mM Tris-HCl (pH 7.8 or 9.0), 1.5 mM 2-mercaptoethanol, 0.5 mg/ml bovine serum albumin, and 6.6 mM MgCl₂). Appropriate ranges of dNTP concentration and incubation time were determined in preliminary experiments, then the parameters were measured using these ranges (see below). The percent of the primer was generally less than 30% under these conditions. For the correct incorporation (i.e. fdUTP and dTTP opposite template A, dCTP opposite template G), the dNTP incorporation concentration was typically 0.025–2 μM, and incubation time was 3 min. For the incorrect incorporation (i.e. fdUTP and dTTP opposite template G), they were typically 10–1000 μM and 10–30 min under these conditions. After incubation, the reaction was terminated by the addition of gel loading buffer consisting of 0.05% xylene cyanol, 0.05% bromphenol blue, 20 mM EDTA, and 98% formamide. The sample was separated by 16% polyacrylamide gel electrophoresis under denaturing conditions. In the gel electrophoresis, TPE (90 mM Tris-phosphate and 2 mM EDTA, pH 6.6) buffer was used in stead of TBE (90 mM Tris borate and 1 mM EDTA, pH 8.2) because electrophoresis of the reaction products containing 3’-terminal fU in TBE resulted in smearing of the band (data not shown), probably due to the acid-base equilibrium of the fU moiety (pK₆ = 8.6) (4, 7). The smearing of the band could be minimized by electrophoresis in TPE buffer. After electrophoresis, the radioactivity of the extended and unextended primer was quantitated by Fuji BAS 2000. Kinetic parameters for incorporation of dNTP were calculated from Lineweaver-Burk plots.

AlkA Reaction—Duplex oligonucleotide substrates 25FU/30A and 25FU/30G (Table I) were incubated with AlkA protein in Buffer B (typically 25 μl) at 37 °C for 30 min. Buffer B for AlkA reactions consisted of 70 mM Hepes-KOH (pH 7.8), 1 mM EDTA, 5 mM 2-mercaptoethanol. The substrate concentration and the amount of AlkA used in the reaction are indicated in the figure legends. After incubation, DNA was purified by phenol extraction and ethanol precipitation. The purified DNA was annealed with endonuclease Pol I Kf (exo−) and incubated with 1 unit of Pol I Kf (exo−) (0.012 unit) and dNTP (fdUTP, dTTP or dCTP) in Buffer C (10 μl), mixed with gel loading buffer, and finally subjected to gel electrophoresis. Buffer C was composed of 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, and 1 mM EDTA.

25FU/30A and 25FU/30G were also treated with AlkA in the presence of MutS protein to elucidate the effect of competitive action of these proteins on the substrates. The substrates (2 μl) were preincubated with MutS protein (0.049–0.39 μg, approximately 0.5–4 pmol) in Buffer B (9 μl) at 0 °C for 10 min, and then AlkA (10 ng) diluted in Buffer B (1 μl) was added to the reaction mixture. The reaction mixture was incubated at 37 °C for 10 min. Product analysis was performed as described above.

MutS Binding Assay—The binding of MutS protein to oligonucleotide duplexes was analyzed by the gel electrophoretic mobility shift assay as described by Jiricny et al. (8). Binding reactions were performed by mixing 3 μl of 5’-labeled oligonucleotide duplexes (25FU/30A, 25FU/30G, 25T/30A, and 25T/30G, 0.1 pmol, Table I) in Buffer D with 2 μl of MutS protein (0.2–0.82 μg, approximately 2.1–8.4 pmol) appropriately diluted in Buffer E. The composition of Buffer D was 20 mM Tris-HCl (pH 7.6), 0.5 mM ethidium bromide, and that of Buffer E was 50 mM Hepes-KOH (pH 7.2), 100 mM KCl, 1 mM dithiothreitol, 1 mM MgCl₂, and 50% glycerol. The reaction mixture was incubated on ice for 30 min and then supplemented with 2 μl 50% sucrose. Samples (2 μl) were loaded onto a 6% non-denaturing polyacrylamide gel and electrophoresed in TBE buffer at 4 °C. Gels were dried and autoradiographed at −80 °C. Free and bound DNA species were quantitated by Fuji BAS 2000.

Attempt to Detect fdUTP in E. coli Cells Exposed to Hydrogen Peroxide—E. coli AB1157 cells were grown at 37 °C in M9 medium supplemented with 0.4% glucose, 0.5% casamino acids (Difco), 2 mM MgSO₄, 0.1 mM CaCl₂. At an absorbance of 0.6, cells were harvested by centrifugation, and resuspended in phosphate-buffered saline (PBS) (pH 7.8, 5 mM EDTA, and lysozyme (final concentration, 1 mg/ml). Cells were lysed by repeated freeze-thaw cycles. The lysis solution was mixed with 3 volumes of cold 5% trichloroacetic acid, mixed vigorously, and clarified by centrifugation. The supernatant was taken and extracted by 2 volumes of trioctylamine/1,1,1-trichloroethane (1:1 v/v) to remove trichloroacetic acid (9). The water phase (400 μl) was passed through a C18 Sep-Pak cartridge (Waters). An aliquot of the flow-through fraction (100 μl) containing nucleotides was mixed with 5 μl of 500 mM Tris-HCl (pH 9.0, 80 mM NaCl, and 1 mg/ml protein phosphatase) and incubated at 37 °C for 2 h. The sample was filtered with an ultrafiltration filter (molecular weight cut, approximately 10,000) and the filtrate was subjected to HPLC analysis. Alternatively, the filtered sample (100 μl) was mixed with 100 mM cysteamine (5 μl) for derivatization to a thiazolinedione derivative (10), left at room temperature for 8 h, and analyzed by HPLC. The HPLC system consisted of Jasco Model PU-980 pumps, a Hitachi Model L-4200 UV-VIS detector, a Hitachi model D-2500 integrator, and a column oven (40 °C). The nucleotides resulting from alkali phosphatase digestion were separated on a C18 WS-DNA column (0.46 × 15 cm, Wako) using a linear methanol gradient in 10 mM phosphate buffer (pH 7.4): 0% methanol (0–5 min), 50–20% (5–35 min). The flow rate was 0.8 ml/min. The monitoring wavelength was 290 nm for 5-formyluridine (fdU) and 265 nm (λmax) for the thiazolinedione derivative for the samples without and with the derivatization, respectively. To determine the recovery efficiency, the extraction procedure was performed on a standard sample containing authentic fdUTP and dTTP.

RESULTS

Base Pairing Properties of fU—In our previous study, it was shown that fdUTP efficiently substitutes for dTTP and to the less extent for dCTP, indicating that fU forms a mispair with guanine as well as a correct pair with adenine (4). To clarify more quantitative aspects of correct and incorrect base pairing capabilities of fU, steady state kinetic parameters for incorporation of fdUTP and dTTP were determined using a set of defined template and primer. For correct incorporation, template/primer 25TA/13A (Table I) was incubated with Pol I Kf (exo−) in the presence of varying concentrations of fdUTP or dTTP, and initial velocities of their incorporation opposite template A were measured by gel electrophoresis. Parameters (Vmax and Kₘ) were determined at pH 7.8 and 9.0 to elucidate the effects of the acid-base equilibrium of the base unit of fdUTP. The pk₆ values of the base units of fdUTP and dTTP are 8.6 and 10.0, respectively (4, 7). The ratio of the correct incorporation efficiencies (f = Vmax/Kₘ) for fdUTP and dTTP were approximately 1/2 (0.52/1) at pH 7.8 and 1/4 (0.06/0.23) at pH 9.0 (Table II).
indicating that fU retained the base pairing capability similar to parent thymine but with somewhat reduced efficiencies. At pH 9.0, ionization of the base unit of fdUTP (pKa = 8.6) led to a notable increase in Km (=1.64 μM) relative to that at pH 7.8 (Km = 0.12). Consequently, the incorporation efficiency was reduced significantly (f = 0.06), although the deviation from the optimum pH for the DNA polymerase appeared to be an additional factor as judged from the moderate decrease in the f value for dTTP: f = 1 (pH 7.8) and 0.23 (pH 9.0).

Mispairing capabilities of fU were studied based on the incorporation of fdUTP opposite template guanine. A set of template and primer containing all four possible nearest neighbor base pairs at the primer terminus (27TG/13A, 27AG/13T, 27CG/13G, and 27GG/13C, Table I) was used to elucidate their effects on the mispairing frequency. The parameters are summarized in Table III along with those for dTTP and dCTP. The mispairing frequencies (f) of fU were lower than the correct pairing frequency of C by factors of 10^2-10^4 at pH 7.8 and 10^5-10^6 at pH 9.0. The major discrimination factor was the large increase in Kmax (10^2-10^5-fold), and the contribution of the reduction of Vmax was relatively minor (at most by a factor of 10). To compare mispairing frequencies of fdUTP and dTTP more clearly, f values in Table III were plotted against the nearest neighbor pairs in Fig. 1. Mispairing frequencies were clearly nearest neighbor-dependent and pH-dependent for both fdUTP and dTTP, and they were consistently higher for fdUTP than dTTP except the T(template):A(primer) pair (see also Fig. 2). The mispairing frequency increased with the pH shift from 7.8 to 9.0 for both fdUTP and dTTP. In this pH range, the dominant form of the base unit of fdUTP changes from the keto to enolate (ionized) form due to the acid-base equilibrium of the dominant form of the base unit of fdUTP changes from the keto to enolate (ionized) form due to the acid-base equilibrium of the base unit of fdUTP.

**Table II**

| Template/primer | dNTP | Vmax | Km | Vmax/Km | f% |
|-----------------|------|------|----|---------|----|
| 3'-TA- | dTTP | 6.4 | 0.05 | 128 | 1.00 |
| 5'-A | fdUTP | 8.0 | 0.12 | 67 | 0.52 |
| pH 9.0 | 3'-TA- | dTTP | 6.0 | 0.2 | 30 | 0.23 |
| 5'-A | fdUTP | 12.0 | 1.64 | 7.3 | 0.06 |

* DNA polymerase reactions were performed using template/primer 27TA/13A.

* Relative values of Vmax/Km.

* Percentage of extended primer per min.

**Table III**

| Template/primer | dNTP | Vmax | Km | Vmax/Km | f% |
|-----------------|------|------|----|---------|----|
| pH 7.8 | 3'-TG- | dTTP | 5.3 | 0.04 | 133 | 1.00 |
| 5'-A | fdUTP | 8.07 | 150 | 5.8 × 10^-3 | 4.4 × 10^-5 |
| pH 9.0 | 3'-AG- | dTTP | 0.65 | 550 | 1.2 × 10^-3 | 9.0 × 10^-6 |
| 5'-T | fdUTP | 0.70 | 210 | 3.3 × 10^-3 | 2.5 × 10^-5 |
| % | 5'-G | dTTP | 0.32 | 95 | 3.4 × 10^-3 | 2.6 × 10^-5 |
| Kg | 5'-C | fdUTP | 0.11 | 70 | 7.0 × 10^-3 | 5.3 × 10^-5 |
| f% | 3'-GG- | dTTP | 0.22 | 90 | 4.7 × 10^-3 | 3.5 × 10^-5 |
| 5'-C | fdUTP | 1.4 | 25 | 5.6 × 10^-2 | 4.2 × 10^-4 |

* DNA polymerase reactions were performed using template/primer 27TG/13A, 27AG/13T, 27CG/13G, and 27GG/13C, respectively.

* Relative values of Vmax/Km.

* Percentage of extended primer per min.

* Correct incorporation.

**Fig. 1.** Nearest neighbor and pH effects on the mispairing of fU and T. The mispairing (fU:G and T:G) and correctly pairing (C:G) frequencies were determined by Pol I Kf (exo-) catalyzed incorporation of fdUTP, dTTP, and dCTP opposite template G at pH 7.8 and 9.0. The mispairing frequency (f, relative to the correct C:G pair) shown in Table III was plotted against the nearest neighbor base pairs in the primer terminus.

3-methyladenine glycosylase II (AlkA) that was shown to recognize correct fU:A pairs and remove fU from DNA in the accompanying paper (5). Substrates containing an fU:G mispair (25FU/30G, Table I) and an fU:A pair (25FU/30A) were incubated with AlkA, followed by endonuclease IV to cleave abasic sites generated by the N-glycosylase activity of AlkA.
Products were analyzed by polyacrylamide gel electrophoresis. Fig. 3A shows typical results of the product analysis. AlkA recognized 5FU placed opposite G (lane 6) as well as A (lane 3), so that products incised at the lesion were observed for both substrates containing 5FU and 5FU:A pairs. To compare the activity of AlkA for 5FU:A and 5FU:G pairs quantitatively, similar reactions were performed with different concentrations of the substrates or different amounts of AlkA. 25FU/30A and 25FU/30G were equally incised at the 0.2 and 10 nM substrate concentrations (Fig. 3B). In addition, the yields of nicked products for 5FU:A and 5FU:G pairs were essentially similar with varying amounts of AlkA and increased in parallel with the amount of AlkA (Fig. 4). These results clearly indicate that 5FU:G and 5FU:A pairs are recognized by AlkA with comparable efficiencies.

Recognition of 5FU:G Mispair by MutS Protein—Base mispairs generated by DNA replication are primarily restored by the methyl-directed (MutHLS) system in E. coli, and similar mechanisms are present in eukaryotic cells (reviewed in Refs. 11 and 12). These postreplication mismatch repair pathways are initiated by binding of MutS or its homologues to mispaired bases. Thus, binding of MutS protein to an 5FU:G mispair was examined using a gel electrophoretic mobility shift assay. Fig. 5 shows the result when homoduplexes (25FU/30A, 25T/30A) and heteroduplexes (25FU/30G, 25T/30G) were incubated with MutS. Shifted bands were observed for 25FU/30G (lane 4) and 25T/30G (lane 8), containing 5FU and T:G mispairs, respectively, but not for 25FU/30A (lane 2) and 25T/30A (lane 6), containing correct 5FU:A and T:A pairs, respectively. To compare the binding affinities of MutS to the mispaired substrates, the concentration of MutS protein was varied in the assay and the fraction of the bound substrates was determined by quantifying free and bound DNA species (Fig. 6). The amounts of MutS required for 50% binding for T:G and 5FU:G mispairs were virtually similar (5.1 and 5.7 pmol, respectively). These values are also translated into approximately 1 μM as a MutS concentration. Accordingly, despite the difference in the C5 substituents of the bases, MutS binds to 5FU:G and T:G mispairs with comparable affinities.

Effects of MutS Protein on the Repair of 5FU by AlkA—The specificity of the two E. coli repair proteins AlkA and MutS shown in the above experiments suggested that 5FU:A pairs were preferentially repaired by AlkA, whereas 5FU:G mispairs were recognized by both AlkA and MutS. Possible consequences of the coexistence of the two proteins in the repair of 5FU were investigated. 25FU/30G and 25FU/30A were preincubated with varying amounts of MutS, then treated with AlkA. The repair efficiency of AlkA to homoduplex 25FU/30A was slightly enhanced by the addition of MutS (Fig. 7). The enhancement was dependent on the amount of MutS and reached a plateau with an approximately 1.8-fold increase in the repair efficiency. A similar enhancement was observed by the addition of bovine serum albumin in place for MutS protein (data not shown), implying that general (or nonspecific) protein-protein interactions resulted in stabilization of the AlkA activity during the assay. In contrast to the homoduplex, the repair activity of AlkA to heteroduplex 25FU/30G was markedly inhibited by the presence of MutS. The repair activity of AlkA was inhibited by 50% with approximately 2 pmol of MutS (or 0.2 μM as a MutS protein concentration) and further decreased by the addition of more MutS. The concentration of MutS required for 50% inhibition of the AlkA activity to 25FU/30G (approximately 0.2 μM) was considerably lower than that for 50% binding to the same substrate in the gel mobility shift assay described above (approximately 1 μM), probably reflecting the latent differences in the two assays. Such discrepancies were also observed when the binding affinity of MutS to
heteroduplexes was measured by gel mobility shift and DNase I protection assays (8, 13).

Atmpted Detection of fdUTP in E. coli Cells—Because a part of the present study concerning genotoxic effects of fU is based on incorporation of fdUTP potentially formed in the intracellular nucleotide pool, we attempted to measure the level of fdUTP in E. coli cells after oxidative stress as described under “Experimental Procedures.” E. coli AB1157 cells were treated with 20 and 40 mM hydrogen peroxide at 37 °C for 1 h and lysed by repeated freeze-thaw cycles. The nucleotides released from cells were converted to the corresponding nucleosides by alkali phosphatase treatment and quantified by HPLC. The standard sample containing authentic fdUTP and dTTP gave reasonable results with respect to the chromatographic separation and their recovery after the extraction procedure used. The retention times of fdU and 2′-deoxythymidine were 14.9 and 18.9 min, respectively. However, the putative fdU peak was obscured by a large unknown co-migrating peak when the extracted sample was directly analyzed. We have previously shown that fdU can be quantitatively converted to a thiazolidine derivative by incubation with cysteamine (10). This modification results in a large shift of the retention time. Thus, the sample after the alkali phosphatase treatment was incubated with cysteamine and analyzed by HPLC. However, the peak corresponding the thiazolidine derivative of fdU (retention time, 26.0 min) was not detected over the background noise. This result suggests that the level of fdUTP in the hydrogen peroxide-treated cells was below the
Recognition of 5-Formyluracil:G Mispairs by AlkA and MutS

In the present and accompanying (5) papers, it has been shown that fU, a major methyl oxidation product of thymine, is recognized by two repair proteins, i.e., AlkA and MutS. fU correctly paired with adenine is excised from DNA by AlkA, an enzyme responsible for base excision repair of alkylated bases such as 3-methyladenine and 7-methylguanine (15, 16). AlkA also recognized fU mispaired with guanine, and the repair efficiencies for fU:A and fU:G pairs were comparable (Figs. 3 and 4). Interestingly, an fU:G but not fU:A pair was also recognized by MutS, which initiates methyl-directed DNA mismatch repair by binding to base mispairs and small insertion/deletions (11, 12). The binding efficiency of MutS to an fU:G pair was comparable to that to a T:G pair, which is most tightly bound by MutS in vitro and most efficiently corrected by the methyl-directed mismatch repair system in vivo (13, 17). These results suggest that fU present in DNA may be subjected to two independent repair pathways, i.e., base excision repair initiated by AlkA (fU:A and fU:G pairs) and methyl-directed mismatch repair initiated by MutS (fU:G), although subsequent repair reactions involving MutL and MutH proteins were not demonstrated in this work.

AlkA has a broad substrate specificity, particularly base lesions with the labile N-glycosidic bond (18–20), and is suggested to use a base flipping mechanism for excision of damaged bases (21, 22). Granted this mechanism, fU, whether placed opposite adenine or guanine, may flip out the DNA duplex into the hydrophobic cleft of the AlkA active site and is removed with comparable efficiencies as judged from the present results (Fig. 4). The methyl-directed mismatch repair initiated by MutS recognizes and corrects all single-base mismatches (except for C:C) and small insertion/deletion mispairs (11, 12). Moreover, it has recently been shown that MutS also binds to genetic lesions such as O6-methylguanine:T and 1,N2-ethenoadenine:T (23, 24). In addition, hMutSα, an eukaryotic functional homologue of MutS and a heterodimer of hMSH2 and hMSH6 (GTBP), binds to base pairs containing damage, such as cisplatin-1,2-d(GpG) cross-links, O6-methylguanine:T, O6-methylguanine:C, O6-methylthymine:A, and 2-aminofluorene- and N-acetyl-2-aminofluorene adducts of guanine (25–28). To our knowledge, the present paper is the first report on the binding of MutS proteins to oxidative DNA lesions, in this case fU. The underlying mechanism of the recognition of an fU:G mispair by MutS is likely an overall structural similarity between fU:G and T:G mispairs. The mispair initially formed during DNA replication contains an fU(enolate):G pair because the enolate (ionized) form of fU is responsible for the mispairing with G (4). After incorporation, the enolate turns into the keto form, a dominant form under physiological pH, due to the equilibrium. According to the pKₐ value for the acid-base equilibrium of fU, the equilibrium ratio of the keto versus enolate form in fU:G mispairs is expected as 96:4 at pH 7.2 (4). Thus, MutS is likely to bind to fU(keto):G mispairs, which closely resemble the mispaired T:G structure. Footprinting and photocross-linking studies on Thermus aquaticus (Tag) MutS protein and GTBP have suggested that a highly conserved N-terminal region of MutS proteins at least interacts with the major groove of mispaired or unpaired sites (29–31). Therefore, the comparable affinities of MutS to fU:G and T:G mispairs indicate that substitution of the thymine methyl group protruding in the major groove by the formyl group (fU) does not alter the binding affinity.

fU is formed in DNA by oxidation of DNA base thymine (Fig. 8, path I) or incorporation of fdTTP generated in the nucleotide pool (paths III and IV). According to the present results, fU in fU:A pairs is removed by AlkA via paths a and c, whereas fU in fU:G pairs formed by incorporation of dGTP (Fig. 8, path II) or fdUTP (path IV) is removed by AlkA or corrected by methyl-directed mismatch repair (paths b and d). These repair pathways, except path c, can operate independently or cooperatively to avoid the genotoxic effects of fU. Conversely, repair of an fU:G mispair formed by miscoding of template fU (path II) by AlkA results in preferential removal of fU (path c), thereby fixing mutation after repair synthesis. Although biological relevance of the two competing repair pathways (b and c) is not known, it is rather puzzling that such a mutagenic repair path c is potentially present in cells. In this regard, we have shown in this study that the activity of AlkA to fU:G mispairs is preferentially inhibited by the prior complex formation between MutS and the heteroduplex substrate containing fU:G (Fig. 7). Accordingly, it is tempting to speculate that preferen-
tial binding of MutS to υU:G mispairs may have dual roles in cells, i.e. recruitment of MutL and MutH to the genetic lesion for methyl-directed repair and prevention of mutagenic repair of υU by AlkA (Fig. 8, path c). The MutS protein binds to heteroduplexes as a dimer (17, 32). The number of MutS molecules in an exponentially growing E. coli cell has been estimated to be 186 as a dimer, which is translated into 260 nM as the MutS dimer concentration (34). Although the number of AlkA molecules in an E. coli cell is not known, this enzyme accounts for roughly 1/40 of the 3-methyladenine glycosylase activity in uninduced cells. The remainder is due to Tag protein, the number of which in a cell has been also estimated around 200 molecules/cell, or 280 nM (35). Granting a simple enzyme distribution proportional to the repair activity to 3-methyladenine, cellular concentration of AlkA will be roughly 20 molecules/cell, or 28 nM. Because the present experiment, shown in Fig. 7, employed 25–200 nM MutS dimers (0.5–4 pmol/10 μl) and approximately 30 nM AlkA (10 ng/10 μl) for the competitive reaction, we feel that the putative second role of MutS (prevention of mutagenic repair of υU:G pairs) has a certain rational basis. According to the DNase I footprinting study (32), MutS asymmetrically protects about 22 nucleotides on each strand around the G:T mismatch. Thus, MutS bound to the υU:G mismatch can inhibit the access of AlkA protein to the lesioned site.

Mutagenic repair similar to path c has been suggested in the repair of 8oxoG:A mispairs that is formed by misincorporation of 8oxoG opposite template A (36). In this case, excision of correct A by MutY DNA glycosylase results in A:T to G:C transitions. In light of the possible dual roles of MutS in the repair of damage-containing mispaired bases (discussed above for υU:G mispairs), we measured the binding affinity of MutS to a duplex containing 8oxoG:A by gel electrophoretic mobility shift assay. However, the affinity of MutS to the 8oxoG:A pair was low (data not shown), thereby ruling out the involvement of MutS in the processing of the 8oxoG:A lesion.

The present work has shown that υU forms mispairs with template guanine in a sequence context-dependent manner when dUTP is incorporated by DNA polymerase, although the detailed mechanism of the nearest neighbor effect is unknown. For example, we feel that the putative second role of MutS in the processing of the 8oxoG:A lesion.

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