Site Specificity of Incisions at G:T and O^6-Methylguanine:T Base Mismatches in DNA by Human Cell-Free Extracts†

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ABSTRACT: Cell-free extract from human tumor cell line A1235 (lacking O^6-methylguanine-DNA methyltransferase) was employed to compare incision at G:T base mispairs with that at O^6-methylguanine (^{m6}G):T pairs at two different sites (sites 20 and 25) in 45-bp heteroduplexes. To study the effect of neighboring bases on the activity(ies), the base pair immediately 5′ to the mismatched G at each site was varied to provide four contexts: CpG:T, TpG:T, ApG:T, and GpG:T (and two analogous series for ^{m6}G:T pairs). At site 20, cell-free extract produced observable incision only in the 45-bp DNA with the G:T mispair in the CpG:T context, giving a product with incisions immediately 5′ and 3′ to the mismatched T. We observed incision of neither the strand containing the mismatched G nor the DNAs with the site 20 ApG:T, GpG:T, and TpG:T mismatches. By contrast, at site 25, incision specificity was different. All four G:T mismatched DNAs were incised, and the ApG:T-25, GpG:T-25, and TpG:T-25 DNAs were incised 1−3 bonds 3′ to the mismatched T, while similar in other respects to the CpG:T-25 DNA, which showed a pattern like the CpG:T-20 DNA. CpG:T-20 specific incision activity in the extract was strongly inhibited by both CpG:T (sites 20 and 25) DNAs, but at least 10-fold more poorly by DNAs with ApG:T-25 and GpG:T-25 pairs. When the G:T mismatches were replaced by O^6-methylguanine:T (^{m6}G:T) mismatches, the extract incised only DNAs with Cp^{m6}G:T and Tp^{m6}G:T pairs at both sites to nearly the same extent, but showed no detectable incision of DNAs having Ap^{m6}G:T or Gp^{m6}G:T pairs. Together, the results suggest that the human cell-free extract may process G:T base mismatches produced by spontaneous deamination of ^{m5}C and ^{m6}G:T pairs by more than one mechanism.

Base:base mismatches in DNA are produced by errors during semiconservative DNA replication, during recombination, and by spontaneous hydrolytic deamination of 5-methylcytosine in DNA (Duncan & Miller, 1980). Unrepaired base mismatches lead to high rates of spontaneous mutations in *Escherichia coli* (Glickman & Radman, 1980; Modrich, 1991). In humans, the cellular lack of a mismatch repair process has been linked to cancer predisposition (Leach et al., 1993; Parsons et al., 1993; Fishel et al., 1993; Papadopoulos et al., 1994; Bronner et al., 1994) as well as to elevated spontaneous mutation rates (Branch et al., 1993; Kat et al., 1993). In *E. coli*, the base mismatches produced during replication and recombination are repaired by the *mutHLSU* repair pathway (Modrich, 1991). In humans, an analogous pathway has been characterized *in vitro* (Holmes et al., 1990; Thomas et al., 1991) and genetically (Parsons et al., 1993; Kat et al., 1993; Palombo et al., 1994). Thus all eight possible base:base mismatches are restored to normal base pairs by this pathway, albeit with widely different efficiencies, in a long repair patch mode involving the removal of the mismatch and replacement of ca. 3000 nt (Wagner & Meselson, 1976).

Hydrolytic deamination of 5-methylcytosines produces only G:T base mismatches in DNA. In *E. coli*, these are repaired to G:C pairs by a very short patch (VSP) mismatch repair mechanism involving the functions of *vsr* and *pol1* genes (Hennecke et al., 1991). VSP repair shows strong site specificity to restore CC(A/T)GG sequences in which the internal C is normally methylated at its 5 position by the deoxycytidine methylase in *E. coli* K strains (Hennecke et al., 1991).

In mammalian cells, the CpG sequence is the target for deoxycytidine methylation (Razin & Riggs, 1980). Thus, the hydrolytic deamination of 5-methylcytosine at these sequences produces only CpG:T mismatches. It has been shown previously *in vivo* that G:T mispairs are repaired efficiently in a strand-specific manner in which the mismatched T is removed and the G:C pair is restored. However, repair of G:T mismatches *in vivo* was not restricted to G:T mismatches occurring within the CpG sequence context (Brown & Jiricny, 1987). Human cell-free extract can carry out the repair of G:T mispairs to G:C pairs in a short patch mode (Wiebauer & Jiricny, 1989, 1990; Neddermann & Jiricny, 1993). The repair is initiated by a thymine-specific DNA glycosylase (Wiebauer & Jiricny, 1990; Neddermann & Jiricny, 1993) followed by AP endonuclease (or AP lyase) action and DNA repair synthesis by DNA polymerase β (Wiebauer & Jiricny, 1990) and ligation. The thymine glycosylase activity of the human repair system has been purified as a 55-kDa protein (Neddermann & Jiricny, 1993). The sequence requirements reported for G:T mismatch repair activity by human extracts have been ambiguous (Sibghat-Ullah & Day, 1993; Griffin & Karran, 1993). One study showed that incision at a DNA G:T mismatch at one site occurred irrespective of the base just 5′ to the mismatched G, but that at another site, incision of a G:T mismatch in the GpG:T context was not detected

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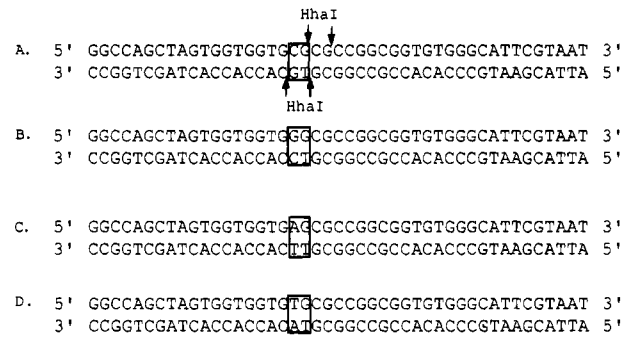

FIGURE 1: Structures of 45-bp DNAs with G:T mismatches at site 20. In A, a single G:T mispair located at site 20 and two *Hha*I sites in the DNA are shown by arrows. (*Hha*I can digest only DNA in which the mismatched T has been replaced by C.) Structures of the remaining DNAs, differing only in the base pair 5′ to the mismatched G, are shown in B−D.

(Sibghat-Ullah & Day, 1993). By contrast, in another DNA sequence, mismatches in the CpG:T context were preferentially incised relative to TpG:T, ApG:T, and GpG:T (Griffin & Karran, 1993).

In this study, we have assayed the incision step of repair of G:T base mismatches by characterizing the incision by cell-free extract of 45-bp DNAs having single G:T mispairs located in either of two sites on the DNA molecule. At each site, the base just 5′ to the mismatched G was altered to produce mismatches in four contexts: CpG:T, TpG:T, ApG:T, and GpG:T, creating eight total DNAs.

O^6-Methylguanine:T (^{m6}G:T[1]) pairs have been shown previously either to serve as substrates for a G:T mismatch type of incision (Sibghat-Ullah & Day, 1992; Griffin et al., 1994) or not to serve as such substrates (Leonard et al., 1990; Karran & Bignami, 1992). To study of the effect of O^6-methylation of guanine on mismatch incision, ^{m6}G:T mismatches were also constructed in all eight configurations.

EXPERIMENTAL PROCEDURES

Cell Extracts. Cell line A1235 was grown in cell culture in DMEM supplemented with 10% FBS at 37 °C (Sibghat-Ullah & Day, 1992). Freshly confluent cells were harvested, and extracts were prepared by the method of Manley et al. (1980) as previously described (Sibghat-Ullah et al., 1989). The extract was quick-frozen in aliquots in liquid nitrogen and stored at −80 °C. The protein concentration was ~10 mg/mL.

DNA Substrates. 45-mer DNAs were constructed following the oligonucleotide sequence first used by Voigt et al. (1989). Bases 10−35 are codons 7−15 of the normal c-*Hras*-1 gene. The 45-mer oligonucleotides, including the 5-methylcytosine-containing oligonucleotide, were synthesized by the DNA Synthesis Laboratory, Department of Microbiology, University of Alberta, by conventional phosphoramidite technology. For convenience, these strands are designated "top" or "bottom" according to their position in Figures 1 or 5. O^6-Methylguanine-containing oligonucleotides were prepared by the Regional DNA Synthesis Laboratory, University of Calgary, using ^{m6}G-phosphoramidite obtained from American Bionetics (Emeryville, CA) and deprotected with 10% 1,8-diazabicyclo[5.4.0]undec-7-ene for 2 weeks at room temperature as previously described (Sibghat-Ullah & Day, 1992). Individual 45-mer oligonucleotides were purified on 12% denaturing polyacrylamide gels followed by electroelution from gel slices. Control DNAs as well as the heteroduplexes shown in Figures 1 and 5 were prepared by mixing and annealing the appropriate strands in equal amounts as described (Sibghat-Ullah & Day, 1992, 1993). 5′-Terminal labeling (of individual strands prior to annealing) was achieved with the help of [γ-^{32}P]ATP and T4 polynucleotide kinase. DNAs were labeled in their 3′ termini using [α-^{32}P]cordycepin triphosphate and TdT as described previously (Sibghat-Ullah & Day, 1992).

Incision Assay and MGMT Treatment. DNA incision was performed by the method of Wiebauer and Jiricny (1989). Incision reactions (50 μL) were carried out by incubating 1−2 ng of 5′- or 3′-end labeled 45-bp DNA substrates with cell-free extract (5−20 μg of protein) in 20 mM Hepes, pH 7.9, 0.5 mM EDTA, 1 mM DTT, 0.01 mM $ZnCl_2$, and 50 mM KCl (incision reaction buffer) at 30 °C for 16−20 h. A total of 5−10 ng of non-mismatched double stranded DNA [45-bp, M13mp9 polylinker-based sequence; a non-mismatched, G:C version of the sequence in Figure 1A of Sibghat-Ullah and Day (1993)] was included in each reaction to reduce the nonspecific degradation of substrate DNA. Following the reaction, DNAs were extracted with phenol and chloroform and precipitated by ethanol. Incision products were analyzed by electrophoresis through 12% denaturing acrylamide gels. In one experiment, the DNAs (prior to being assayed for incision) were pretreated with *E. coli* MGMT (the 19-kDa portion of the *E. coli* Ada protein, a kind gift of Dr. Daniel Yarosh; Applied Genetics, Inc., Freeport, NY) following the protocol used previously (Sibghat-Ullah & Day, 1993).

Substrate Competition Experiments. 5′-Terminally labeled DNA substrate was mixed in incision reaction buffer with increasing concentrations of selected DNAs that were not radiolabeled. Following this, an aliquot of extract was added to initiate the reaction under the conditions of incision assay specified above. After an appropriate time of incubation, DNA was extracted and analyzed by electrophoresis as above. In some cases, the incision product bands were quantified using an LKB densitometer and XGELSCAN software in order to assess the inhibitory effect of individual substrates on the incision activity(ies) in the extract.

RESULTS

Incision of DNAs with G:T or ^{m6}G:T Base Pairs at Site 20. The 45-bp DNAs having single G:T mispairs preceded by different bases 5′ to the mismatched G at site 20 (including those shown in Figure 1A−D) were used as substrates in incision assays performed with cell-free extract from the A1235 cell line. All DNAs were labeled in the 5′ termini of the bottom strands (see Figure 1) containing the mismatched T. The result is shown in Figure 2. As shown in lanes 3 and 4, only DNAs containing either CpG:T or ^{m5}CpG:T base pairs yielded incision fragments (25 nt). Because the DNAs were labeled in the 5′ terminus of their bottom strands and the size of the incision product was 25 nt, we infer that an incision into the phosphodiester bond immediately 5′ to the mismatched T occurred during the reaction with extract. Incision of DNAs containing site 20 G:T mismatches in the ApG:T (lane 2), GpG:T (lane 5), or TpG:T (lane 6) contexts was not detectable under the

[1] Abbreviations: AP, abasic; m6G, O^6-methylguanine; MGMT, O^6-methylguanine-DNA methyltransferase.

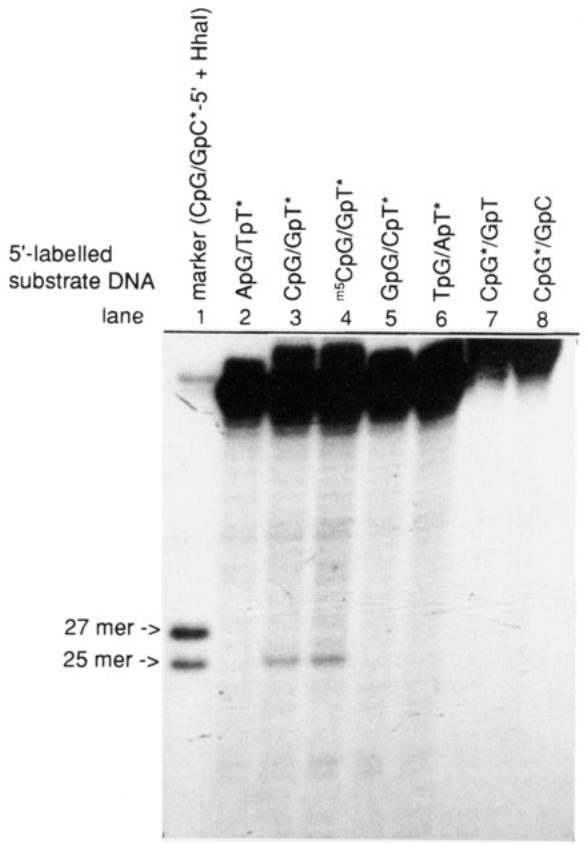

FIGURE 2: Incision of 45-bp DNAs containing single G:T mismatches at site 20 and 5′ terminally labeled. Cell-free extract (10 μg of protein) was incubated with 5′-terminally labeled DNAs, and samples were processed as described under Experimental Procedures. In lanes 2−6, DNAs with ApG:T, CpG:T, ^{m5}CpG:T, GpG:T, and TpG:T mismatches were labeled in the bottom (T-containing) strand (Figure 1) as designated by the asterisk. In lanes 7 and 8, DNAs with CpG:T and CpG:C pairs were labeled in the top strand. The marker sample electrophoresed in lane 1 was a *Hha*I digest of non-mismatched 5′-terminally labeled CpG:C* DNA.

conditions of the experiment. Similarly, incision of DNAs 5′-terminally labeled in the top strand (with or without a mismatched G) was not observable (lanes 7 and 8). Neither did we observe detectable incision of non-mismatched control DNAs having the four NpA:T or four NpG:C base pairs at site 20. (See DNAs 1−12 of Table 1, which summarizes the results of tests of DNAs for incision that were obtained in this study.)

When the incision assay was performed with DNAs labeled in their 3′ termini, the same two DNAs were incised by the extract (Figure 3, lanes 3 and 4). The 20 nt size of the 3′-labeled incision product was consistent with its length being 19 nt (after correction by subtracting the 1 nt added to the 3′ end by the cordycepin/TDT labeling protocol), which indicates that the bond immediately 3′ to the mismatched T was incised by the extract. As in the experiment in which the 5′ termini were labeled (Figure 2), incision was not observed in the ApG:T (lane 2), GpG:T (lane 5), or TpG:T (lane 6) DNAs. Incision of the control non-mismatched DNA having a G:C pair was also not detectable (lane 7). DNAs 3′-labeled in the top strand were not observed to serve as substrates (lanes 8 and 9).

All four DNAs in Figure 1 (A−D) were then prepared with ^{m6}G:T base pairs at site 20, as were the corresponding four controls having ^{m6}G:C pairs. These were labeled in the 5′ termini of their bottom strands and tested for incision by

Table 1: Incision of 40 DNAs with Base Pairs or Mismatches at Sites 20 and 25[a]

DNAs with natural bases			DNAs with m6G	
site 20	incision (5′ 25-mer)	competition (G:T incision)	site 20	incision (5′ 25-mer)
1. ApG/TpT	−	nd	25. Apm6G/TpT	−
2. CpG/GpT	+	strong	26. Cpm6G/GpT	+
3. TpG/ApT	−	nd	27. Tpm6G/ApT	+
4. GpG/CpT	−	weak	28. Gpm6G/CpT	−
5. ApG/TpC	−	nd	29. Apm6G/TpC	−
6. CpG/GpC	−	nd	30. Cpm6G/GpC	−
7. TpG/ApC	−	nd	31. Tpm6G/ApC	−
8. GpG/CpC	−	nd	32. Gpm6G/CpC	−
9. ApA/TpT	−	nd		
10. CpA/GpT	−	nd		
11. TpA/ApT	−	nd		
12. GpA/CpT	−	nd		
DNAs with natural bases			**DNAs with m6G**	
site 25	incision (5′ 20-mer)	competition (G:T incision)	site 25	incision (5′ 20-mer)
13. ApG/TpT	+	weak	33. Apm6G/TpT	−
14. CpG/GpT	+	strong	34. Cpm6G/GpT[c]	+
15. TpG/ApT	+	strong[b]	35. Tpm6G/ApT	+
16. GpG/CpT	+	weak	36. Gpm6G/CpT	−
17. ApG/TpC	−	nd	37. Apm6G/TpC	−
18. CpG/GpC	−	none[b]	38. Cpm6G/GpC	−
19. TpG/ApC	−	nd	39. Tpm6G/ApC	−
20. GpG/CpC	−	nd	40. Gpm6G/CpC	−
21. ApA/TpT	−	nd		
22. CpA/GpT	−	none		
23. TpA/ApT	−	nd		
24. GpA/CpT	−	nd		

[a] DNAs were prepared 5′-labeled in their bottom strands, and 2 ng (70 fm) of these was incubated with 10 μg of A1235 extract protein for 16 h and analyzed for incision as described in Experimental Procedures. A "+" indicates incision to extent of ∼2−3 fm product; "−" indicates that no detectable product was produced. The bases on the left of the "/" represent bases 19 and 20 (site 20) or bases 24 and 25 (site 25) of the top strands of the DNAs; the bases to the right are those bases in the bottom strand that would form base pairs with the designated top strand bases. [b] Sibghat-Ullah & Day (1993). [c] Strong competition for incision with G:T mismatched DNA (Sibghat-Ullah & Day, 1992); nd, not done.

the extract. The result is shown in Figure 4. Like the specificity for site 20 CpG:T mismatches in Figure 2, lane 3, the DNA that contained Cp^{m6}G:T pairs was a substrate for incision (Figure 4, lane 4). However, unlike the site 20 TpG:T DNA, Tp^{m6}G:T pairs at site 20 were substrates for the activity (Figure 4, lane 3). No incision of the DNAs in which the mismatched G was preceded 5′ by A or G was observed (Figure 4, lanes 2 and 5). Neither was incision observed after incubation of the four control DNAs having Np^{m6}G:C pairs with extract (Figure 4, lanes 6−9 and Table 1, DNAs 29−32).

Incision at G:T and ^{m6}G:T Pairs in DNAs with Mismatches at Site 25. Figure 5 (A−D) shows DNAs with G:T mismatches at site 25 and with each of the four DNA bases 5′ to the mismatched G. To compare incision of G:T mispairs with that of ^{m6}G:T pairs at site 25, these four DNAs as well as four DNAs with ^{m6}G replacing G at site 25 were all 5′-terminally labeled in their bottom strands and incubated with the extract and analyzed for incision products. The results are shown in Figure 6. Lanes 7−10 show that all four DNAs yielded fragments (20 nt) in the incision assay in accordance with an earlier report (Sibghat-Ullah & Day, 1993). Incubation of eight control DNAs without mismatches (NpG:C or NpA:T) with the extract gave rise to no

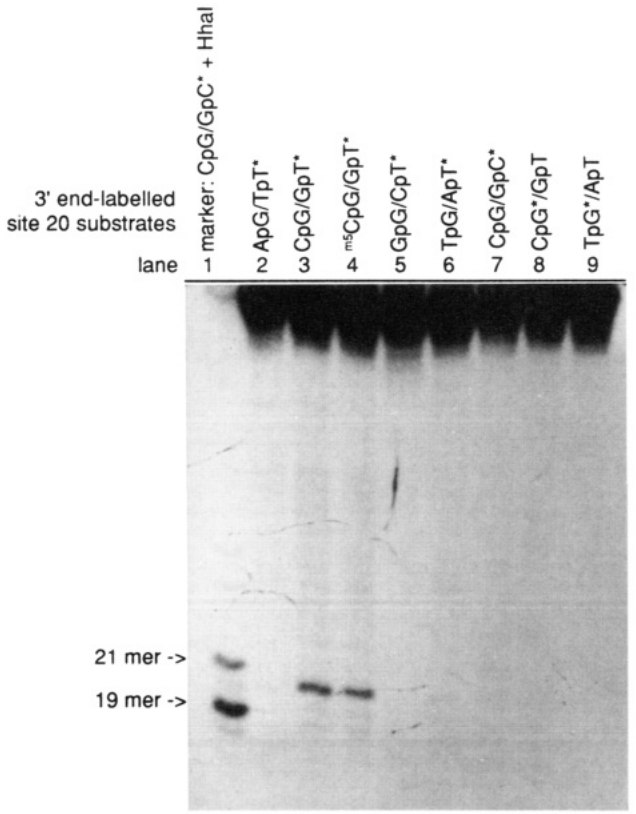

FIGURE 3: Incision of 45-bp DNAs containing single G:T-20 mismatches and 3′-terminally labeled. Cell-free extract (10 μg of protein) was incubated with DNAs shown, and the incision products were analyzed. Lanes 2−7 show DNAs with ApG:T, CpG:T, ^{m5}CpG:T, GpG:T, TpG:T, and CpG:C pairs labeled in the 3′ end of the bottom strand (designated by the asterisk) with cordycepin triphosphate and TDT and in the top strands of DNAs having CpG:T and TpG:T mismatches in lanes 8−9. (The 3′-labeling process adds 1 nt to the 3′ terminus.)

observable incision (Table 1, DNAs 17−24). Previous control experiments with the site 25 CpG:T substrate showed that incision of the strand containing the mismatched G was not detectable (Sibghat-Ullah & Day, 1992).

When G:T was replaced with ^{m6}G:T at site 25, only DNAs containing Cp^{m6}G:T and Tp^{m6}G:T pairs served as substrates in the incision reaction as shown by the production of the 20 nt fragment during incubation with extract (Figure 6, lanes 4 and 5). Neither DNA in which the mismatched G was preceded 5′ by A or G (lanes 2 and 3) showed evidence of being incised. These results are very similar to those obtained with the site 20 ^{m6}G:T mismatched DNAs in Figure 4. (The prior treatment of all four DNAs containing ^{m6}G:T pairs at site 25 with MGMT, to convert ^{m6}G to G, rendered all four substrates susceptible to incision as expected [data not shown].) Incubation with extract of the four control DNAs, in which C replaced the mismatched T in the bottom strand (Figure 6, lanes 1 and 6; Table 1, DNAs 37−40), gave rise to no observable incised product.

The mismatched DNAs shown in Figure 5 were then labeled in the 3′ termini of their bottom (mismatched T) strands and tested for incision by the cell-free extract as shown in Figure 7. Incision of the 3′-terminally labeled DNA having the CpG:T mismatch produced the single band 25 nt in length (24 after correction for the cordycepin added to the 3′ terminus as descibed in the preceding section) seen in lane 3. This length implies an incision site just 3′ to the mismatched T. But the incision of 3′-terminally labeled

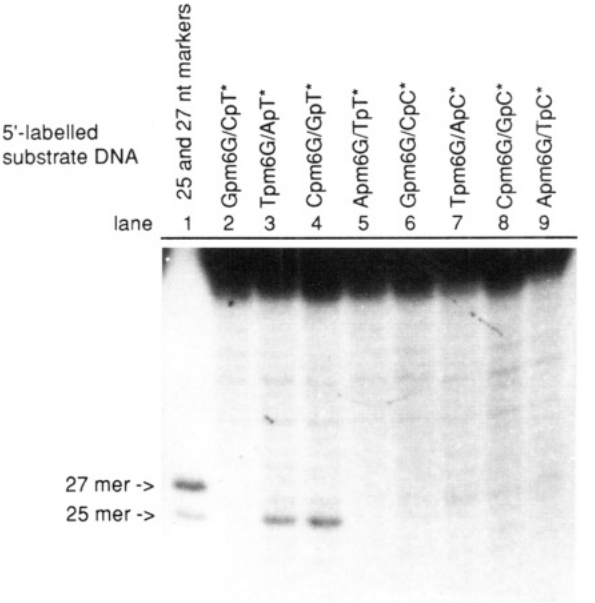

FIGURE 4: Incision of 45-bp DNAs containing ^{m6}G:T mispairs at site 20. Cell-free extract (10 μg of protein) was incubated with the DNAs shown in the figure, all 5′-terminally labeled in the bottom strand (as in Figure 1) as indicated (*). Reactions and analyses were carried out as described under Experimental Procedures. Lanes 2−5 show results obtained with DNAs having Gp^{m6}G:T, Tp^{m6}G:T, Cp^{m6}G:T, and Ap^{m6}G:T pairs. Lanes 6−9 show results obtained with the corresponding control DNAs having Np^{m6}G:C pairs. Incision products (25 nt) are indicated by an arrow.

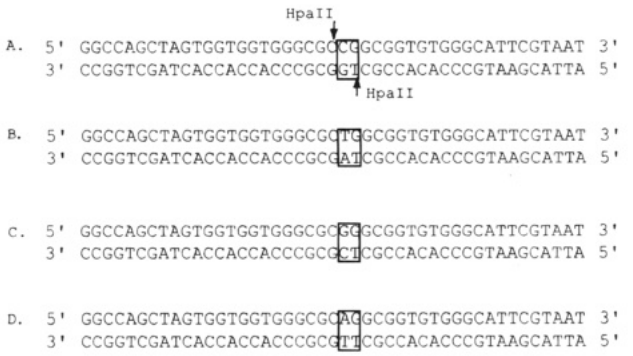

FIGURE 5: Structures of 45-bp DNAs having single G:T mismatches at site 25. DNAs with G:T mismatches at site 25 are shown in A−D. *Hpa*II sites that would exist in the DNAs if the mismatched T's were replaced by C's are shown by arrows.

DNAs containing TpG:T, GpG:T, and ApG:T mismatches (lanes 4−6) was accompanied by the production of 23 and 24 nt fragments in addition to the 25 nt product. These 23 and 24 nt fragments are not observed as incision products of DNAs having CpG:T mismatches either at site 25 (Figure 7, lane 3) or at site 20 (Figure 3, lane 3). By contrast, when the 5′ termini of the bottom strands were labeled, incision produced only a single 20 nt fragment from the GpG:T site 25 DNA (Figure 7, lane 1) as well as from the site 25 CpG:T DNA (lane 2).

Competition Experiments. We wished to determine whether the site-related incision activities in the extract could be inhibited by site-specific mispaired DNAs. For this purpose, unlabeled CpG:T-25, GpG:T-25, and GpG:T-20 and non-mismatched CpA:T-25 DNAs were allowed to compete for incision activity with CpG:T-20 DNA that had been 5′-terminally labeled in the bottom strand. The results of the experiment are shown in Figure 8. The data were quantified by densitometer, and best fit lines were drawn through the

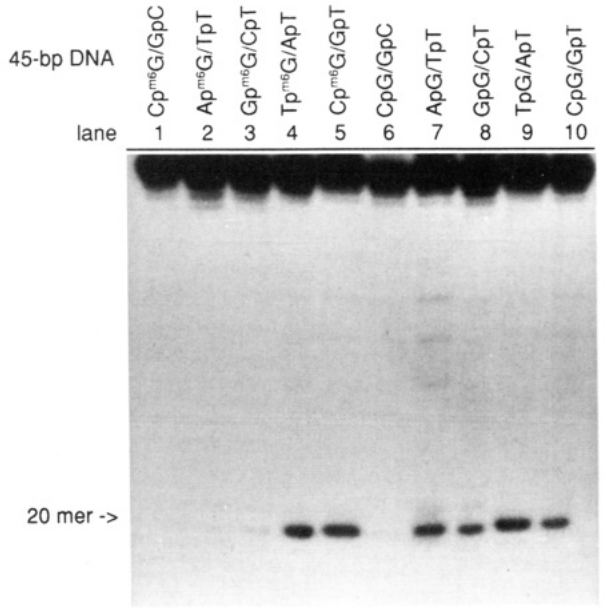

FIGURE 6: Incision of 45-bp DNAs containing G:T base mismatches at site 25. DNAs were labeled in their 5′ termini and incubated with cell-free extract (10 μg of protein). The incision reaction and analyses were carried out as described under experimental procedures. Lanes 1−5 show the results obtained with DNAs containing Cp^{m6}G:C, Ap^{m6}G:T, Gp^{m6}G:T, Tp^{m6}G:T, and Cp^{m6}G:T bases. Lanes 6−10 show results obtained with DNAs having structures shown in Figure 5. All DNAs were labeled in the 5′ termini of their bottom strands. The position of the 20-mer incision product is indicated by an arrow.

FIGURE 7: Incision of 45-bp DNAs containing G:T at site 25. Lanes 2−6 represent incision products from DNAs with G:T mismatches at site 25 in CpG:T, TpG:T, GpG:T, and ApG:T contexts. These DNAs were labeled in the 3′ termini of their bottom strands (as designated in Figure 5). The incision assays, employing 10 μg of extract protein, were performed as described in Experimental Procedures. For comparison, DNAs with GpG:T and CpG:T mismatches similar to the above but labeled in the 5′ termini of their bottom strands were used in the incision assay (lanes 1−2). Incision fragment sizes are indicated by arrows.

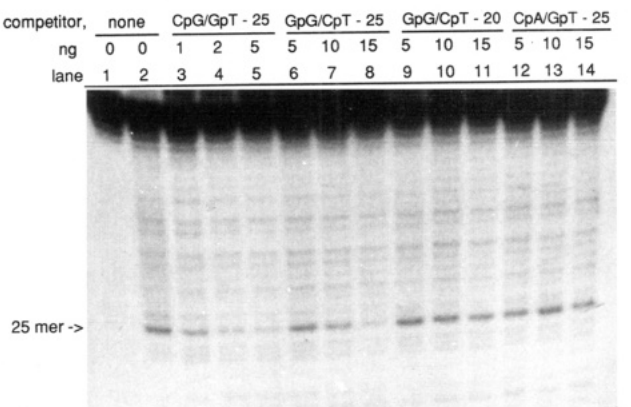

FIGURE 8: Inhibition of activity that incises DNA bearing a CpG:T mismatch at site 20. 45-bp DNA with a CpG:T mismatch at site 20 (Figure 1A) was labeled in the 5′ terminus of its bottom strand and mixed with increasing concentrations of unlabeled substrates. The incision assay was performed, and the products were analyzed as described in Experimental Procedures. Lanes 1 and 2 show the products of the incision reaction without and with extract (and without competing DNAs). Lanes 3−14 show products obtained in the presence of competing DNAs. Competing DNAs: lanes 3−5, CpG:T-25 (Figure 5A); lanes 6−8, GpG:T-25 (Figure 5D); lanes 9−11, GpG:T-20 (Figure 1B); (lanes 12−14) a non-mismatched control DNA CpA:T-25.

data to determine the extent of inhibition. Only the CpG:T-25 DNA competed strongly for the incision of CpG:T-20 activity (compare lane 2 with lanes 3−5; 50% inhibition at ~1 ng). The GpG:T-25 substrate was less inhibitory (lanes 6−8; 50% inhibition at ~10 ng), and the non-incised GpG:T-20 DNA was the least effective (lanes 9−11; 25% inhibition at ~15 ng), about as effective as the CpA:T-25 non-mismatched DNA. Inhibition by these same unlabeled DNAs was similar when labeled CpG:T-25 DNA was used as the substrate in this experiment (data not shown).

In a similar experiment, the incision of labeled GpG:T-25 DNA was only weakly inhibited by the ApG:T-25 or GpG:T-25 substrates (Figure 9, compare lane 2 with lanes 3−6). However, the activity was strongly inhibited by CpG:T-20 or CpG:T-25 DNAs (Figure 9; compare lane 2 with lanes 7−10). The results in Figures 8 and 9 led us to question whether the product was produced as rapidly from the GpG:T-25 DNA as from the CpG:T-25 DNA. The reaction with the CpG:T-25 DNA was shown previously to be slow and linear for 24 h (Sibghat-Ullah & Day, 1992). In an experiment in which the reaction products were assayed after 0, 2, 4, 6, 8, and 12 h of incubation at 30 °C, the reaction course was linear with time, and the amounts of product from the GpG:T-25 and CpG:T-25 substrates were equal to within a factor of 2 (data not shown).

DISCUSSION

Cell-free extract from the A1235 human malignant glioma cell line was used previously to study the sequence requirement of G:T mismatch activity (Sibghat-Ullah & Day, 1993). The prior investigation showed that incision at G:T mismatches was not affected by the base 5′ to the mismatched G in agreement with earlier results of an *in vivo* study (Brown & Jiricny, 1987). Here, we have extended our previous study by comparing the context requirement for G:T mismatch incision by A1235 extract at two DNA sites in the DNA substrate, sites 20 and 25.

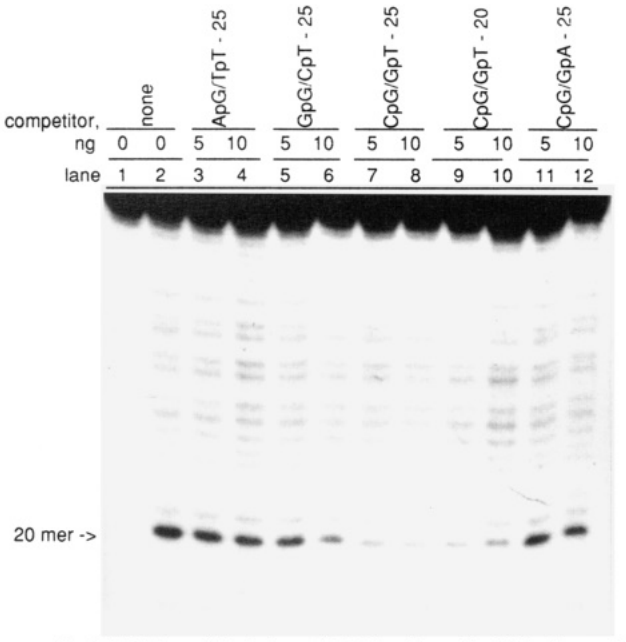

FIGURE 9: Inhibition of incision of DNA with a GpG:T mismatch at site 25. DNA having a GpG:T mismatch at site 25 (Figure 5D) was labeled in the 5′ terminus of its bottom strand (containing the mismatched T) and mixed with selected amounts of unlabeled DNAs. Extract (10 μg of protein) was added, and the incubations and analyses were performed as described in Experimental Procedures. Lanes 1 and 2 show the products of the incision reaction without and with extract (and without competing DNAs). Lanes 3−12 show the reaction products obtained in the presence of competing DNAs. Competing DNAs: lanes 3−4, ApG:T-25 (Figure 5C); lanes 5−6, GpG:T-25 (Figure 5D); lanes 7−8, CpG:T-25 (Figure 5A); (lanes 9−10) CpG:T-20 (Figure 1A); (lanes 12−14) a DNA with a G:A mismatch, CpG:A-25.

The cell-free extract showed mismatch incision activity at site 20 in a very restricted manner, i.e., incision only in the CpG:T sequence context, a specificity also determined in a recent study that employed a different DNA sequence as substrate (Griffin & Karran, 1993). The incision sites in that study and in our study of DNA with the site 20 mismatch were the bonds immediately 3′ and 5′ to the mismatched T. This pattern is a feature that is characteristic of an incision-initiating activity previously described by Wiebauer and Jiricny (1989, 1990) and purified by Neddermann and Jiricny (1993). By contrast to site 20, but in agreement with our previous work (Sibghat-Ullah & Day, 1993), DNA carrying a G:T mismatch at site 25 was incised in a manner independent of the base 5′ to the mismatched G. While CpG:T-25 DNA was incised similarly to the CpG:T-20 DNA, in that the incisions both 5′ and 3′ to the mismatched T were at single sites, the incision of ApG:T-25, GpG:T-25 and TpG:T-25 DNAs occurred at multiple sites 3′ to the mismatched T (and at a single site 5′ to the mismatched T). This broader activity may indicate that the abasic products from the latter three substrates have structures different from that of the former and that a different mode of AP endonuclease (or AP lyase and/or exonuclease) digestion may occur to produce the incised product. Alternatively, the properties such as specificity for CpG:T mismatches at site 20, but sequence independence at site 25, together with the different incision pattern 3′ to the mismatched T can be interpreted as favoring the idea that the cell extract has more than one activity that processes G:T mismatches in DNA.

To determine whether the activity that incises G:T mismatches at site 25 (independently of which base is 5′ to the mismatch) may be distinguished from an activity specific for CpG:T mismatches at both sites, we resorted to substrate competition experiments. The results demonstrated that CpG:T specific activity was much more strongly inhibited by DNA containing a G:T mismatch in a CpG:T context than by DNAs having ApG:T-25 or GpG:T-25 mismatches. This suggests weak protein binding to ApG:T and GpG:T site 25 mismatches relative to binding at CpG:T mismatches. However, the rate of product formation from CpG:T-25 and GpG:T-25 DNAs was very similar, so the weak binding does not influence overall reaction rate. By contrast, the activity interacts strongly with CpG:T-20 or CpG:T-25 DNAs. Because competition experiments assay binding to the competitor (not reactivity with the competitor), we cannot discern whether one or more glycosylases produce our results. Two activities may exist, one able to incise the ApG:T-25, GpG:T-25, and TpG:T-25 DNAs and possibly the CpG:T-25 DNA, and the other with reaction specificity limited to CpG:T-20 and possibly CpG:T-25 mismatches. To account for the competition data, both enzymes would have to have high affinity for both site 20 and site 25 CpG:T mismatches and relatively low affinity for the site 20 and site 25 GpG:T and/or site 25 ApG:T mismatches. Or one enzyme, having site-dependent incision either inherently or by virtue of complexing to the DNA through binding to site-specific G:T-mismatch binding proteins already attached to the DNA, could account for the data. G:T binding proteins have been found in human cell-free extracts (Stephenson & Karran, 1989; Jiricny et al., 1988; Hughes & Jiricny, 1992), one of which is the product of the human homologue of the *E. coli mutS* gene (Palombo et al., 1994). Fractionation of our activities will be required to answer this question definitively.

It is interesting that ^{m6}G:T base pairs at the two sites are incised with very similar specificity, i.e., only when the base 5′ to the ^{m6}G is a pyrimidine. This unique pattern could again be suggestive of multiple incision activities, one specific for ^{m6}G:T pairs and the other(s) for G:T pairs. We reported incision at ^{m6}G:T pairs in the Cp^{m6}G:T-25 context (Sibghat-Ullah & Day, 1992) following two reports that mismatch incision at ^{m6}G:T pairs did not occur (Leonard et al., 1990; Karran & Bignami, 1992; neither reported the structure of their substrate DNA). A more recent study that employed a DNA having a sequence different from ours reported that incision at CpG:T or Cp^{m6}G:T pairs occurred with strong preference over incision either at GpG:T, ApG:T, or TpG:T mismatches or Gp^{m6}G:T, Ap^{m6}G:T, and Tp^{m6}G:T pairs (Griffin et al., 1994). In that work, O^6-methylation of the mismatched G did not render the TpG:T site incisable. One explanation (among others) is that our extract preparation (Manley et al., 1980) may have contained more than one G:T mismatch incision activity, and the extract prepared (Li & Kelly, 1985) in the other study (Griffin et al., 1994) did not.

The observation that guanine O^6-methylation renders certain G:T mismatches refractory or accessible to incision by mismatch repair mechanisms may have more basic physicochemical underpinnings. Cotton et al. (1993) have compiled data showing that the T's of G:T mismatches in various DNA contexts are differentially oxidized by OsO_4 treatment. For example, of nine mismatches having the form

5′-NpGpG-3′/3′-N′pTpC-5′, corresponding to the site 25 structures used here (Figure 5), eight mismatched T's were reactive and one was not. Of five mismatches having the form 5′-NpGpC-3′/3′-N′pTpG-5′, corresponding to site 20 (Figure 1), which is incisable only when N:N′ is C:G, the reverse was true: all T's were unreactive. The authors argued that stacking forces, greatest for 5′-GpC-3′ and second greatest for 5′-GpT-3′ (Ornstein & Rein, 1978), would protect against oxidation. If accessibility to enzyme (or G:T binding proteins) paralleled susceptibility to OsO_4, O^6-methylation to produce 5′-Ap^{m6}GpG-3′/3′-TpTpG-5′ at site 25 would be supposed to increase the stacking forces in this scenario, thereby protecting the ^{m6}G:T pair against enzymatic incision. Such an increase in stacking forces upon O^6-methylation could be effected by the transition of G:T pairs in the wobble configuration (Kennard, 1985), to m6G:T pairs in the Watson−Crick configuration (Swann, 1990; Kalnick et al., 1989a,b; Leonard et al., 1990). The finding that unlabeled site 25 Cp^{m6}G:T DNA competes well for incision of site 25 CpG:T DNA (and vice versa; Sibghat-Ullah & Day, 1992) is consistent with the idea that the glycosylase that incises G:T mismatches also incises ^{m6}G:T mismatches. If this turns out to be so, then the effect on mismatch incision of O^6-methylating the G involved in a G:T mismatch would presumably be caused by the altered conformational and/or energetic states in the substrate. In summary, our data show that there is much that remains to be understood about the substrate and sequence specificity of G:T-type mismatch repair systems in human extracts.

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