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ARTICLE *in* BIOCHEMISTRY · AUGUST 2002

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Substrate Specificity and Sequence Preference of G:T Mismatch Repair: Incision at G:T, O^6-Methylguanine:T, and G:U Mispairs in DNA by Human Cell Extracts†

Sibghat-Ullah Lari,* Fahad Al-Khodairy, and Malcolm C. Paterson

Department of Biological and Medical Research (MBC 03), King Faisal Specialist Hospital and Research Center (KFSH&RC), P.O. Box 3354, Riyadh 11211, Saudi Arabia

Received March 26, 2002; Revised Manuscript Received May 15, 2002

ABSTRACT: Extracts of two human glioma cell lines (lacking O^6-methylguanine DNA-methyltransferase) (i.e., A1235 and its alkylation-resistant derivative A1235-MR4) were examined for their ability to execute strand incision at different base mismatches in model (45-bp) DNA. These heteroduplex substrates were of the same sequence except for the presence, at the same site, of one of three mispairs: G:T, O^6-methylguanine:T (m6G:T), and G:U. The parental (A1235) extract, when supplemented with ATP and human thymine DNA glycosylase (TDG), acted proficiently on all three substrates, incising immediately 5′ to the mismatched thymine or uracil residue. In contrast, the derivative extract, under the same conditions, recognized only the G:U substrate. The activity of the A1235 extract toward the G:T (or m6G:T) substrate was markedly reduced in the absence of ATP, whereas the G:U substrate was incised rapidly by both extracts irrespective of the addition of ATP. These combined data confirm and extend our earlier findings demonstrating that human cells possess two G:T incision activities, one efficient and ATP-dependent and the other inefficient and ATP-independent. The derivative extract lacks the former activity but retains the latter activity. In substrate competition assays, the G:U substrate inhibited the ATP-dependent G:T incision activity to a greater extent than did the G:T substrate itself. Given the well-known substrate preference of TDG for G:U as compared to G:T, this unexpected result implies that TDG may be an integral component of the ATP-dependent G:T incision machinery in human cells. Finally, the base 5′ to the mismatched G in the G:T mispair conferred sequence preference on the A1235 extract in the presence of ATP and TDG, with a pyrimidine (especially cytosine) being much favored over a purine. This latter observation suggests that the ATP-dependent G:T incision activity is designed to repair deaminated 5-methycytosine lesions in CpG islands, the methylation of which is linked to control of gene expression.

G:T base mismatches are produced by the spontaneous hydrolytic deamination of m5C[1] in DNA (*1*). *Escherichia coli* strain K12 possesses a specific repair process designed to recognize the mutagenic thymine in a G:T mismatch and to restore the site to a normal G:C pair. This process is carried out in part by the combined actions of endonuclease *vsr* and DNA pol I gene products (*2*, *3*). An analogous repair pathway exists in humans (*4*−*6*). In vitro studies have suggested that a TDG activity initiates G:T mismatch repair, catalyzing the release of the mismatched thymine base (*4*, *5*). The phosphodiester bonds immediately 5′ and 3′ to the resultant AP site are then cleaved by a dual backbone-incision reaction, initiated by AP endonuclease 1, thus generating a one-nucleotide gap in one strand (*7*); the complementary strand containing the mismatched guanine is not severed. Subsequently, a cytosine base is inserted opposite the guanine by DNA pol β, and finally, a DNA ligase seals the opening so as to reinstate a normal G:C pair in the double helix (*7*). Nedderman and Jiricny (*8*) and Hang et al. (*9*) independently purified a 55-kDa protein from human cells that exhibits the aforementioned TDG activity, and the cognate cDNA has been subsequently isolated (*10*). The purified human TDG protein shows a distinct preference for a G:T mismatch occurring in a 5′-CpG dinucleotide (i.e., a G:T mismatch with cytosine 5′ to the mismatched guanine (CpG:T)), raising the distinct possibility that TDG participates in the repair of G:T mismatches arising via spontaneous deamination of m5C to thymine (*11*, *12*). MBD4, a m5CpG sequence-binding protein, exhibits an activity similar to that of TDG and interacts with the general mismatch repair protein MLH1 (*13*, *14*). A highly purified preparation of TDG also displays affinity for a G:U mismatch resulting from the spontaneous deamination of cytosine. In fact, TDG appears to be much more efficient at removing uracil from a G:U mispair than in releasing thymine from a G:T mispair (*12*, *15*). It is therefore reasonable to postulate that, under normal circumstances, TDG, by itself, may not be primarily responsible for initiating correction of G:T mispairs to G:C base pairs in human cells (*8*−*12*). Instead, the first step in G:T mismatch repair in vivo may be performed by a multihet-

† This work was supported by Research Proposal No. 2000 006 from the Research Advisory Council, KFSH&RC, Saudi Arabia.

* To whom correspondence should be addressed. Phone: 966-1-464-7272 ext. 31752. Fax: 966-1-442-7858. E-mail: lari@kfshrc.edu.sa.

[1] Abbreviations: m5C, 5-methylcytosine; TDG, thymine DNA glycosylase; AP, apurinic or apyrimidinic; m6G:T, O^6-methylguanine:T; *MLH1*, human homologue of yeast MutL gene; MGMT, O^6-methylguanine DNA-methyltransferase; MNNG, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine; UDG, uracil DNA glycosylase; G:T, m6G:T, or G:U substrate, denoting a model 45-bp DNA containing a single G:T, m6G:T, or G:U mispair, respectively, at a specific site; m6G:C, O^6-methylguanine:C.

10.1021/bi020239n CCC: $22.00
Published on Web 06/21/2002

eromeric complex of proteins comprising several mismatch-recognition factors including, for example, MBD4, and possibly TDG as well (*10*, *13*, *16*). In short, TDG, when acting alone, may be relegated to a "back-up" role.

In keeping with the aforementioned prediction, we have recently provided evidence that the repair of G:T mismatches in human cells may be initiated by a reaction distinct from that mediated solely by purified human TDG (*17*). This evidence was obtained by monitoring the initiation of strand incision at an m6G:T mismatch in a model (45-bp) DNA substrate after the addition of purified TDG and ATP to extracts of the human glioma cell line A1235 (lacking MGMT) and the alkylation-tolerant derivative cell line A1235-MR4 (in addition to lacking MGMT, also resistant to killing by MNNG and phenotypically reminiscent of colon carcinoma cell lines harboring a deficiency in one or more known mismatch repair factors (*16*, *17*)). Augmentation of the A1235 cell extract with TDG, along with ATP, led to a severalfold increase in m6G:T incision activity, suggesting a stimulatory interaction between the parental cell extract and TDG. In contrast, substitution of the A1235 cell extract with the A1235-MR4 cell extract in an otherwise identical reaction mixture was found to inhibit incision totally. Intriguingly, when both ATP and TDG were omitted from the reaction mixture, the time course of incision at m6G:T sites was indistinguishable for the parental (A1235) and derivative (A1235-MR4) cell extracts (*17*). These unforeseen findings prompted us to hypothesize that human cells contain at least two m6G:T (or G:T) incision activities (i.e., a "TDG-like" ATP-independent activity and an ill-defined ATP-dependent activity (*17*)). Our ongoing attempts to identify the precise mismatch recognition factor(s) comprising the ATP-dependent m6G:T (or G:T) incision complex and the substrate specificity of this complex have met with limited success thus far, perhaps due to either dissociation or inactivation of the putative factors during cell extract fractionation.

In this paper, we have examined the effects of different base mismatches, and of the specific base pair 5′ to the site of the respective mismatches in the model (45-bp) DNA substrate used in our earlier studies (*17*, *18*), on the initiation of strand incision by A1235 and A1235-MR4 cell-free extracts (henceforth denoted as A1235 and A1235-MR4 extracts, respectively). In brief, model duplexes containing a single G:T, m6G:T, or G:U mispair at one and the same site each served as a suitable substrate for lesion-recognizing incision activities present in the A1235 extract. In contrast, the A1235-MR4 extract operated on a G:U mispair but failed to act on either a G:T or m6G:T mispair. Unlike that observed for a G:T mispair, the kinetics of incision at G:U in the model substrate was reminiscent of that of the well-delineated TDG-like incision activity, including the observed inability of the addition of ATP to the A1235 extract to stimulate incision at a G:U site. Together, these data offer further support for the existence of two G:T (or m6G:T) incision activities in human cells. In addition, the efficiency at which the A1235 extract incised at a G:T (or m6G:T) site was enhanced considerably if the mismatched guanine (or m6G) was flanked on the 5′ side by a pyrimidine (notably cytosine) as opposed to a purine base, signifying a degree of sequence preference for the ATP-dependent mode of G:T incision activity.

FIGURE 1: Basic structure of the prototype (45-bp) DNA homoduplex from which all base mismatch-containing DNA substrates used herein were derived. The location, corresponding to 21 nt in the bottom strand, of the G:T, m6G:T, and G:U mismatches in the respective model substrates is denoted in boldface.

MATERIALS AND METHODS

Preparation of Cell-Free Extracts and Enzymes. As alluded to previously, the human extracts used here were prepared from the glioma tumor-derived cell line A1235 and its MNNG-resistant derivative line A1235-MR4. The origins and pertinent properties of these two lines have been described elsewhere (*17*, *19*). Both lines were kindly supplied by Rufus S. Day III (University College of the Cariboo, Kamloops, BC, Canada). After seeding in 150-mm dishes, cells were cultivated in Ham's F12 medium supplemented with 15% fetal bovine serum, 50 U/mL penicillin, and 50 mg/mL streptomycin. Cultures were routinely incubated at 37 °C in a humidified atmosphere of 95% air/5% CO_2. All culture supplies were purchased from Gibco Laboratories (Grand Island, NY). For each cell line confluent cultures were harvested from 10 dishes by conventional trypsinization. Extracts (each equivalent to $(2-5) \times 10^8$ cells) were prepared by the procedure of Manley et al. (*20*). Each extract, containing 20 mg of protein/mL, was divided into 40 aliquots, which were immediately snap-frozen in liquid nitrogen and stored at −80 °C. Human recombinant TDG was generously provided by Dr. Josef Jiricny (Institute of Medical Radiobiology, Zürich, Switzerland). T4 polynucleotide kinase was supplied by Pharmacia Biotech (Piscataway, NJ) and DNA Pol I (Klenow fragment) was furnished by Boehringer Mannheim (Mannheim, Germany).

Oligonucleotides and DNA Duplexes: Preparation and End-Labeling. A total of 21 oligonucleotides, each 45 nt in length and modeled after the *c-Hras-1* base sequence originally constructed by Voigt et al. (*21*), were employed in this study (Figure 1). Sixteen oligonucleotides, including those containing a mismatched thymine or uracil, were prepared by the DNA Synthesis Laboratory, Department of Microbiology, University of Alberta (Edmonton, AB, Canada), adopting conventional β-cyanoethyl phosphoramidite methodology. The remaining five oligonucleotides, each harboring a m6G residue, were prepared by the Regional DNA Synthesis Laboratory, University of Calgary (Calgary, AB, Canada). The latter polymers were constructed from m6G-phosphoramidite (American Bionetics, Emeryville, CA) and were deprotected with 10% 1,8-diazabicyclo [5.4.0] undec-7-ene for 2 weeks at ambient temperature (*18*). Individual polymers were purified by 12% denaturing polyacrylamide gel electrophoresis and subsequent electroelution from gel slices. DNA substrates, both homoduplex (normal base pair-containing) and heteroduplex (base mispair-containing), were routinely prepared by mixing and annealing equal quantities of the desired strands (*18*). For referral purposes, the two strands of a given DNA substrate are denoted "top" and "bottom" depending upon their location in Figure 1. When required, either strand of a 45-bp DNA substrate was labeled

at its 5′ terminus by incubation with T4 polynucleotide kinase and [γ-^{32}P]-ATP prior to annealing of its complementary strand (*18*). Substrates containing a radioactive label at the 3′ terminus of the bottom strand were generated with the aid of DNA 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. The 44-mer bottom strand was annealed to a complementary 45-mer top strand prior to carrying out the DNA Pol I reaction. The concentration of individual unlabeled oligonucleotides was determined spectrophotometrically at a wavelength of 260 nm, whereas the concentration of labeled oligonucleotides was approximated assuming 100% recovery from gel purification and ethanol precipitation of the electroeluted material.

ATP-Dependent G:T Mismatch Incision Assay. This incision assay was performed as described earlier (*17*). Briefly, the reaction mixture (total volume, 50 μL) contained cell-free extract (20 μg of protein), 5′ end-labeled and base mismatch-containing heteroduplex substrate (2 ng), 20 mM Hepes (pH 7.9), 50 mM NaCl, 0.5 mM EDTA, 2 μM ATP, 1 mM $MgCl_2$, 0.01 mM $ZnCl_2$, 40 μg phosphocreatine, 1 U creatine phosphokinase, and 100 μg/mL BSA. Unlabeled 45-mer DNA containing a normal G:C pair (5 ng) was added to act as a competitive substrate for any nonspecific endonucleolytic activity toward G:T- and m6G:T-containing DNAs. Human recombinant TDG protein (1−2 ng) was added to the reaction mixture, wherever mentioned, for augmenting G:T incision activity. The reaction mixture, when performed with G:U-containing substrate, was augmented with unlabeled A:U-containing DNA (10 ng) to block nonspecific UDG activity. Each reaction was routinely carried out at 30 °C for 0−4 h, and the DNA reaction products were analyzed by 12% sequencing gel electrophoresis followed by autoradiography on Kodak X-ray film (*18*).

ATP-Independent G:T Mismatch Incision Assay. In short, the reaction mixture (50 μL) contained cell-free extract (10−20 μg of protein), 5′ terminus-labeled 45-bp DNA containing a base mispair (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. As noted previously, the reaction mixture, when conducted with G:U-containing substrate, was supplemented with excess unlabeled A:U-containing DNA (10 ng), thus competing effectively for nonspecific UDG activity while at the same time not affecting TDG activity (*22*). The reaction mixture was incubated at 30 °C for appropriate times ($\leq$2 h), and the DNA reaction products were analyzed as indicated previously.

RESULTS

Intrastrand Incision of Base Mispair-Containing Substrates by Cell Extracts. As a prelude to gaining insight into the substrate specificity of the two human G:T mismatch incision activities, three model DNA substrates, each containing a single base mismatch (i.e., m6G:T, G:T, or G:U), were first constructed to our specifications (Figure 1). These three substrates are known to be recognized by purified TDG (*11*, *12*) and are hereafter abbreviated simply as G:T, m6G:T, and G:U substrates, respectively. Each substrate was incubated in turn with the A1235 and A1235-MR4 extracts supplemented with purified TDG and ATP so as to monitor maximal mispair-specific strand incision by the ATP-dependent G:T repair activity (see ref *17* for details). As illustrated in Figure 2A (lanes 1−3), all three model substrates, when labeled at the 5′ end of the bottom strand, were incised by the A1235 extract, yielding a 20-mer fragment of the bottom strand on a sequencing gel. The generation of this common reaction product is consistent with extract-induced cleavage immediately 5′ to the mismatched T or U, depending on the substrate under study. Incidentally, when the m6G:T substrate was incubated with the A1235 extract, the amount of 20-mer product was not enhanced by adding the four normal dNTPs to the reaction mixture, suggesting that (i) ATP serves as the sole cofactor in mediating the strand incision reaction and (ii) this activity is not dependent upon the execution of the subsequent gap-filling step. The m6G-containing strand in the m6G:T substrate was not cleaved by the A1235 extract, since the substrate, when labeled at the 5′ end of the top strand, remained intact upon analysis of the reaction products (Figure 2A, lane 4). The G:T and m6G:T substrates proved refractory to strand fragmentation when the A1235 extract was replaced with the derivative A1235-MR4 extract in the reaction mixture (lanes 5 and 6). In contrast, the G:U substrate was readily incised, as revealed by the appearance of 20-mer comparable in yield to that produced by the parental extract (compare lanes 3 and 7). As expected, all three substrates, when incubated without any extract, did not give rise to smaller fragments (lanes 8 and 9; unpublished data). The observed incision at G:T and m6G:T mismatches by the A1235 extract but not the A1235-MR4 extract is compatible with the existence of an ATP-dependent G:T mismatch incision activity that is present in the former (parental) but absent from the latter (derivative) extract. The observation that strand incision at the G:U mismatch by the A1235-MR4 extract occurs with kinetics indistinguishable from that of the A1235 extract argues against the involvement of the ATP-dependent G:T incision activity in this incision reaction. Instead, the fact that the addition of excessive quantities of A:U-containing DNA (i.e., substrate of UDG but not TDG) to a reaction mixture containing the G:U substrate with either extract still yielded a 20-mer fragment in normal amounts suggests that initiation of the incision reaction may involve G:U/G:T-specific TDG rather than UDG. This suggestion is further supported by our unpublished finding that incision at mismatch sites was much more competitively inhibited by either unlabeled G:U- or G:T-containing DNA in comparison to unlabeled A:U-containing DNA. The presence of several minor bands (i.e., 19-, 18-, and 17-mers of decreasing intensity) in lanes 3 and 7 deserves mention. These bands vary considerably in intensity from one extract preparation to another and presumably arise from attack on the major (20-mer) product by a nonspecific 3′→5′ exonuclease.

To characterize further the ATP-dependent G:T incision activity and the accompanying enzymatic removal of the mispaired dTMP nucleotide from the model heteroduplex, we proceeded to measure the lengths of the two extract-generated strand fragments. To this end, two radioactive m6G:T substrates were prepared, one containing the label at the 5′ terminus and the other at the 3′ terminus of the bottom strand, and each substrate was incubated with the

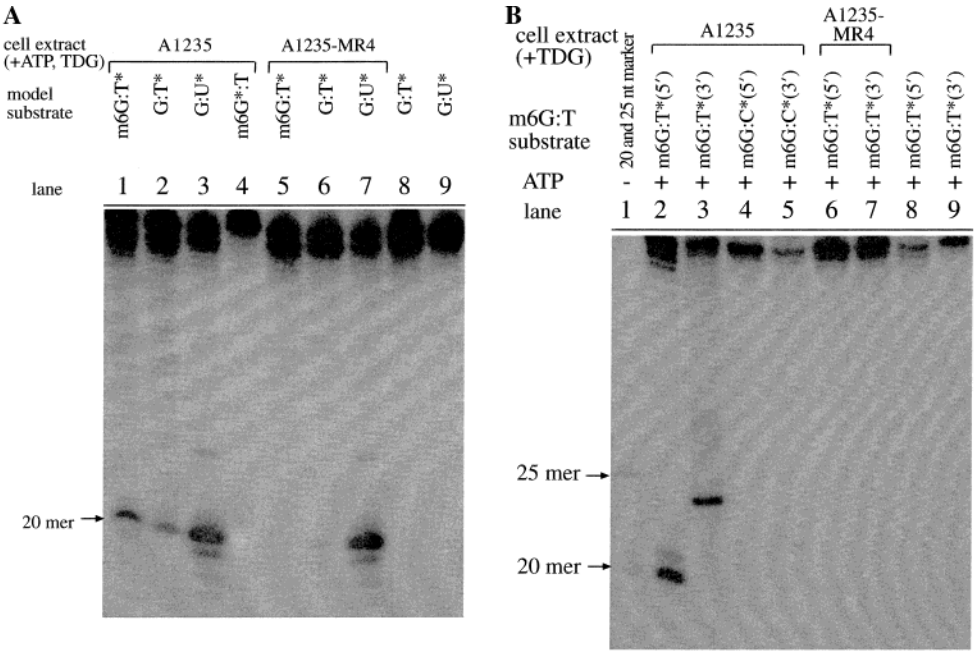

FIGURE 2: (A) Strand incision at base mismatches in the m6G:T, G:T, and G:U substrates by human cell-free (A1235 and A1235-MR4) extracts. Extract, heteroduplex substrate, purified TDG, and ATP were incubated in the ATP-dependent G:T incision assay buffer, and the reaction products were analyzed as described in Materials and Methods. Lanes 1 and 5, 2 and 6, and 3 and 7 correspond to incision of the m6G:T, G:T, and G:U substrates, respectively, each labeled at the 5′ end of the bottom strand (indicated as m6G:T*, G:T*, and G:U*). (Lane 4) Incision of the m6G:T substrate labeled at the 5′ terminus of the top strand (indicated as m6G*:T). (Lanes 1−4) A1235 extract-treated substrates. (Lanes 5−7) A1235-MR4 extract-treated substrates. (Lanes 8 and 9) Sham-treated substrates. (B) Incision sites in the m6G:T substrate. The A1235 or A1235-MR4 extract, supplemented with TDG and ATP, was incubated with the m6G:T substrate in order to conduct the ATP-dependent G:T incision assay as described in Materials and Methods. (Lane 1) 20- and 25-mer markers, which were obtained by *Hpa*II digestion of the 45-bp DNA containing a normal G:C pair, labeled at the 5′ and 3′ termini (bottom strand), respectively. (Lanes 2 and 6) Incision of the m6G:T substrate labeled at the 5′ end of the bottom strand (designated as m6G:T* (5′)). (Lanes 3 and 7) m6G:T substrate, labeled at the 3′ end of the bottom strand (designated as m6G:T* (3′)). (Lanes 2−5) DNA substrates incised by the A1235 extract. (Lanes 6 and 7) DNA substrates treated with the A1235-MR4 extract. (Lanes 4 and 5) Incision of the 45-bp DNA containing a single m6G:C instead of m6G:T and labeled at the 5′ and 3′ termini (bottom strand) (denoted as m6G:C* (5′) and m6G:C* (3′)), respectively. (Lanes 8 and 9) Sham-treated DNA.

A1235 and A1235-MR4 extracts supplemented with TDG and ATP. Figure 2B vividly demonstrates that the A1235 extract (lane 2), but not the A1235-MR4 extract (lane 6), acted on the 5′ end-labeled substrate of the bottom strand to produce a 20-mer fragment, along with a 21-mer as a minor product. Similarly, the 3′ end-labeled m6G:T substrate yielded a 24-mer fragment when treated with the parental cell line extract (lane 3), whereas no fragment was observed in the presence of the derivative cell line extract (lane 7). A model substrate containing a m6G:C mismatch (i.e., replacement of thymine with cytosine at the mispair site) was not incised in the bottom strand by the A1235 extract (lanes 4 and 5) and neither strand of the m6G:T substrate was cleaved when this model substrate was sham-treated (i.e., incubated in the absence of any extract) (lanes 8 and 9; unpublished data). The observed sizes of the 5′ and 3′ end-labeled incision fragments of the bottom strand of the m6G:T substrates (i.e., 20- and 24-mer, respectively) under our electrophoresis conditions suggest that the sequence of repair steps involving the ATP-dependent G:T incision activity may produce two intrastand incisions, one immediately 5′ and the other 3′ to the mismatched thymine in the m6G:T substrate, thus creating a one-nucleotide gap. This result resembles that of the combined actions of human TDG and associated AP endonuclease 1, which also produce dual incisions in mismatch-containing DNA (*7*, *10*, *18*). The presence of a minor but significant amount of a 21-mer fragment from the 5′ end-labeled bottom strand (lane 2) presumably reflects the action, to a limited extent, of the subsequent (gap-filling) repair step in which a nucleotide has been added opposite to the m6G residue in the top strand.

Comparative Incision Kinetics at G:T and G:U Mispairs by the Two G:T Incision Activities. To detail further the distinct substrate preferences of the two G:T incision activities, we elected to determine the relative rates of strand incision introduced into the G:T and G:U substrates by the A1235 extract, first without and then with ATP added to the reaction mixture. As illustrated in Figure 3A (lanes 1−4), the G:T substrate, labeled at the 5′ terminus of the bottom strand, proved to be a poor substrate for the A1235 extract in the absence of the supplementary ATP, as only a small amount of 20-mer fragment was in evidence even when the reaction was carried out for 120 min. In contradistinction, in the presence of ATP, the parental extract incised the G:T substrate, with the yield of 20-mer product increasing steadily from 30 to 120 min incubation (lanes 5−7). These findings unequivocally demonstrate that the G:T mismatch-containing duplex serves as a suitable substrate for the ATP-dependent, but not the ATP-independent, G:T incision activity residing in the A1235 extract. Parallel data for the G:U substrate, also labeled at the 5′ terminus of the bottom strand, are presented in Figure 3B. In addition to being independent of ATP, the kinetics of extract-induced incision events at the G:U base mismatch was both rapid and robust, nearing completion after only 30 min, such that the yield of 20-mer product after this short incubation time in the absence of

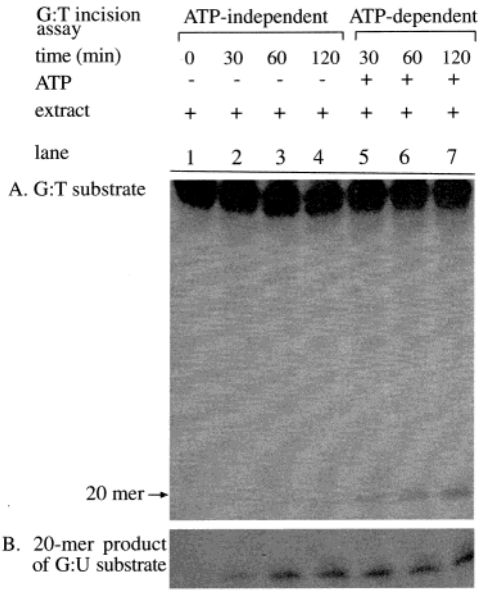

FIGURE 3: Kinetics of incision of the G:T and G:U substrates by assaying the ATP-independent and -dependent G:T mismatch repair activities. (Panel A) G:T substrate, labeled at the 5′ end of the bottom strand, was incubated with the A1235 extract for the times indicated in the absence (lanes 2−4) or presence of supplementary ATP (lanes 5−7). (Panel B) G:U substrate, labeled at the 5′ end of the bottom strand, was incubated with the A1235 extract in the absence (lanes 2−4) or presence of ATP (lanes 5−7). In both panels, the reaction products were analyzed as described in Materials and Methods. Incision fragment, a 20-mer, is denoted by an arrow. Lane 1 shows sham-treated G:T and G:U substrates.

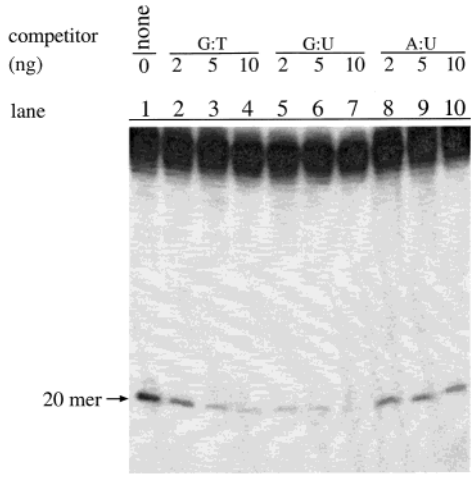

FIGURE 4: Inhibition of ATP-dependent incision of the G:T substrate by the addition of unlabeled competitor mismatch-containing substrates. Aliquots (2 ng each) of G:T substrate (labeled at the 5′ terminus of the bottom strand) were mixed with indicated amounts of unlabeled G:T, G:U, and A:U heteroduplexes, respectively, in the ATP-dependent G:T incision assay buffer, after which the incision reaction was carried out upon the addition of the A1235 extract and ATP (see Materials and Methods for details). (Lane 1) No competitor. (Lanes 2−4) Varying amounts of unlabeled G:T substrate. (Lanes 5−7) Varying amounts of G:U substrate. (Lanes 8−10) Varying amounts of A:U substrate. (G:T incision product) A 20-mer is identified by an arrow.

ATP was substantially greater than that found for the G:T substrate, even after 120 min in the presence of ATP. Accordingly, the G:U mispair site appears to be a model lesion for the ATP-independent incision activity in the A1235 extract, an observation consistent with earlier reports (*12*, *15*).

Competitive Inhibition of the ATP-Dependent Incision at G:T Mispairs by G:U and A:U Mispairs. We next examined the ability of substrates containing each of the three mispairs, namely, G:T, G:U, and A:U, to inhibit competitively the ATP-dependent incision of the G:T substrate by the A1235 extract in order to ascertain the lesion specificity of the ATP-dependent G:T incision activity. Thus, the G:T substrate, labeled at the 5′ end of the bottom strand, was incubated with increasing quantities (0−10 ng) of each of the three unlabeled base mismatch-containing substrates in the presence of extract and ATP. As can be seen in Figure 4 (lane 1), the labeled G:T substrate, when treated with the extract and ATP in the absence of any unlabeled competitive substrate, yielded a significant amount of 20-mer fragment. When the quantity of unlabeled G:T substrate in the reaction mixture was increased from 2 to 10 ng, the yield of 20-mer product decreased gradually (lanes 2−4). Intriguingly, use of unlabeled G:U substrate (2−10 ng) as a competitor in the reaction mixture served to suppress the G:T incision activity much more effectively (lanes 5−7) than did the unlabeled G:T substrate itself. In sharp contrast, the A:U substrate, even at the highest concentration used (10 ng), caused little reduction in the yield of 20-mer fragment (compare lane 1 with lanes 8−10), implying that a A:U mispair is essentially refractory to the ATP-dependent G:T incision activity. The unexpected finding that a G:U mispair, which is known to be the preferred lesion of TDG (*12*), acts as a particularly effective inhibitor of the ATP-dependent G:T incision activity is noteworthy. Accordingly, it is tempting to speculate that either TDG itself or perhaps an ill-defined component of the ATP-dependent G:T incision activity can be rate-limiting for the ATP-dependent G:T incision activity present in the A1235 extract.

Sequence Preference of the ATP-Dependent G:T Incision Activity: Effect of the Base Pair 5′ to G:T or m6G:T in the Model Substrate. To explore the influence of the DNA sequence flanking a G:T or m6G:T mispair on the efficiency of the ATP-dependent G:T incision activity, two series of 45-bp heteroduplex substrates were synthesized in which each of the four possible normal base pairs were placed 5′ to the mismatched guanine or m6G located at position 25 in the top strand of the standard substrate (i.e., ApG:TpT, GpG:CpT, TpG:ApT, and CpG:GpT) (see Figure 5A and ref *11*). The results for G:T mismatch-specific incision of the four model substrates, each labeled at the 5′ terminus of the bottom strand, by the A1235 extract augmented with TDG and ATP, are presented in Figure 5B. Interestingly, the base 5′ to the G:T mispair proved to be highly significant, with the relative efficiency of the G:T incision event, as measured by 20-mer yield, decreasing in the following order: CpG/GpT ∼ TpG/ApT ≫ GpG/CpT ≫ ApG/TpT (see lanes 1−4). When the A1235 extract was replaced with the A1235-MR4 extract in the reaction mixture, all four G:T substrates of the same sequence but with different base pairs 5′ to the mismatch remained intact, as deduced from the total absence of 20-mer fragment (lanes 5−8). As illustrated in Figure 5C, similar results were obtained for the corresponding four m6G:T hetroduplex substrates (i.e., only the Cpm6G/GpT- and Tpm6G/ApT-containing substrates generated significant quantities of 20-mer product (lanes 3 and 4)), and this only occurred when the reaction mixture contained

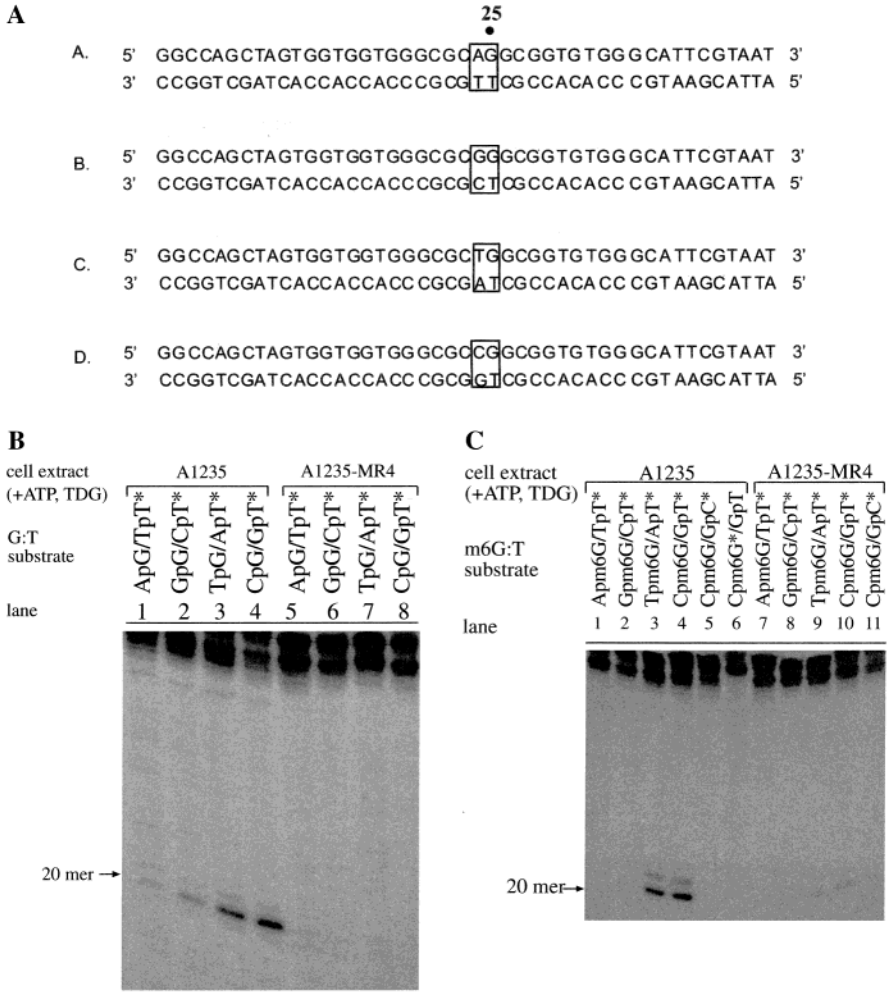

FIGURE 5: (A) Structure of a panel of G:T substrates, each containing a G:T mispair at site 25 in the upper strand flanked on the 5′ side by one of the four possible normal base pairs (A−D). (B) Effect of different normal base pairs 5′ to the G:T mispair in the G:T substrate on the efficiency of the ATP-dependent G:T incision activity. The G:T substrates containing G:T in four different sequence contexts (5′-ApG-3′/3′-TpT-5′, 5′-GpG-3′/3′-CpT-5′, 5′-TpG-3′/3′-ApT-5′, and 5′-CpG- 3′/3′-GpT-5′) were prepared with the radioactive label in the 5′ terminus of each bottom strand and incubated with each extract to monitor ATP-dependent G:T incision activity (refer to Figure 2A for the experimental procedure). Lanes are designated according to the specific model substrate present in the reaction mixture. (Lanes 1−4) A1235 extract-treated substrates. (Lanes 5−8) A1235-MR4 extract-treated substrates. (C) Effect of different normal base pairs 5′ to the mispair in the m6G:T substrate on the efficiency of the ATP-dependent G:T incision activity. The m6G:T substrates in four different sequence contexts, namely, 5′-Apm6G-3′/3′-TpT-5′, 5′-Gpm6G-3′/3′-CpT-5′, 5′-Tpm6G-3′/3′-ApT-5′, and 5′-Cpm6G-3′/3′-GpT-5′, were prepared with the radioactive label at the 5′ terminus of each bottom strand and mixed with each extract and ATP (consult Figure 2A for details). Lanes are designated Npm6G:T or Npm6G:C according to the heteroduplex in the reaction mixture. (Lanes 1−6) Strand incision by the A1235 extract. (Lanes 7−11) Incision by the A1235-MR4 extract. (Incision product) A 20-mer is shown by an arrow.

the A1235 extract rather than the A1235-MR4 extract. Moreover, a model substrate harboring a m6G:C mispair was not recognized by the ATP-dependent G:T incision activity in the A1235 extract (lane 5) and neither was the m6G-bearing (top) strand in the Cpm6G/GpT-containing substrate (lane 6). Finally, once again, all four substrates were refractory to the A1235-MR4 extract (lanes 7−10), as was the bottom strand in the Cpm6G/GpC-containing substrate (lane 11).

To determine the extent to which the base pair 5′ to the site of the mispair (m6G:T or G:T) may influence the relative efficiency of the ATP-dependent G:T incision activity observed in the previous experiments, a second location in the 45-bp duplex, namely, site 20 in the top strand, was selected for the introduction of a m6G:T mispair. As before, four model substrates were prepared with each of the four possible normal base pairs 5′ to the m6G:T lesion (see Figure 6A for details). In short, the results were essentially the same as noted previously (Figure 6B). Once again, under these reaction conditions, the preferred substrate for the incision activity in the A1235 extract was the Cpm6G/GpT-containing duplex, followed by the one containing Tpm6G/ApT and, to a limited extent, the Gpm6G/CpT-harboring substrate (lanes 1−4). As witnessed in the previous experiments, none of the four m6G:T-containing duplexes were acted upon by the A1235-MR4 extract (lanes 7−10) and neither were the Cpm6G/GpC (i.e., m6G:C) -harboring substrate (lanes 5 and 11) and the top (i.e., m6G-containing) strand in the substrate containing Cpm6G/GpT (lanes 6 and 12). Together, these data in Figures 5 and 6 unequivocally demonstrate that the ATP-dependent G:T incision activity may be designed primarily to participate in the repair of G:T (or m6G:T) base mismatches arising from the spontaneous deamination of m5C, given that cytosine methylation in higher eukaryotes, including humans, is largely confined to CpG dinucleotides (*23*).

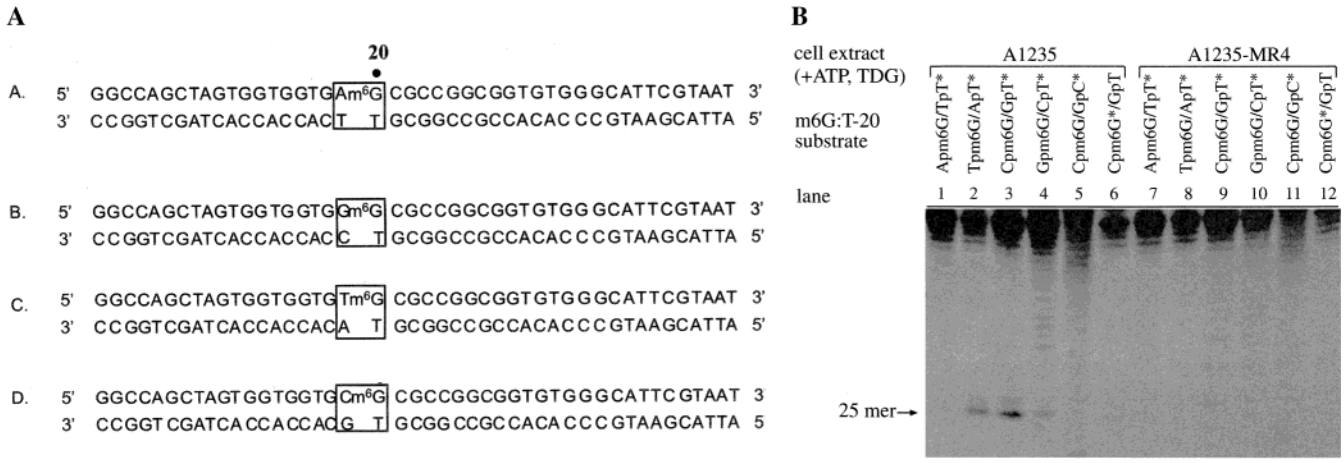

FIGURE 6: (A) Structure of a series of m6G:T substrates, each containing the mispair at site 20 in the top strand flanked on the 5′ side by each of the four possible normal base pairs (A−D). (B) Effect of the different 5′ base pairs on the m6G:T substrate (m6G:T being at site 20) on the ATP-dependent G:T incision activity. The m6G:T substrate containing m6G:T at nt 20 in four different sequence contexts (5′-Apm6G-3′/3′-TpT-5′, 5′-Tpm6G-3′/3′-ApT-5′, 5′-Cpm6G-3′/3′-GpT-5′, and 5′-Gpm6G-3′/3′-CpT-5′), each prepared with radioactive label at the 5′ end of the bottom strand. Reactions were carried out and the resultant products were analyzed as described in Figure 2A. Lanes are designated Npm6G:T or Npm6G:C according to the specific substrate in the reaction mixture. (Lanes 1−6) A1235 extract-treated substrates. (Lanes 7−12) A1235-MR4 extract-treated substrates. (Potential incision product) A 25-mer is indicated by an arrow.

DISCUSSION

Evidence presented herein corroborates and expands our earlier findings (*17*) attesting to the presence in human cell extracts of two distinct activities for incising DNA at G:T (or m6G:T) base mismatches: a "TDG-resembling" ATP-independent activity and a poorly characterized ATP-dependent activity. Data in Figure 2A demonstrate that all three (i.e., G:T, m6G:T, and G:U) model substrates employed are subject to incision by the parental (glioma cell line A1235) extract supplemented with ATP and purified TDG protein, whereas the derivative (alkylation-resistant cell line A1235-MR4) extract under the same reaction conditions operates on only one of the three heteroduplexes (i.e., the G:U substrate). The efficiency of strand incision at the G:T and m6G:T mispairs by the A1235 extract is critically dependent on the addition of ATP (Figure 3), consistent with the postulate that these two types of mismatched base pairs are preferentially recognized by the ATP-dependent G:T incision activity. Further support for this hypothesis derives from the observed ability of increasing amounts of unlabeled G:T heteroduplex to inhibit, in a competitive manner, the ATP-dependent incision of the G:T substrate by the A1235 extract (compare lanes 1−4, Figure 4).

While G:T and m6G:T mispairs are handled by the ATP-dependent G:T incision activity, G:U mispairs appear to be recognized by the TDG-like ATP-independent incision activity. Several lines of evidence are in keeping with this prediction. First and foremost, the A1235-MR4 extract, which is devoid of the ATP-dependent G:T incision activity, readily incises at a G:U site in the 45-bp model DNA (Figure 2A). Second, the much enhanced rate of incision at G:U as compared to G:T sites (Figure 3), together with the competitive inhibition of incision in the G:U substrate by unlabeled heterduplex containing G:T or G:U (but not A:U) mispairs (data not shown), are reminiscent of the properties of the human incision activity initiated by TDG alone (*10*, *12*). Third and finally, the fact that the incision kinetics displayed by the A1235 extract on incubation with the G:U substrate (Figure 3) is essentially insensitive to supplementary ATP supports our contention that the initiation of incision at a G:U mispair is mediated solely by TDG residing in the A1235 extract. We thus conclude that human cells harbor different activities for initiating the repair of base mismatches in their DNA; G:T and m6G:T mispairs are mainly targeted by an ATP-dependent incision-inducing complex and a G:U mispair is operated on by a TDG-like ATP-independent activity.

In this investigation, additional insight into the primary function of the ATP-dependent G:T incision activity was obtained by monitoring the influence of the precise base pair 5′ to a G:T or m6G:T mismatch on the efficiency of the incision reaction by the A1235 extract in the presence of TDG and ATP. As vividly shown in Figures 5B,C and 6B, the efficiency of the mismatch-induced incision event was noticeably dependent on the base 5′ to the mismatch G (or m6G), with unequivocal predilection for a pyrimidine base, especially cytosine. Accordingly, we are led to hypothesize that the ATP-dependent G:T incision complex constitutes the initial component of a dedicated repair mechanism for deaminated m5C lesions at CpG islands in DNA (*23*).

Perhaps the most unexpected, and potentially insightful, result emerging from the present studies is the observation that an unlabeled G:U substrate is in fact a more effective inhibitor of the ATP-dependent G:T incision activity than an unlabeled G:T substrate itself (compare lanes 2−4 with 5−7, Figure 4). Given that a G:U mispair is known to be a better substrate for TDG than is a G:T mispair (*24*, *25*), it seems reasonable to postulate that TDG may be an integral constituent of the ATP-dependent G:T incision apparatus. Other data support this view. As a case in point, the A1235 extract, when supplemented with ATP and TDG, produces two incisions, one directly 5′ and the other directly 3′ to the mismatched thymine, in the m6G:T substrate (Figure 2B), thereby creating a one-nucleotide gap opposite to the m6G lesion. This dual incision event, which is presumably initiated by the ATP-dependent G:T incision activity, imitates the

action of the human TDG protein in concert with AP endonuclease 1 (*7*), implying that these two incision activities may overlap functionally. Additionally, there is the aforementioned sequence effect in which the ATP-dependent G:T incision activity, like the TDG protein, displays a distinct preference for a pyrimidine base (especially cytosine) 5′ to the G (or m6G) base at the mismatch site. And last but not least, data presented both herein (Figures 2 and 3) and elsewhere (*17*) reveal that the ability of the A1235 extract to incise at a G:T (or m6G:T) mismatch is greatly enhanced when a limited amount of purified TDG is present in the reaction mixture, implying that TDG may be rate-limiting in the execution of the multistep process leading to the restoration of the mismatch site to a normal configuration.

There is compelling evidence from several independent lines of investigation (*7*, *10*−*12*) to suggest that in human cells the repair of G:T mispairs arising from the deamination of m5C is initiated by TDG, resulting in the release of the mismatched thymine base. When performing the reaction in vitro, purified TDG removes an equimolar quantity of thymine, such that the glycosylase behaves as a single-turnover enzyme, with each molecule binding tightly to the resultant apurinic residue opposite to the guanine base once the target base has been released (*12*). In contemplating a plausible explanation for the noncatalytic behavior of TDG, others have advanced the notion that the formation of a stable enzyme−AP site complex may act as a scaffold to recruit other proteins that are required to execute later steps in the base excision repair process acting on a G:T mispair (*12*). Additional support for this notion derives from our finding (see lanes 5 and 6, Figure 2A, and ref *17*) that the A1235-MR4 extract lacks the ATP-dependent G:T incision activity, even when TDG is included in the reaction mixture. Undoubtedly, other factors, in addition to TDG, are therefore necessary to introduce an intrastrand nick at a G:T site in the ATP-dependent mode of incision. A clue into the identity of some of these putative proteins can be gleaned from our earlier report (*17*) documenting the similarity in the mismatch repair hallmarks of the derivative extract and those of several colon tumor-derived cell lines known to be deficient in certain mismatch repair-recognition proteins (*26*, *27*). By applying standard protein fractionation methods to extracts of ATP-dependent G:T incision-proficient (A1235) and deficient (A1235-MR4) cells, our laboratory is making rapid progress in identifying the supposed factors comprising the ATP-dependent G:T incision complex in human cells.

ACKNOWLEDGMENT

The authors thank Dr. Sultan Al-Sediary for encouragement and support throughout this study. We also thank Peter D. Sell, Head of Photographics, for expert art work.

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BI020239N