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Multiplicity of Strand Incision at G:T Base Mismatches in DNA by Human Cell Extracts†

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ABSTRACT: Cell extract from the HT29 human colon carcinoma cell line (lacking mutator phenotype) was used to study the ATP-dependent G:T mismatch repair. We found that when a 45-bp (model) DNA with a single CpG/TpG mispair was incubated with the cell extract and ATP, it was incised immediately 5′ and 3′ to the mismatched T, and we noted that the actual 5′- and 3′-labeled fragments were similar to the cleaved products of thymine DNA glycosylase (TDG). This TDG-like cleavage product was enhanced (5-fold) with stimulation of several novel fragments, as inferred from the effect on incision at CpG/TpG site of the addition of G:U competitor DNA and ATP to the HT29 extract. The novel fragments were compatible with a strand incision on both sides of the mismatch (the third phosphodiester bond 5′ and the second phosphodiester bond 3′ to the mismatched T) and an incision 3′ to the mismatched T, respectively. This suggests that while the ATP-dependent (TDG-like) incision activity, contrary to expectation, shows a lack of substrate competition, its catalytic property is likely modified by an interaction with G:U mispair. These multiple ATP-dependent incision events were not detected when extracts of the mismatch repair (MMR) defective HCT15 or HCT116 cell line were augmented with ATP and G:U. We postulate that these multiple ATP-dependent incision events possibly require the same MMR factors, and together they constitute a modified single ATP-dependent G:T incision activity. This activity toward the CpG/TpG was competitively inhibited by a 45-bp DNA with an ApG/TpT mispair; incision at a single site 5′ to the latter mismatch compares with one of the multiple sites incised 5′ to the former mismatch. These results suggest that one of several mismatch-incision factors is required by the human ATP-dependent G:T incision activity, in addition to MMR factors and ATP.

G:T base mismatches are produced in the DNA of all cells by errors of semiconservative DNA replication, within the heteroduplexes that arise during recombination, and by the spontaneous (hydrolytic) deamination of m5C[1] residues to thymines, preferentially at the sites targeted by DCM proteins (*1*−*5*). If G:T mispairs are not repaired to G:C normal pairs prior to DNA replication, they result in G:C→A:T transition mutations (*6*). In vivo correction of G:T mispairs to normal G:C pairs shows that a G:T mismatch occurring within the CpG sequence context, and one in which the mismatched G was 3′ to an A, were equally recognized by the human G:T mismatch repair (*7*). Several studies have suggested that the G:T mismatches arising from the spontaneous deamination of m5C are recognized by TDG, which has been purified and corresponding cDNA of which has been isolated and expressed (*8*−*11*). The recombinant 46-kDa protein binds to G:U, G:T, and other inappropriate mismatched base pairs, and a G:T mismatch in the 5′-CpG-3′/5′-TpG-3′ sequence context acts as the best substrate of TDG, as compared with any other base pair on the 5′ side of the G:T mismatch (*10*, *12*, *13*). Moreover, purified TDG removes uracil from a G:U mispair faster than T from a G:T mismatch (*12*, *13*). The fact that G:U is readily acted on by purified TDG argues that TDG alone does not primarily remove the T mispaired with the dG and thus G:T → G:C repair in vivo may be initiated by protein(s) other than TDG.

In keeping with the aforementioned prediction, our previous study showed that the repair of G:T mismatches in human cells may be initiated by a reaction distinct from that mediated solely by purified TDG (*14*). The evidence was obtained by monitoring the initiation of strand incision at m6G:T in a 45-bp DNA incubated with cell-free extracts of the MMR$^+$ (glioma A1235 and colon carcinoma HT29) and the MMR$^-$ (glioma A1235-MR4 and colon carcinoma HCT15) cell lines. Accordingly, augmentation of the extract of an MMR$^+$ cell line with ATP served to enhance strand incision at m6G:T site in the DNA, whereas that of the MMR$^-$ cell extract with ATP blocked incision completely. Intriguingly, when ATP was omitted from the reaction

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[1] Abbreviations: m5C, 5-methylcytosine; DCM, deoxycytosine methylase; TDG, thymine DNA glycosylase; m6G:T, *O*^{6}-methylguanine:T; MMR, mismatch repair; MMR$^+$/MMR$^-$, designating cell lines that are proficient (MMR$^+$) or deficient (MMR$^-$) in mismatch repair; Ap, apyrimidinic/apurinic; CG/TG or AG/TT substrate, denoting a synthetic 45-bp DNA with a single G:T mismatch in 5′ CpG 3′/5′ TpG 3′ or 5′ ApG 3′/5′ TpT 3′ context; *MSH6* or *MSH2*, human homologue of yeast MutS gene; *MLH1*, human homologue of yeast MutL gene; G:U competitor, denoting a 45-bp DNA containing a G:U mispair; single multisite G:T incision activity, denoting a modified ATP-dependent G:T incision activity in human cell extract.

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FIGURE 1: The 45-bp (model) DNA substrates. In panel A, the sequence is based on human H-ras oncogne used earlier (14,15). A single G:T base mismatch flanked on the 5′ side by a normal C:G pair is shown in the box, and the G:T mispair is located at position 25 bases from the 5′ terminus of the top strand. In panel B, a single G:T mismatch is flanked on the 5′ side by an A:T base pair in the same 45-bp model DNA.

mixture, the rate of the incision at m6G:T was indistinguishable for the MMR^+ and MMR^- cell extracts (*14*). Put together, these results suggested that the human cells contain two G:T mismatch incision activities, namely, an ATP-independent "TDG-like" activity and an ATP-dependent G:T incision complex activity (*14*). Furthermore, the sizes of the incision fragments were similar to that produced by TDG-based incision activity when the DNA containing a G:T mispair was incubated with MMR^+ cell extracts after augmentation with ATP plus limited TDG or with ATP alone (*14*, *15*). There are certain points that are unclear: is TDG in the extract engaged in a high-affinity complex with proteins, such as the MMR recognition factors, and ATP and the G:T mismatch; is strand incision at G:T mismatch in DNA similar to the cleavage by purified TDG (*15*)? Hence, the actual site of strand incision by the constitutive ATP-dependent G:T incision activity needs to be confirmed.

In this paper, we assayed strand incision in a 45-bp (model) DNA with single G:T mispair produced by the ATP-dependent G:T incision activity, using HT29 (MMR^+) cell extracts (*14*, *15*). The 45-bp DNAs containing CpG/TpG mispairs gave rise to incision fragments consistent with TDG-like activity when incubated with the HT29 extract and ATP (*14*). Paradoxically, we found a lack of effect of G:U competitor on TDG-like activity coupled with the stimulation of new strand incision events when HT29 extract was augmented with ATP and low amounts of G:U. Augmentation of several MMR^- cell extracts with ATP and G:U caused no multiple ATP-dependent incision events toward CpG/TpG mispairs. We conclude that these incision events catalyzed by the HT29 extract constitute a modified single ATP-dependent G:T incision activity (denoted as a multisite G:T incision activity). We also showed that strand-specific incision at an ApG/TpT mispair in a 45-bp DNA by the modified single ATP-dependent G:T incision activity occurred mainly at a single site 5′ to the mismatch. This site is similar to one of the multiple sites incised on each side of the CpG/TpG mispair by the enzyme.

MATERIALS AND METHODS

Preparation of Cell Extracts. Colon carcinoma cell lines HT29, HCT15, LoVo, DLD-1, and HCT116 were grown in Ham F12 medium supplemented with 15% fetal bovine serum, 50 U/mL penicillin, and 50 μg/mL streptomycin at 37 °C as described earlier (*14*). For each cell line, confluent cultures in 10 (150-mm) dishes were harvested by the standard cell trypsinization. Extracts (each equivalent to (2−5) × 10^8 cells) were prepared by the procedure of Li and Kelly (*16*, *17*). Briefly, the cell pellet was washed in 10−15 mL of ice-cold hypotonic buffer (20 mM Hepes, pH 7.5, 5 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT) containing 0.25 M sucrose followed by 10 mL of ice-cold hypotonic buffer alone. The washed cell pellet was suspended in about 0.5 mL of hypotonic buffer, and the cells were allowed to swell on ice for 30 min and were disrupted with 10 strokes of a tightly fitting pestle in a Dounce homogenizer. After 30 min on ice, the lysate was centrifuged at 10 000 × *g* for 30 min at 4 °C. Supernatant was carefully recovered and adjusted to a final concentration of 100 mM NaCl by adding a desired portion of 5 M NaCl solution and was again centrifuged at 100 000 × *g* for 1 h at 4 °C. The supernatant containing the cell extract was recovered, aliquoted, snap frozen in liquid nitrogen, and stored at −80 °C. The protein content of the extracts was measured and found to be 8−10 mg/mL.

Preparation of DNA Substrates. The 45-bp (model) DNA substrates shown in Figure 1A,B were prepared using the desired 45-mer top and bottom strands (*18*, *19*). Thus, several 45-mer top strands and the complementary bottom strands were synthesized by the DNA Synthesis Laboratory, Department of Microbiology, University of Alberta, Edmonton, AB, Canada, so as to prepare heteroduplexes containing CpG:T, CpG:U, ApG:T mispairs and duplexes with normal CpG:C, ApG:C and ApA:T base pairs at the known sites in the 45-bp DNAs (*18*, *19*). When required, either strand of a 45-bp DNA substrate was labeled at its 5′ terminus by incubating with T4 polynucleotide kinase and [γ-^{32}P]-ATP prior to annealing with its complementary strand (*18*, *19*). Substrate containing a radioactive label at the 3′ terminus of the bottom strand was generated with the help of Pol I (Klenow fragment) in the presence of [α-^{32}P]-dCTP such that only a single radioactive dCMP was added to the 3′ terminus of the 44-mer bottom strand (*15*). The 44-mer bottom strand was annealed to a complementary 45-mer top strand prior to carrying out the DNA Pol I reaction (*15*). T4 polynucleotide kinase was supplied by Pharmacia Biotech (Piscataway, NJ), and DNA Pol I was furnished by Boehringer Mannheim (Mannheim, Germany). [α-^{32}P]-dCTP and [γ-^{32}P]-ATP were purchased from Amersham International (Little Chalfont, U.K.).

ATP-Dependent G:T Mismatch Incision Activity. The incision assay was performed as described earlier (*14*). Briefly, the reaction mixture (total volume, 50 μL) contained cell extract (10−20 μg of protein), 5′-end-labeled, base-mismatch-containing heteroduplex substrate (2 ng), 20 mM Hepes (pH 7.6), 50 mM KCl, 0.5 mM EDTA, 2 μM ATP, 1 mM $MgCl_2$, 0.01 mM $ZnCl_2$, 25 μg of phosphoenolpyruvate, 1 U of pyruvate kinase, and 100 μg/mL BSA. In some reactions varying amounts of the unlabeled 45-bp DNA containing a G:U mispair was added to the extract to serve as a TDG inhibitor. Each reaction was routinely carried out at 30 °C for 4 h, and reaction products were analyzed by running DNAs on 12% sequencing gels followed by autoradiography on Kodak X-ray film (*19*).

ATP-Independent (or TDG) Incision Assay. In short, the reaction mixture (50 μL) contained cell extract (10 μg), 5′-terminus-labeled 45-bp DNA containing a base mismatch (2 ng), 20 mM Hepes (pH 7.9), 0.5 mM EDTA, 1 mM DTT, 0.01 mM $ZnCl_2$, and 100 μg/mL BSA. The reaction was carried out at 30 °C for 4 h. DNAs were extracted after conducting incision reactions and were analyzed for products on 12% sequencing gels as described before (*19*).

RESULTS

Strand Incision at G:T Mispair in DNA by the HT29 Extract Supplemented with ATP: Effect of the Addition of G:U Competitor to Extracts. To study the effect of G:U competitor on the ATP-dependent incision at G:T base mismatch, a 45-bp (model) DNA containing a single CpG/TpG mispair (hereafter referred to as CG/TG substrate) was first prepared as specified in Figure 1A. The CG/TG substrate was labeled at the 5′ terminus of the strand containing the mismatched T, and was incubated with the extract of colon carcinoma HT29 cell line (lacking mutator phenotype, ref *20*), ATP, and increasing amounts of G:U competitor (an unlabeled 45-bp DNA containing a G:U mispair) as an inhibitor of the G:T mismatch binding protein TDG (*13*). Thus, strand incision in the model CG/TG substrate before and after the addition of G:U competitor in the reaction mixture was monitored on sequencing gels by characterizing sizes of the incision product(s). Figure 2A shows that the labeled CG/TG substrate was incised by the HT29 extract, supplemented with ATP alone or together with a low amount (0.2 ng) of unlabeled G:U competitor, yielding a labeled 20-mer fragment (lanes 2 and 3), whereas the substrate incubated without the extract gave no such incision product (lane 1). The size of the 5′-labeled 20-mer fragment is indicative of incision of the immediate phosphodiester bond 5′ to the mismatched T in the substrate and is similar to the cleaved product of purified TDG (*12*, *13*). The addition of a small amount of G:U competitor (0.4−1 ng) in the reaction did not inhibit the TDG-like (20-mer) incision product due to lack of substrate competition between the CpG/TpG and G:U mispairs. In addition, a 21-mer (or a slow moving 20-mer) fragment was also obtained when CG/TG substrate was co-incubated with 0.4 or 0.6 ng of G:U competitor (lanes 4 and 5). The size of the 5′-labeled 21-mer intermediate is consistent with a strand incision at a unique site 3′ to the mismatched T in the CG/TG substrate, a feature similar to a TDG with 3′ AP endonuclease activity, which first removes the mispaired T and at the same time hydrolyzes the phosphodiester bond 3′ to T (S.-U. Lari and R. S. Day, unpublished data). More intriguingly, the addition of increasing G:U competitor (1 ng) to the reaction mixture did not reverse the lack of substrate competition with CpG/TpG mispair. Instead, in addition to elevated TDG-like 5′ 20-mer fragment (4−5-fold greater than 20-mer in lane 2) and the reduced 5′ 21-mer, a new 5′-labeled 18-mer fragment resulting from a unique strand incision at the third phosphodiester bond 5′ to T in the CG/TG substrate was obtained (lane 6). The multiplicity of strand incision at a single G:T mismatch in DNA, that is, observation of three 5′-labeled incision fragments as a result of the repair of G:T mismatch, is an unusual finding (lanes 4−6). The lack of incision at G:T mismatch in the CG/TG substrate (absence of fragments

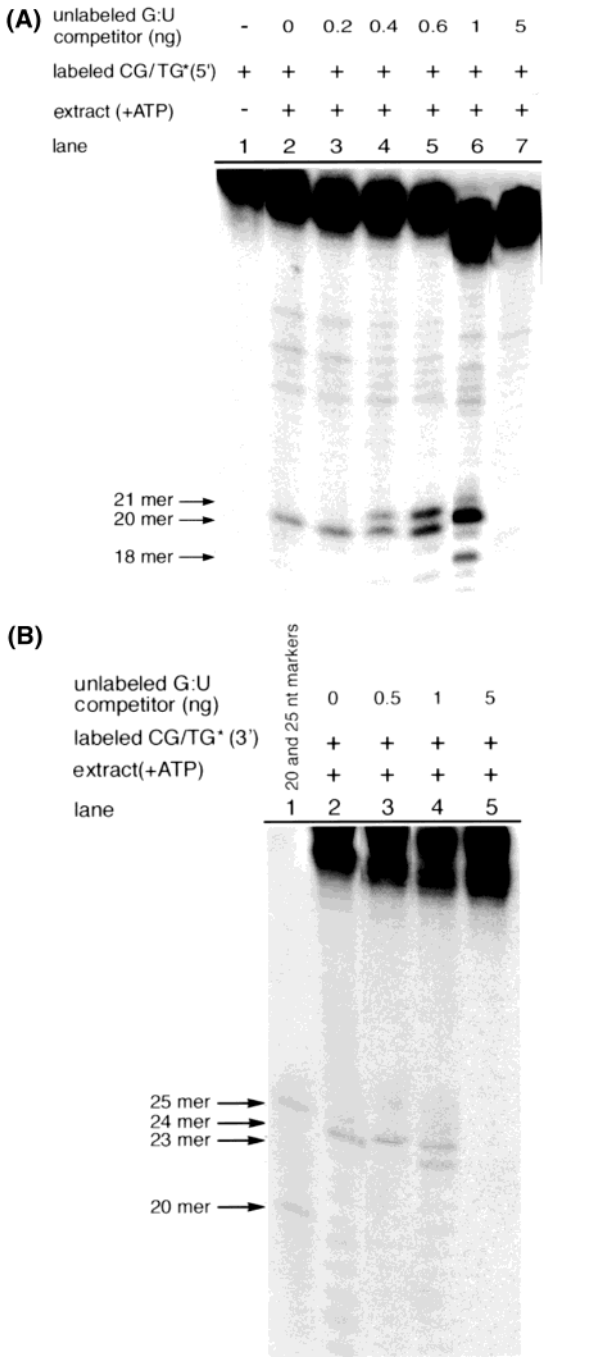

FIGURE 2: Panel A shows the effect of G:U competitor on the ATP-dependent G:T mismatch specific incision activity. Extract from the HT29 cell line (20 μg protein), CG/TG substrate (2 ng) labeled at the 5′ terminus of the strand containing the mismatched T (see Figure 1, noted as CG/TG*(5′)), ATP, and unlabeled G:U competitor were incubated in the ATP-dependent G:T incision assay buffer. The reaction mixture was incubated at 30 °C for 4 h. The 45-bp DNA extracted from the reaction mixture was subjected to electrophoresis as described under Materials and Methods. The 5′-labeled 18-mer, the 20-mer, and the 21-mer incision fragments are marked by arrows. Lane 1 represents sham-treated CG/TG* substrate. Panel B shows the effect of G:U competitor on the incision of the 3′-end-labeled CG/TG substrate. The 3′ terminus in the bottom strand (designated as CG/TG*(3′)) was labeled and incubated with the HT29 extract, ATP, and varying concentrations of the unlabeled G:U competitor DNA. Reactions were performed as described in the legend to panel A. The amounts of the unlabeled G:U competitor used in the reactions are shown in lanes 2−5. The labeled 3′ 23-mer and 3′ 24-mer incision products are indicated by arrows. The 20- and 25-mer markers in lane 1 were prepared as before (*19*).

in lane 7) in the presence of substantially high G:U competitor (5 ng DNA) is attributed to substrate competition.

The results of the incision reaction using the CG/TG substrate labeled at the 3′ terminus in the strand containing the mismatched T and the HT29 extract supplemented with ATP at different unlabeled G:U competitor concentrations are illustrated in Figure 2B. The 3′-end-labeled CG/TG substrate incubated with the extract and ATP gave rise to a 3′-labeled 24-mer fragment in the absence of G:U competitor (lane 2) or in the presence of G:U competitor (10 ng/mL) (lane 3). The 3′ 24-mer fragments obtained in lanes 2 and 3 are similar to TDG-cleaved product (*15*) and to that of a TDG with 3′ AP endonuclease-like activity (S.-U. Lari, and R. S. Day, unpublished data), respectively. The incision of the 3′-labeled CG/TG substrate co-incubated with higher levels of G:U competitor (20 ng/mL) yielded a TDG-like 3′-labeled 24-mer and a 3′-labeled 23-mer product (lane 4). The novel 3′ 23-mer fragment indicates that the second phosphodiester bond 3′ to T in the CG/TG substrate is hydrolyzed (lane 4) and is expected to occur together with hydrolysis of the third phosphodiester bond 5′ to T of the CpG/TpG (see 5′ 18-mer in Figure 2A, lane 6). Note that the latter incisions on both sides of the CpG/TpG mispair are catalyzed by the extract incubated with CG/TG substrate DNA and G:U competitor DNA in a ratio of 2:1.

Lack of Multiple Strand Incisions in the CG/TG Substrates by MMR⁻ Cell Extracts. The model CG/TG substrates labeled at the 5′-termini of the bottom strands (Figure 1A) were incubated with various MMR⁻ cell extracts, each supplemented with ATP and G:U competitor (20 ng/mL), to determine whether the multiple strand incisions in the CG/TG substrate required the same MMR factor(s). As shown in Figure 3, lanes 2 and 8, the labeled CG/TG substrates treated with the HT29 extract containing ATP and G:U competitor gave rise to the elevated TDG-specific 5′ 20-mer, the 5′ 18-mer, and the reduced 5′ 21-mer fragments, as expected, whereas a sham-treated CG/TG substrate failed to give any incision fragment (lane 1 or 7). In contrast, the 5′-labeled incision fragments, that is, 20-mer, 21-mer, or 18-mer, were not stimulated and were produced in very little amounts when the CG/TG substrates were incubated with extracts of the colon carcinoma cell lines (HCT15 and HCT116) harboring deficiency in various MMR factors (*1*−*3*) despite augmentation with ATP and G:U competitor (lanes 3 and 4). Note that some TDG-like 20-mer fragment was produced by the HCT116 extract in lane 4, possibly due to weak interaction between DNA substrate and TDG. The two MMR⁻ cell extracts tested positive for the presence of TDG activity (*14*). It is noticeable that the site-specific strand incisions in the CG/TG substrates were stimulated by mixing the HCT15 and HCT116 cell extracts, as shown clearly by the presence of labeled 5′ 20-mer or the 5′ 21-mer but poorly by the 5′ 18-mer fragment; the amount of each fragment in lanes 5 and 6 is relatively more than that in lanes 3 or 4. In addition, the yield of each of these fragments in lane 5 or 6 was about 10−12-fold less than the counterpart fragment produced by the HT29 extract in the control reaction (lane 2). Incidentally, these ATP-dependent G:T incision events were detected in a group of MMR⁻ cell lines (i.e., LoVo and DLD-1, refs *1*−*3*), extracts of which were efficient in incising the 5′-end-labeled CG/TG substrates, producing substantial 5′ 20-mer, 5′ 18-mer and reduced 21-mer

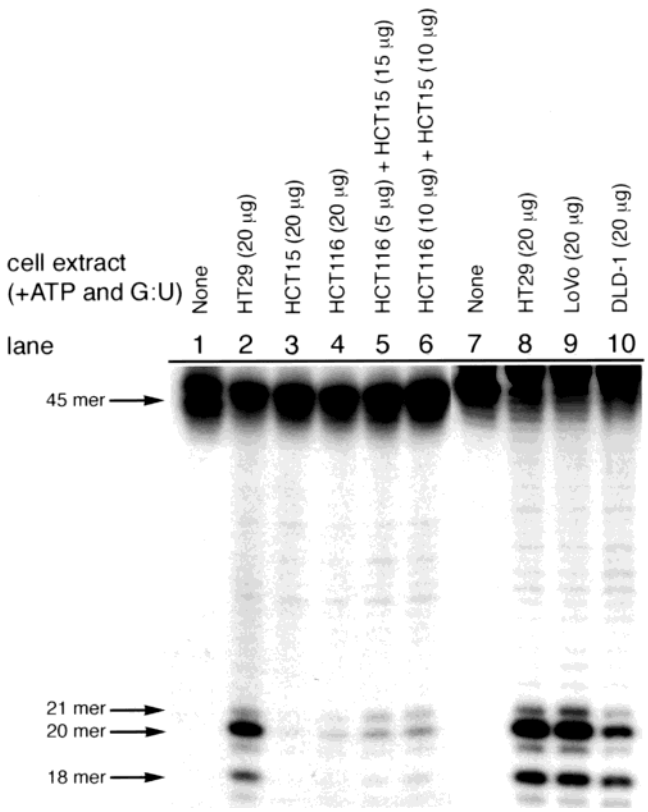

FIGURE 3: Efficiency of the extracts of different human tumor cell lines in the catalysis of multisite G:T mismatch specific incision in DNA with a single CpG/TpG mispair. The model CG/TG substrate labeled at the 5′ terminus in the bottom strand containing the mismatched T (2 ng) was incubated with different cell extracts. Each contained ATP and 1 ng of G:U competitor for strand incision. The amount of the extracts is indicated in the figure, and reaction products were analyzed as described in Figure 2A. Lanes 1 and 7 contained sham-treated CG/TG substrates; lanes 2 and 8 contained CG/TG substrate treated with HT29 cell extract; lanes 3 and 4 contained CG/TG substrates treated with HCT15 and HCT116 cell extracts, respectively; lanes 5 and 6 contained CG/TG substrates treated with a mixture of HCT15 and HCT116 extracts; lanes 9 and 10 contained CG/TG substrates treated with LoVo and DLD-1 cell extracts, respectively. The 18-mer and 20-mer products are indicated by arrows.

products, respectively (lanes 9 and 10). Collectively, these results are consistent with the view that each of the ATP-dependent G:T incision events catalyzed by human cell extracts may depend on the same MMR recognition factors.

Competitive Inhibition of Multiple Site-Specific Strand Incision at CpG/TpG Sites. We next assessed the competitive inhibition of the multiple strand incision at a CpG/TpG mismatch site catalyzed by the HT29 extract supplemented with ATP and 1 ng of G:U competitor, using particularly a 45-bp DNA with an ApG/TpT mispair, denoted as AG/TT substrate (Figure 1B) (*18*). The CG/TG substrate, labeled at the 5′ end of the bottom strand, was incubated with increasing quantities (0−10 ng) of each of the 45-bp unlabeled DNA containing CpG/TpG, ApG/TpT, and control CpG/CpG or ApA/TpT in the presence of the HT29 extract, ATP, and 1 ng of G:U competitor. As can be seen in Figure 4 (lane 2), the labeled CG/TG substrate yielded predominantly 5′ 20-mer and 5′ 18-mer fragments in the presence of ATP and 1 ng of G:U competitor and no unlabeled DNA substrate. The changes in 20-mer and 18-mer bands at 5 ng of unlabeled CG/TG substrates were very small (compare lane 2 with lane

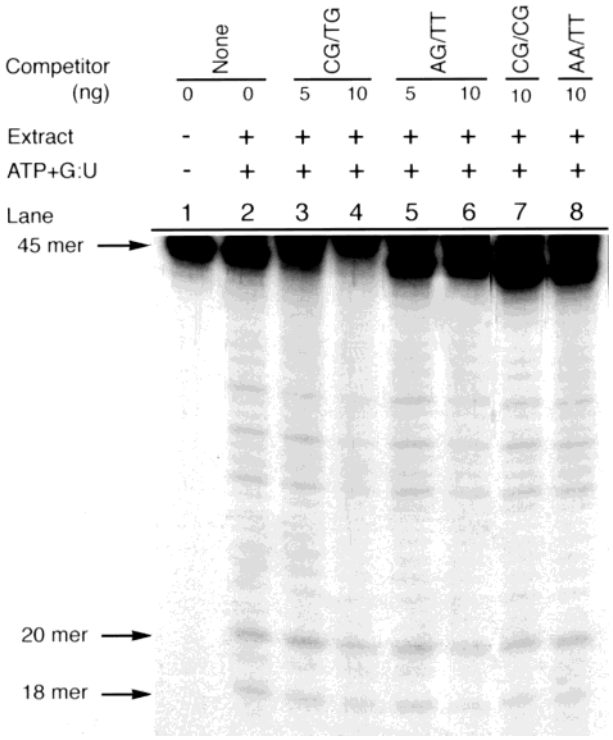

FIGURE 4: Inhibition of multisite G:T incision activity by the unlabeled competitor 45-bp DNA substrates. Aliquots (2 ng) of the CG/TG substrate (labeled at the 5′ terminus of the bottom strand) were mixed with the indicated amounts of the unlabeled CG/TG, AG/TT, CG/CG, and AA/TT substrates in the ATP-dependent G:T incision assay buffer, and the incision reaction was carried out upon the addition of the HT29 extract, ATP, and 1 ng of G:U competitor DNA (see Material and Methods for details). Lane 1 contained sham-treated CG/TG substrate; lane 2 contained reaction with no competitor; lanes 3 and 4 contained reactions with varying amounts of the unlabeled CG/TG substrate; lanes 5 and 6 contained reactions with varying amounts of the unlabeled AG/TT substrate; lane 7 contained the normal CG/CG pair containing 45-bp DNA; lane 8 contained the normal AA/TT pair containing DNA. The 18-mer and 20-mer products are indicated by arrows.

3), whereas 10 ng of unlabeled competitor caused relatively more diminution in the multisite incision activity found in the extract (see the reduced 18-mer and 20-mer in lane 4). Like the unlabeled CG/TT substrate, unlabeled AG/TT substrate at 10 ng (lane 6), as compared with 5 ng (lane 5), served to reduce the multisite G:T incision activity-specific 18-mer and 20-mer products effectively (lane 6 versus lane 4). In contrast, unlabeled CG/CG (replacing CpG/TpG in the 45-bp DNA) or unlabeled AA/TT (replacing ApG/TpT in the 45-bp DNA) at 10 ng of DNA caused little reduction in 20-mer and 5′ 18-mer fragments (lanes 7 and 8). Note that the marginal incision of the labeled substrate (i.e., high molecular-weight fragments as the products of nonspecific endonucleases; lanes 4 and 6) was unusually suppressed when substrate competition on the G:T incision activity was assayed in the extract. Altogether, these results show for the first time that the ApG/TpT mismatch in DNA is possibly recognized by the ATP-dependent multisite G:T incision activity. Recognition of the ApG/TpT mismatch compared with the CpG/TpG mismatch by purified TDG has not been reported (*12*, *13*).

Incision at CpG/TpG and ApG/TpT Mispairs by the HT29 Extract. Because both ApG/TpT and CpG/TpG mismatches serve as substrates of the same multisite G:T incision activity, we questioned whether the AG/TT substrate is incised by the activity in a manner similar to the CG/TG substrate. To this end, each substrate was labeled at the 5′ terminus of the bottom or the top strand and incubated with the HT29 extract supplemented with ATP and unlabeled G:U competitor to monitor strand incisions. Figure 5A, lane 3, illustrates that the CG/TG substrate labeled at 5′ terminus of the bottom strand gave a 5′ 20-mer product in agreement with TDG activity when incubated with the HT29 extract alone (*12*, *19*). As expected, the CG/TG substrate labeled in the bottom strand was incised as a result of the action of the G:T mismatch activity and its interaction with G:U and ATP, each present in the HT29 extract. The resulting predominant 5′ 20-mer and 5′ 18-mer fragments identified on a sequencing gel are shown in lane 4. This is consistent with multiple strand-specific incisions, namely, a predominant incision at the first phosphodiester bond 5′ to T and a reduced incision at the third phosphodiester bond 5′ to T, both in the same bottom strand of the CG/TG substrate. The activity did not cleave the other strand containing the mismatched G, since the CG/TG labeled at the 5′ end of the strand, gave no incision fragments (lane 6). Similarly, a DNA with CpG/CpG normal base pair, labeled in the bottom strand, was not incised by the activity (lane 5). Figure 5B shows the result of the incision of the AG/TT substrate. Incubation of the AG/TT substrate labeled in the 5′ end of the bottom strand with the HT29 extract alone gave a very poor yield of 20-mer fragments because it did not serve as a substrate for endogenous TDG (lane 2; *12*, *15*). In sharp contrast when the AG/TT substrate labeled at the 5′ terminus in the bottom strand was incubated with the HT29 extract, ATP and G:U competitor, it yielded a greater amount of 5′-labeled 18-mer fragments than any other, suggesting that the ApG/TpT is processed by the multisite G:T incision activity with the help of a factor other than TDG (lane 3). The AG/TT substrate labeled in the top strand (lane 4), as well as a 45-bp DNA with a normal ApG/CpT or ApA/TpT base pair (lane 5 or 6), gave no incision fragments.

DISCUSSION

The ATP-dependent incision at a single CpG/TpG mismatch site located in a 45-bp (model) DNA by the extract of the HT29 (MMR^+) cells assayed at increasing G:U competitor concentrations is shown in Figure 2A,B. The observation that the ATP-dependent G:T incision activity (i.e., primarily a TDG-like 20-mer incision product) increased significantly with the G:U competitor DNA in lanes 2−6 (Figure 2A) suggests that a TDG (possibly a subtype) along with other proteins, such as MMR recognition factors, ATP, and CpG/TpG mismatch constitutes a high-affinity G:T mismatch repair complex (*14*, *15*).

The effect of G:U mismatch on the ATP-dependent incision at CpG/TpG mismatch discussed in the preceding is inadequate because the CG/TG substrate was incised at TDG-sensitive and other sites, notably a site 2 bases away 5′ to the mismatched T in the same strand, and the two incision products (the 5′-labeled 20-mer and the 5′-labeled 18-mer fragments) were simultaneously observed (see Figure 2A). If the CG/TG substrates were targeted by a TDG-multiprotein complex, as postulated elsewhere (*14*), concurrently with a separate putative 5′ 18-mer producing mismatch incision activity, one would expect predominantly the smaller

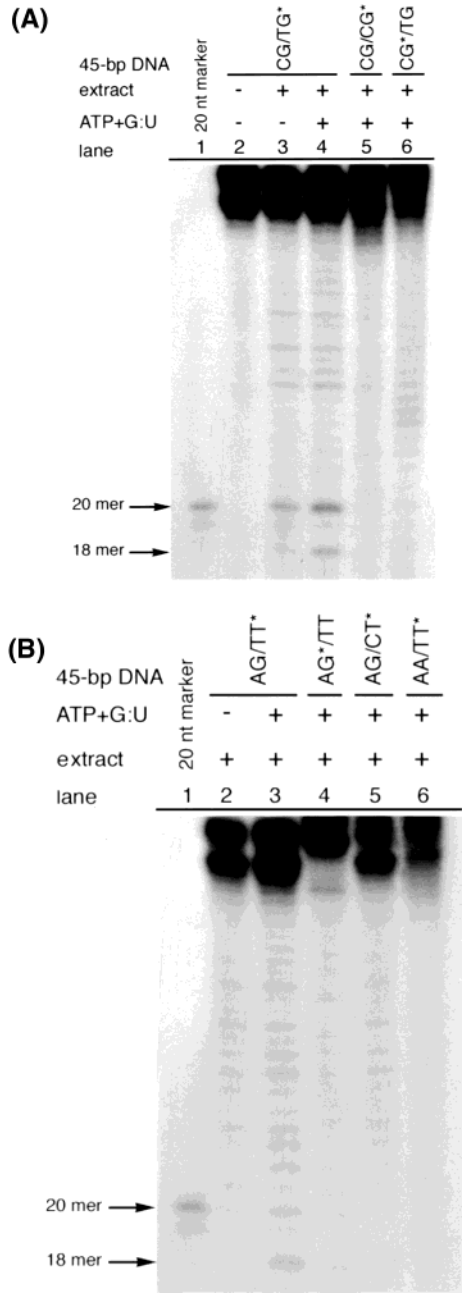

FIGURE 5: Panel A shows multiple site-specific incision of 45-bp DNA containing a single CpG/TpG mismatch. The 45-bp CG/TG substrate labeled in the bottom strand (denoted as CG/TG*, 2 ng) was treated with the HT29 extract, 20 μg of protein (lane 3), or with extract, ATP, and 1 ng of G:U competitor (lane 4). The 45-bp DNA with a G:C pair and bottom strand labeled (as CG/CG*) or with a CpG/TpG mispair and top strand labeled (as CG*/TG) was used with extract supplemented with ATP and G:U competitor (lanes 5 and 6). Reaction products were analyzed as described in Figure 2A. The 45-bp CG/TG* alone is given in lane 2 and the 20-mer marker in lane 1. Panel B shows site-specific strand incision at ApG/TpT mismatch. The incision reaction was performed as described in panel A. The 45-bp DNA having an ApG/TpT mispair (Figure 1B) was labeled at the 5′ terminus of the bottom strand (denoted as AG/TT*) and treated with HT29 extract alone (lane 2) or with extract containing ATP and G:U competitor (lane 3). In lane 4, the AG/TT substrate was labeled in the top strand (denoted as AG*/TT) and was treated as described for lane 3. In lanes 5 and 6, the 45-bp DNA with a normal ApG/CpT pair (as AG/CT*) or ApA/TpT pair (as AA/TT*) was treated as described in lane 3. Lane 1 shows the 20-mer markers.

5′ 18-mer product from the 5′-labeled CG/TT substrate with a single G:T mismatch. The results in lanes 4−6 do not support this notion. Taken in conjunction with the effect of G:U competitor on strand incision at CpG/TpG mismatch by the extract and ATP, that is, (i) a lack of competition at the TDG-sensitive site and (ii) simultaneous stimulation of strand incisions at sites distinct from the TDG-sensitive site, each in CG/TG substrate with a single mismatch (Figure 2), the existence of an ATP-dependent G:T incision activity with multiple incision factors including TDG may not be ruled out. It is conceivable that a key catalytic subunit other than the incision factors or substrate-recognition subunit in the ATP-dependent G:T incision machinery is targeted by low G:U competitor leading to accessibility of the G:T mismatch bound by the enzyme to several mismatch-incision factors as components of the single enzyme complex. This is also independent of whether this G:T incision machinery is bound or unbound to CpG/TpG mismatch. As a result, the CG/TG substrate bound by the modified ATP-dependent G:T incision complex is subjected to incision, alternatively, by more than one factor at single mismatch site, although CpG/TpG mispair may not be very well recognized by all the putative factors with higher specificity, except TDG (Figure 2A, lane 2−6). In the same context, we believe that non-TDG-like strand incision(s) in the CG/TG substrate are nonproductive and fortuitous, since the CG/TG bound by unmodified enzyme in the extract lacking G:U competitor DNA provided a TDG-like cleavage product (Figure 2A, lane 2; Figure 2B, lane 2).

As shown in Figure 3, the extracts of two of four MMR$^-$ cell lines tested, namely, HCT15, HCT116, LoVo and DLD-1, were found to lack multiple ATP-dependent G:T incision events. The tumor HCT15 cell line, which is known to harbor deletion mutations in one of the human MutS gene homologues, *MSH6* (also denoted as GTBP), is deficient in in vitro repair of heteroduplexes containing all eight types of single base mismatches (*14*, *21*, *22*, *23*). The HCT116 contains no functional *MLH1* (one of the human MutL homologues) protein due to a single base substitution mutation in cognate gene (*14*, *24*). Defects in mismatch repair genes and loss of mismatch repair activity correlates closely with the loss of each of the ATP-dependent G:T incision events on CpG/TpG mismatches in the same cell lines, that is, HCT15 and HCT116 (Figure 3, lanes 3 and 4). Moreover, when extracts of both cell lines were mixed, HCT15 (*MSH6* deficient) and HCT116 (*MLH1*deficient), they were involved in the limited functional complementation of each of the ATP-dependent incision events toward CpG/TpG, as evidenced by the presence of characteristic 20-mer, 21-mer, and 18-mer products (Figure 3, lanes 5−6). These findings led us to hypothesize that each of these ATP-dependent incision events observed in the HT29 extract may depend on one and the same MMR factors and may constitute a single multisite ATP-dependent G:T incision activity. The presence of the multisite incision activity (Figure 3, lanes 9, 10) in MMR$^-$ cell extracts from LoVo and DLD-1 cell lines is intriguing, given that LoVo and DLD-1 harbor defective MMR genes, respectively, *MSH2* and *MSH6* (*14*, *21*, *23*). However, the finding that the MMR$^-$ cells as a group do not lack the multiple G:T specific incision events shows a correlation between the multisite G:T incision activity and the ATP-dependent G:T incision activity that we reported earlier (*14*).

The identification of a multisite G:T incision activity proved to be very useful to further the characterization of

human ATP-dependent G:T incision activity (*14*). For example, the substrate competition on the multisite G:T incision activity existing in the HT29 extract showed that both the 45-bp DNA with a CpG/TpG mispair and a 45-bp DNA with ApG/TpT mispair acted as the substrates of the same incision activity (Figure 4). The former mismatch in DNA is recognized by purified TDG, whereas the latter mismatch is not a substrate of TDG (*12*−*13*). Taken together with strand incision data of Figure 5A,B, it can be concluded that the strand incision at ApG/TpT or CpG/TpG mismatch is initiated by a multisite G:T incision activity and not by TDG as reported earlier by others (*8*−*10*). More importantly, while CG/TG and AG/TT act as substrates of the same multisite G:T incision activity, the former is primarily incised by a TDG-like factor, and the latter is incised by a distinct G:T incision factor, as characterized by the 5′-labeled 20-mer and the 5′-labeled 18-mer fragments, respectively (Figure 5A,B). These results serve to advance our view that the multisite G:T incision activity depends on several distinct G:T-specific incision factors. Further identification of the novel ApG/TpT-specific incision factor is being carried out in our laboratory to ascertain its glycosylase or endonuclease behavior. We did not observe incision at ApG/TT mispair by the extract and ATP used in a previous study (*15*). The reason the ApG/TT is incised in this study may lie in the type of extracts used. Thus, the extract prepared by the method of Li and Kelly (*16*) with ATP and perhaps G:U competitor provided a relatively better system to detect the multisite G:T incision activity on ApG/TpT DNA (Figures 4 and 5), as compared with the Manley extract used earlier (*15*).

In summary, this study extends our earlier findings on the ATP-dependent G:T repair activity in cell extracts by describing a similar multisite G:T mismatch incision activity. We provide evidence that the strand-specific incision at G:T mispair is initiated by a multisite G:T incision activity that requires, among other proteins, several distinct mismatch-specific incision factors and several MMR recognition factors, as well as ATP as cofactor. Our finding of the two mismatches, namely, CpG/TpG and ApG/TpT, serving as substrates of a single multisite G:T incision activity distinguishes this activity from TDG (*10*−*13*). The CpG/TG is produced due to spontaneous deamination of m5C to T residue at a CpG site in eukaryotic DNA. Unlike the CpG, the ApG site is not a target of DCM, and ApG/TpT mismatch is thus indistinguishable from the products of the error-prone semiconservative DNA replication (*4*−*5*). The recognition of these two distinguishable G:T mismatches in DNA by a single multisite G:T incision repair activity is in good agreement with in vivo G:T mismatch repair data of Brown and Jiricny (*7*).

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