Incision at O^6-Methylguanine:Thymine Mispairs in DNA by Extracts of Human Cells†

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ABSTRACT: Human cell-free extracts were used to detect activities specifically incising O^6-methylguanine (m6G) paired with C or T in DNA. A 45-bp double-stranded DNA containing one m6G across from a T (m6G:T) was the test substrate. Extracts from glioblastoma cell lines A172 and A1235 (lacking the m6G-specific repair protein m6G-DNA methyltransferase, MGMT) and colon carcinoma cell line HT29, containing MGMT, showed incision activities specific for the T strand of m6G:T [and G:T, as reported previously by Wiebauer and Jiricny (1989)] substrates, but did not cleave m6G:C (or G:C) substrates. Competition experiments showed that the activity was similar to, if not identical with, the activity in human cells that incises G:T mismatches. The incision sites were similar to those recognized by human G:T- or G:A-specific mismatch enzymes, i.e., the phosphodiester bonds both 3′ and 5′ to the poorly matched T, suggesting the glycolytic removal of the poorly matched T followed by backbone incisions by class I or II AP endonucleases. Three experiments in which MGMT was inactivated showed that the m6G:T incision activity was not simply due to a two-step mechanisms in which MGMT would first mediate conversion of the m6G:T substrate to a G:T substrate which would serve as a substrate for G:T incision. Extracts from HT29 contained a DNA-binding factor, possibly DNA sequence-specific, that inhibited incision of the m6G:T (but not the G:T) substrate, that was removed by the addition of synthetic DNA to the reaction.

We are interested in understanding the biological effects produced by unrepaired O^6-methylguanine (m6G)[1] in DNA. m6G is produced in DNA when DNA guanine residues react with such SN1 methylating agents as MNNG and MNU (Lawley, 1976) and is repaired in normal cells by the 22-kDa protein MGMT that transfers the m6G methyl group to a specific MGMT residue, thus restoring proper structure to guanine (Olsson & Lindahl, 1980; Harris et al., 1983; Yarosh et al., 1983; Foote et al., 1983). About 20–30% of human solid tumor cell lines show the Mer⁻ phenotype (Day et al., 1980a), lack MGMT [Yarosh et al. (1983, 1984) and Sibghat-Ullah et al., unpublished results], and are hypersensitive to MNNG as indicated by enhanced lethality (Day et al., 1980a,b; Scudiero et al., 1984; Domoradski et al., 1984), SCE formation (Day et al., 1980a; Wolff et al., 1977), and mutation induction (Baker et al., 1979, 1980; Domoradski et al., 1984). That the introduction of the entire or the truncated *Escherichia coli ada* gene, a bacterial MGMT gene (Ishizaki et al., 1986, 1987; Brennand & Margison, 1986; Samson et al., 1986; Kataoka et al., 1986), or the human MGMT gene (Hayakawa et al., 1990) into such m6G-repair-deficient lines prior to MNNG treatment reverses cellular propensity to these DNA damage-related biological effects adds strongly to the evidence implicating m6G in their production. Semiconservative DNA synthesis over m6G-containing parental DNA templates must occur to allow the cell division required for observation of lethality, SCE, and mutations. DNA replication in vitro is more frequently accompanied by the incorporation of TMP residues than by dCMP residues opposite m6G (Singer et al., 1989; Snow et al., 1984), and such potentially mutagenic incorporation in vivo is believed to underlie the elevated rates of MNNG- and MNU-induced mutagenesis of cells lacking MGMT (Loechler et al., 1984), and the observation that m6G is mutagenic and induces G → A transitions in mammalian systems (Ellison et al., 1989). It is believed that mismatch repair processes influence the biological effects of m6G in *E. coli* (Sklar & Strauss, 1980; Karran & Marinus, 1982). We and others have proposed that the enhancement of MNNG-produced biological effects in Mer⁻, in comparison to Mer⁺, cells is due to a DNA mismatch repair system that recognizes m6G-containing mispairs in DNA (Day et al., 1987; Goldmacher et al., 1986; Scudiero et al., 1984). In this paper, we show that extracts prepared from both Mer⁻ and Mer⁺ cells incise double-stranded DNA substrates containing either m6G:T or G:T mispairs, but not m6G:C mispairs or G:C pairs and interpret the biological effects of MNNG treatment of Mer⁻ cells in terms of these results.

EXPERIMENTAL PROCEDURES

Oligodeoxynucleotides. For the preparation of duplex DNA substrates, four 45-base oligodeoxynucleotides (Voigt et al., 1989) were synthesized. For convenience, these are designated "top" or "bottom" strands according to their position in Figure 1. The two top strands contained either m6G or G at position 25 from the 5′ terminus. The two bottom strands contained either C or T at position 21 from the 5′ terminus, the position paired with position 25 of the top strand. The top-strand sequence containing m6G was prepared by the Regional DNA Synthesis Laboratory, University of Calgary,

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[1] Abbreviations: MNNG, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine; MNU, 1-methyl-1-nitrosourea; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; m6G, O^6-methylguanine; MGMT, O^6-methylguanine-DNA methyltransferase; Mer⁺ and Mer⁻, designating cell lines that are able (Mer⁺) or unable (Mer⁻) to support plaquing by MNNG-treated adenovirus as well as do normal human fibroblasts, but which support plaquing of non-treated viruses normally; Mex⁺ and Mex⁻; designating cell lines respectively able and unable to repair O^6-methylguanine in their DNA after treatment with methyl-labeled *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine; SCE, sister chromatid exchange; HS, herring sperm; RH, relative humidity; SN1, nucleophilic substitution type 1, TDT; terminal deoxynucleotidyl transferase; AP, apurinic or apyrimidinic site.

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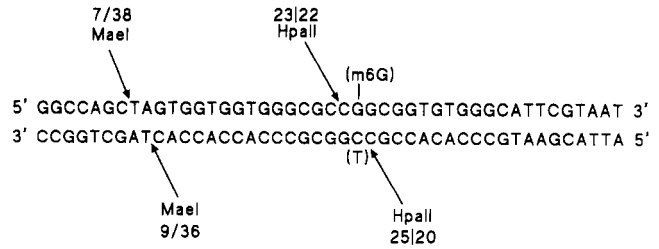
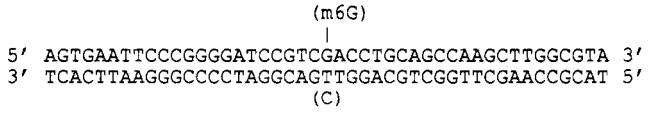


FIGURE 1: Sequence of the 45-bp DNA substrate. The location of the O^6-methylguanine residue is designated by m6G. Across from O^6-methylguanine, T, in parenthesis, replaces C in the bottom strand of the m6G:T and G:T substrates. The cleavage sites for restriction enzymes *Hpa*II and *Mae*I are indicated by arrows, and the sizes in base pairs of the 5′- and 3′-terminated digestion products are indicated. DNAs of the same sequences were used previously (Voigt et al., 1989).

using O^6-methylguanine phosphoramidite obtained from American Bionetics (Emeryville, CA). The modified tritylated oligodeoxynucleotide was released from the solid support, and detritylation was achieved in two ways in order to determine whether the mode of preparation influenced the outcome of our incision experimentation: (i) the oligonucleotide was treated with concentrated ammonia at 50 °C for 24 h (Borowy-Borowski & Chambers, 1987), and (ii) the oligonucleotide was exposed to 5% (v/v) 1,8-diazabicyclo[5.4.0]-undec-7-ene (DBU) in anhydrous methanol for 14 days at room temperature (Leonard et al., 1990; Voigt et al., 1989). The top-strand sequence containing the unmodified base G at position 25, the two bottom strands in Figure 1, and the primer oligodeoxynucleotide 5′-ATTACGAATGCCC-3′ (complementary to the right end of the top strand in Figure 1) were prepared by the DNA Synthesis Lab, Department of Microbiology, University of Alberta. For the competition experiment shown in Figure 12, four oligonucleotides with structures different from those in Figure 1 were used to prepare the competing double-stranded DNAs. The 45-mer structures were derived from the 90-mer substrate of Wiebauer and Jiricny (1989) and were



The m6G-containing strand was prepared by the Regional DNA Synthesis Laboratory in Calgary with 10% DBU deprotection for 2 weeks, but otherwise by the same procedure as the DBU-deprotected m6G-containing oligonucleotide in Figure 1. The other three strands were prepared by the DNA Synthesis Lab, Department of Microbiology, University of Alberta. All oligodeoxynucleotides were purified on 12% denaturing gels and electroeluted from gel slices. The 5′ end labeling of the DNA was achieved by T4 kinase and 5′-[γ-^{32}P]ATP. Terminal deoxynucleotidyl-transferase and cordycepin 5′-[α-^{32}P]triphosphate were used for labeling 3′ termini (Tu & Cohen, 1980).

Preparation of Substrates. The m6G- (or G-) containing 45-base oligonucleotide top strand was mixed in a 1:1 ratio with the (nearly) complementary bottom strand containing either dC or dT across from m6G (Figure 1) in 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, and 1 mM EDTA. The strands were hybridized by heating at 65 °C for 15 min, followed by cooling at room temperature to room temperature. Double-stranded DNA (>90%) was separated from single-stranded DNA on 12% nondenaturing acrylamide gels. Alternatively, duplex DNAs were prepared enzymatically by Pol I (Klenow fragment) using both normal and modified 45-base oligonucleotide top strands (Figure 1) as template. Top strands (containing either m6G or G; 0.13 μmol) were mixed with 0.20 μmol of 13-mer primer, previously labeled at the 5′ terminus as above (in 50 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 0.1 mM DTT, and 50 μg/mL BSA) and heated at 65 °C for 15 min, followed by cooling to room temperature. To this were added 100 μM each of the four normal dNTPs and 5 units of Pol I (Klenow fragment), and DNA synthesis was allowed for 6 h at room temperature. The DNA was extracted with phenol and chloroform and precipitated with ethanol. The double-stranded 45-bp DNA was purified on a 12% nondenaturing polyacrylamide gel. The formation of duplex DNAs was also assayed by cleavage with *Hpa*II (G:C DNA only) or with *Mae*I.

Modification of Herring Sperm DNA with MNNG. The method was based on the finding of Lawley and Thatcher (1970) that *N*-acetyl-L-cysteine or pH 9 treatment enhances in vitro methylation of DNA by MNNG. Herring sperm DNA (100 mg) was dissolved in 20 mL of 10 mM Tris, 1 mM EDTA, pH 8.0 (TE), brought to 1 M in NaCl, precipitated with 2.5 volumes of ice-cold ethanol, wound out on a stirring rod, and resuspended in an equal volume of TE. Two 0.9-mL samples were prepared and treated overnight either with 0.1 mL of ethanol or with 0.1 mL of ethanol containing 20 mg/mL MNNG. Because the yellow color, characteristic of unreacted MNNG, had not entirely disappeared from the MNNG-treated sample, 10-μL portions of 1 M *N*-acetyl-L-cysteine (pH 7) were successively added until the yellow color disappeared (50 μL); then 50 μL more 1 M N-acetyl-L-cysteine was added. A 100-μL aliquot of 1 M *N*-acetyl-L-cysteine was added to the non-MNNG-treated sample. To both samples were then added 1.0 mL of TE, followed by 0.5 mL of 5 M NaCl. After mixing, 8 mL of ice-cold ethanol was layered over the solution, and the DNA was wound out, washed in 70% ethanol/30% TE and resuspended in 0.9 mL of TE.

Enzymes and Chemicals. *Hpa*II, *Mae*I, T4 kinase, and terminal deoxynucleotidyl-transferase, Pol I (Klenow fragment), DNAse I, and 1-kb DNA ladder were purchased from GIBCO BRL. *E. coli* Ada protein was a kind gift from Dr. S. Mitra. MNNG was from Aldrich. Herring sperm DNA and dNTPs were from Sigma. 5-[γ-^{32}P]ATP (5000 Ci/mmol) was from Amersham, and cordycepin 5′-[α-^{32}P]triphosphate, (5000 Ci/mmol) was from NEN Dupont.

Cultured Cells and Extracts. Tumor cell lines A172 and A1235 were from S. A. Aaronson, NIH, Bethesda, MD. HT29 was from the American Type Culture Collection, Rockville, MD. Cells were cultured in 100-mm dishes at 37 °C, 85% RH, and 10% CO_2 in DMEM supplemented with 5% fetal bovine serum plus 50 units of penicillin/mL and 50 μg/mL streptomycin. In some experiments cells were treated with MNNG as follows. Medium was aspirated from the plates, and the cells were washed once with PBS. Fresh medium containing 5% fetal bovine serum, 10 mM Hepes, pH 7.4, and 1 μg/mL (7 μM) MNNG was added to the cells, which were incubated at 37 °C for selected time periods. Extracts were prepared by the method of Manley et al. (1980) as described by Sibghat-Ullah et al. (1989). The extracts were frozen in small aliquots in liquid nitrogen, were stored at −80 °C, and were stable for 1 year. Protein concentrations (Bradford, 1976) were 8–10 mg/mL.

DNA Incision Assay. The incision reactions were performed as follows (Wiebauer & Jiricny, 1989, 1990). The reaction mixtures (50 μL) contained 25 mM Hepes, pH 7.9, 0.5 mM EDTA, 0.01 mM $ZnCl_2$, 0.5 mM DTT, 100 μg/mL BSA, 8

ng (2 ng of ^{32}P-labeled plus 6 ng of unlabeled) of 45-bp duplex DNA, and 0–40 μg of cell-free extract protein. (Whenever 8 ng of substrate is specified, it is always 2 ng of labeled plus 6 ng of unlabeled substrate of the same sequence. Addition of the 6 μg of unlabeled 45-bp duplex DNA reduced the appearance of nonspecific breakdown products in the autoradiograms. In Figures 6, 11, and 12, 40 ng of 1-kb ladder was used for this purpose.) Reactions were at 30 °C for 12–24 h and were stopped by adding 10 μg/mL proteinase K into the reaction mixture for 30 min. Following this, the DNAs were extracted with phenol and chloroform and precipitated with ethanol. Vacuum-dried DNAs were then dissolved in 20 μL of formamide/dyes and analyzed by electrophoresis through 12% acrylamide sequencing gels. Gels were exposed to X-ray film at –80 °C for 1–3 days to obtain autoradiograms.

In the experiments concerning the inhibitor of incision and substrate competition (the results of which are shown in Figures 11 and 12), the protocol for incision assay was altered somewhat. The mixture of 2 ng of ^{32}P-labeled plus 6 ng of unlabeled 45-mer (of the same sequences) had originally been selected to provide sufficient ^{32}P dpm for the observation of product while at the same time reducing the appearance of autoradiographic bands due to nonspecific breakdown of the labeled substrate. For the inhibitor experiments, a high extract:substrate DNA ratio was desired so that the putative inhibitor would entirely prevent the HT29 extract from incising the labeled substrate. To achieve this, the 6 ng of unlabeled m6G:T DNA was not added to the 2 ng of labeled DNA when the substrate was prepared. But, in an attempt to maintain reduced nonspecific breakdown of the labeled 45-mer DNA, various amounts of unlabeled 1-kb ladder DNA were added to the reactions in the belief that the 1-kb ladder DNA might not reverse the effects of the inhibitor if the inhibitor were DNA sequence-specific. It was determined that at least 40 ng of 1-kb ladder DNA could be added without inducing observable incision of the substrate by the HT29 extract. Thus, in the inhibitor experiment of Figure 11, as well as in the competition experiments of Figure 12, 40 ng of 1-kb ladder DNA was added to reaction mixtures in place of the 6 ng of unlabeled 45-mer duplex substrates used in the other experiments.

MGMT Assay. The MGMT assay [similar to that of Wu et al. (1987)] was performed as follows using the 45-bp DNA containing m6G:C base pairs. Reactions were conducted either under the conditions of the incision reaction (above) or in 50 μL of a reaction mixture containing 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM DTT, *E. coli* Ada protein or HT29 cell-free extract, and 45-bp DNA substrate (see figure legends for amounts). Following incubation (see individual figure legends for amounts, temperatures, and times), DNAs were extracted with phenol and chloroform and precipitated with ethanol. The vacuum-dried DNAs were dissolved in 1× *Hpa*II buffer and digested with *Hpa*II for 1 h at 37 °C. The DNAs were recovered by extraction with phenol and chloroform and ethanol precipitation and analyzed on 12% acrylamide DNA sequencing gels. *Hpa*II digestion of CCGG sequences is blocked if the 5′ G is O^6-methylated (CC^{m6}GG); when the sequence is demethylated by *E. coli* Ada protein (or human MGMT), the sequence is digested by *Hpa*II.

RESULTS

Incision of DNA Containing m6G by Human Cell-Free Extracts. 45-bp DNAs containing m6G:C or m6G:T mismatched pairs at position 25 of the top strands (Figure 1), and labeled at the 5′ termini of either their top or bottom strands, were tested as substrates for the in vitro incision. These were incubated with human cell-free extract prepared from the Mer$^-$ cell line A1235. The results of the assay performed with these two substrates as well as with control G:T and G:C substrates are shown in Figure 2. The DNAs containing the m6G:T or G:T pairs labeled on the 5′ terminus of the bottom strand (lanes 4 and 5) were incised as indicated by the presence of a specific incision fragment migrating more rapidly than the full-length DNA substrate near the top of the figure. These incision products were produced when the substrates were radiolabeled on the 5′ termini of their bottom strands (lanes 4 and 5) and were not produced when labeled on the 5′ termini of their top strands (lanes 8 and 9). The incision products are of 20-mer length, based on comigration with the *Hpa*II digestion fragment of the G:C substrate labeled on the 5′ terminus of its bottom strand (lane 1). The result that the substrate containing the G:T mismatched pair is incised in this fashion is in accordance with earlier reports (Wiebauer & Jiricny, 1989, 1990). Substrate DNAs, containing no mismatch (G:C) or the m6G:C mispair, 5′-labeled in either strand, produced no signal in this assay (lanes 2, 3, 6, and 7). These results taken together suggest that the cell-free extract contains an activity that specifically recognizes DNAs containing m6G:T or G:T mismatches and incises the T-containing strand just 5′ to the mismatched T.

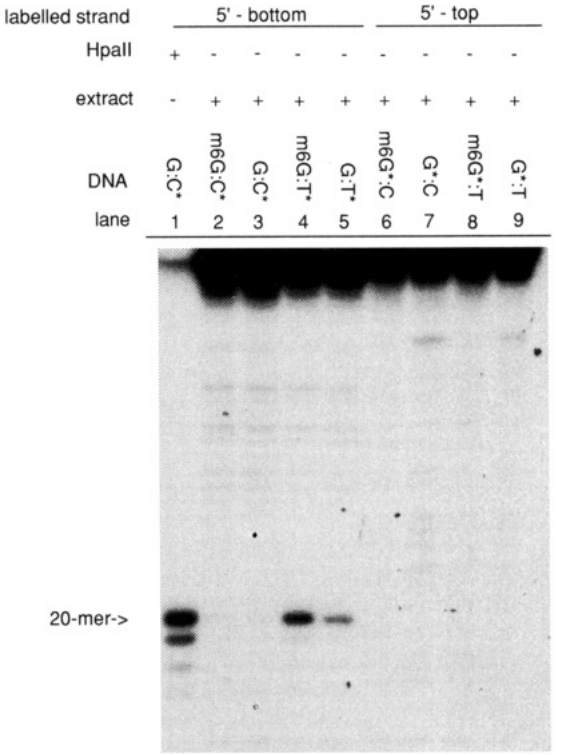


FIGURE 2: Incision of 5′-labeled 45-bp DNA by A1235 cell-free extract. Double-stranded 45-bp substrates, either properly paired (G:C) or poorly paired (m6G:C, m6G:T, and G:T) at position 25 of the top strand (Figure 1), were prepared with ^{32}P label in the 5′ termini of either the bottom strands (lanes 1–5) or the top strands (lanes 6–9). An 8-ng sample of each substrate (2 ng of ^{32}P-labeled plus 6 ng of unlabeled; see Experimental Procedures) was incubated with 10 μg of A1235 cell-free extract protein in 50 μL of DNA nicking buffer for 12 h at 30 °C. Incision products were analyzed on a 12% sequencing gel, followed by autoradiography. "20-mer→" beside lane 1 designates the 20-mer *Hpa*II fragment of the G:C substrate. Note that a small amount of DNA contamination or background is seen in lanes 2–5, 7, and 9, the amounts of which are small in comparison to the signal in lanes 4 and 5.

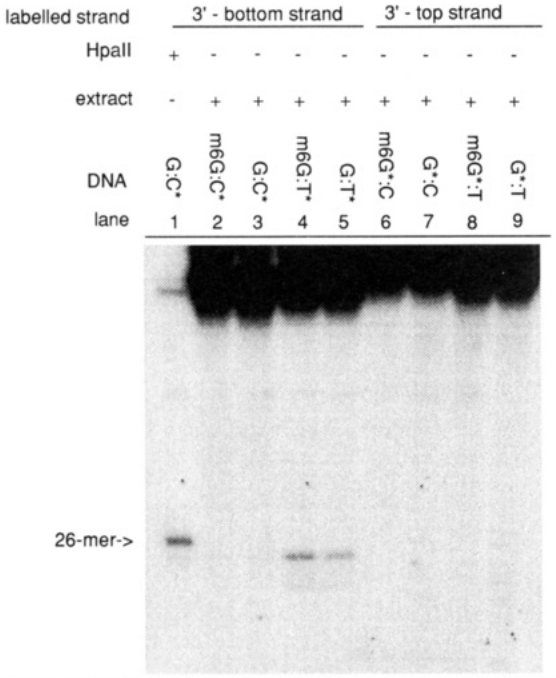


FIGURE 3: Incision of 3′-labeled 45-bp DNA substrates by A1235 cell-free extract. The DNA substrates (8 ng) were assayed separately for incision with 10 μg of A1235 cell-free extract as described under Figure 2. (Note that the labeled strands of the 3′-labeled DNA substrates, as well as the 3′-incision fragments in lanes 4 and 5, and the 3′-labeled *Hpa*II fragment in lane 1 are one base longer than the unlabeled DNA due to the addition of cordycepin monophosphate at the 3′ terminus.)

FIGURE 4: Incision sites on the m6G:T substrate. The incision fragments (prepared by reactions as in Figures 2 and 3) of the m6G:T substrate (either 5′- or 3′-labeled bottom strand) incubated with A1235 cell-free extract were electrophoresed together with *Hpa*II standards prepared from the G:C substrate (either 5′- or 3′-labeled in the bottom strand). In lane 1, a one nucleotide ladder prepared by DNase I treatment of the m6G:T substrate, 5′-labeled in its bottom strand, is shown. The nucleotides at positions 20 and 26 from the 5′ end are designated by arrows, using the *Hpa*II fragments (lanes 2 and 3) as guides. The 5′-incision fragment (20-mer, lane 5) is identical in size to the 5′ *Hpa*II fragment in lane 3. The 3′-incision fragment (lane 4, a 25-mer) is one base shorter than the 26-mer *Hpa*II fragment in lane 2. After correction for the addition on the 3′ end of cordycepin, the size of the 3′-incision fragment is 24 bases, which would imply that the T at position 21 in the bottom strand (and opposite the m6G at position 25 of the top strand) has been removed and that the phosphodiester bonds, designated by the lines bracketing the "C(T)", have been incised. Lanes 6 and 7 show incision signals at about zero time of incubation.

To characterize the 3′-incision product, we conducted the in vitro incision assay using the DNA substrates radiolabeled in their 3′ termini with TDT and cordycepin triphosphate. (This procedure adds one base to the 3′ terminus.) Substrates were again incubated with cell-free extracts from A1235 cells and analyzed. The results are shown in Figure 3. Again, the incubation of extract with only the DNA substrates containing m6G:T or G:T pairs (lanes 4 and 5) resulted in the production of a specific incision fragment. The size of the fragment appeared to be one base shorter than the 26-mer fragment produced by *Hpa*II digestion of the DNA substrate with the G:C pair labeled with cordycepin [^{32}P]monophosphate in the 3′ terminus of its bottom strand (lane 1). Again, the result that the substrate containing the G:T mismatch was incised in this fashion (lane 5) is in accordance with earlier results (Wiebauer & Jiricny, 1989, 1990). When the DNAs were 3′-terminally labeled in their top strands, none served as substrate for the incision reaction (lanes 6–9). Taken together, the results suggest that the activity in the cell-free extract incises the bottom strand of the 3′-terminally labeled DNA substrate containing the m6G:T pair just 3′ to the mismatched T.

In Figure 4, we have characterized the 5′- and 3′-incision fragments obtained (Figures 2 and 3) with DNA substrate containing m6G:T pairs by electrophoresing them together with a DNAse I ladder and size markers (lanes 4 and 5). The *Hpa*II digestion products of substrates containing G:C pairs are shown in lanes 2 (substrate labeled in the 3′ terminus of the bottom strand) and 3 (substrate labeled on the 5′ terminus of the bottom strand), along with a ladder produced by DNase I digestion of the m6G:T substrate. The length of the 5′-incision fragment (lane 5) is confirmed as being the same as that of the 20-mer *Hpa*II fragment (lane 3), and the 3′-incision fragment (lane 4) is one base shorter than the 26-mer *Hpa*II fragment (lane 2). Collectively, the results suggest that the activity in the cell-free extract results in two incisions in the DNA containing the m6G:T pair: just 3′ and just 5′ to the mismatched T [designated by the horizontal lines bracketing the base denoted C(T) along the left side of the ladder in Figure 4]. The T cannot be retained in the substrate after such double phosphodiester bond breakage, and a one-base gap, bounded by 3′ and 5′ termini of undermined structure, is almost certainly produced. Such incision activity could be due either to a true mismatch-specific endonuclease or to the sequential action of a mismatch-specific glycosylase followed by an AP endonuclease.

Characterization of Time and Concentration Dependence of the m6G:T Incision Activity. The effect of extract concentration on incision activity was studied by incubating cell-free extracts from A172 (another Mer$^-$ line) with substrates labeled in the 5′ termini of their bottom strands. Figure 5A shows that the substrates with the m6G:T and G:T mispairs, but not the substrates with m6G:C mispairs or G:C pairs, are increasingly incised with increasing amount of extract. The activity remains linear in the range of 5–20 μg of extract protein/50 μL (0.1–0.4 mg/mL) of reaction mixture in our conditions. Beyond this, at 40 μg/50 μL, the activity is significantly reduced possibly because of an inhibitory factor to be discussed later. Figure 5B shows that the amount of

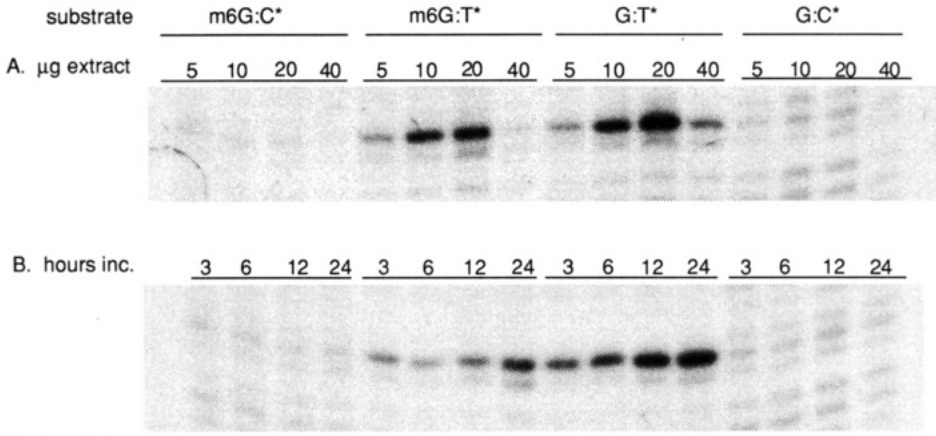


FIGURE 5: Incision of 45-bp DNA substrates as a function of time and extract concentration. Substrates were 5′-labeled on their bottom stands. (A) Increasing amounts of cell-free extract from A172 cells were incubated in 50 μL of DNA nicking buffer for 16 h at 30 °C with 8 ng of the four substrates indicated. Analysis was by electrophoresis in a 12% sequencing gel followed by autoradiography. (B) Aliquots of 20 μg each of A172 extract were incubated with 8 ng of the four substrates for increasing periods at 30 °C.

product produced by 20 μg of extract protein when reacting with either the m6G:T or G:T substrate increases slowly with time up to 24 h. The result that the incision activity is approximately linear for 24 h suggests that it stable in the presence of excess substrate over this period.

Purity of the m6G:T-Containing Substrate. The 45-base, m6G-containing, oligonucleotide used in our nicking assay was synthesized as described (Leonard et al., 1990) employing DBU and anhydrous methanol in the deprotection procedure to minimize the conversion of m6G either to G or to 2,6-diaminopurine and to maximize the removal of the isobutyryl group from the N2 position m6G (Borowy-Borowski & Chambers, 1987). However, the amount of the m6G-containing DNA, as measured by the conversion of m6G to G by *E. coli* Ada protein (followed by *Hpa*II digestion; see Experimental Procedures), was found to be 60–65%. It was conceivable that an impurity in the m6G:T substrate might be responsible for the observation that the DNA containing the m6G:T mispair was a substrate for the nicking reaction. Our approach to this question was based on the fact that Pol I incorporates TMP in preference to dCMP across from template m6G in vitro (Snow et al., 1984; Singer et al., 1989). Pol I extension was performed using as templates the m6G-containing single-stranded DNAs, synthesized either with the ammonia or DBU-deprotection procedures, and with the 13-mer [^{32}P]-5′-pATTACGAATGCCC-3′ as primer. After gel purification, the products were used as substrates for assaying m6G:T incision by extracts from the A1235 cell line. Figure 6 shows that the incubation of the product of primer extension over the control top strand (containing G at position 25; Figure 1) with the extract did not produce any incision product (lanes 2 and 3). By contrast, reactions of extract with the extension products prepared with either m6G-containing top strand (deprotected with ammonia, m6G1, lane 5; deprotected with DBU, m6G2, lane 7) as template gave rise to the 20-mer incision product. The amount of incision product is qualitatively similar to that obtained under similar conditions from DNA containing the m6G:T pair prepared by hybridizing strands totally synthesized from phosphoramidites. This result (along with the care taken in the preparation of the m6G-containing strands) is a strong indication that the activity incises m6G:T pairs.

To characterize whether the Pol I-synthesized DNA substrates contained C or another base across from m6G, we carried out the following experiment. First we treated the above 45-bp gel-purified extension products with *E. coli* Ada

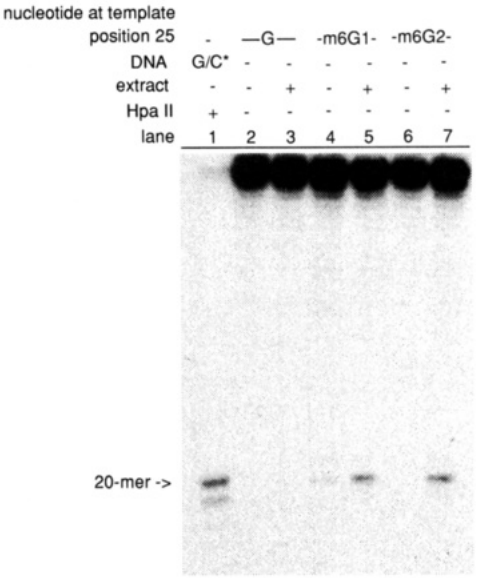


FIGURE 6: Incision by A1235 extract of m6G:T substrate prepared by using DNA polymerase I. Top strands containing either G or m6G (m6G1, m6G2) at position 25 served as templates for extension of the 5′-ATTACGAATGCCC-3′ primer (5′-labeled) by the Klenow fragment of *E. coli* DNA polymerase I. M6G1 was deprotected with ammonia, while m6G2 was prepared by DBU deprotection (see Experimental Procedures). A 10-μg sample of A1235 cell-free extract protein was incubated in 50 μL of DNA nicking buffer with 40 ng of 1-kb DNA ladder and 2 ng of each 45-bp polymerase product, 5′-labeled in the bottom strand. The incubation was at 30 °C for 12 h.

protein to convert m6G to G, then digested the products with *Hpa*II, and analyzed the digestion mixture on a 12% denaturing gel (Figure 7). Only the extension product synthesized from the template with G at position 25 was cleaved by *Hpa*II (lane 6), indicating that a dCMP had been incorporated across from the G at position 25. That no cleavage of the products of Pol I extension over the m6G-containing templates was observed (even after conversion of m6G to G by *E. coli* Ada protein; lanes 8 and 10) indicated both that m6G did not stimulate incorporation of dCMP into the extension product and, by inference, that neither m6G-containing template oligonucleotide carried detectable G at position 25. [From this, we

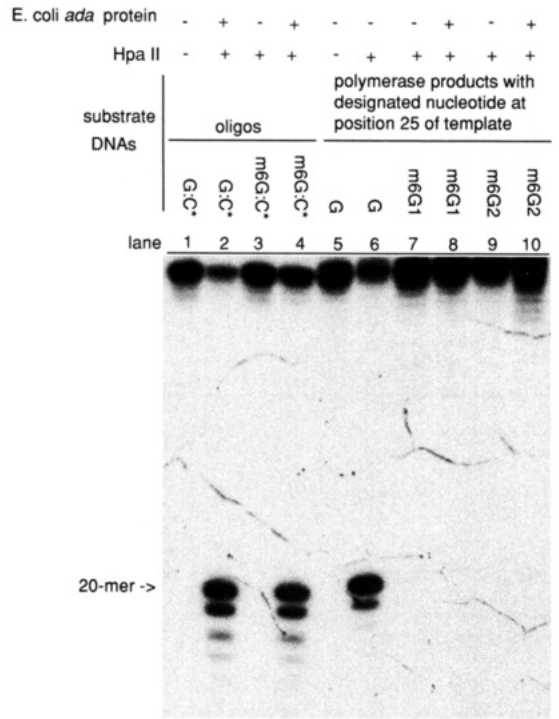


FIGURE 7: m6G, not G, at position 25 in the 45-mer substrates. The m6G was not contaminated by G and did not stimulate Pol I incorporation of dCMP. About 1 ng of the 45-bp DNAs prepared either by strand hybridization (G:C and m6G:C) or by the Klenow polymerase I method (G, m6G1, m6G2) were assayed for whether they contained detectable G at position 25, or C across from m6G, by first incubating with 100 ng of *E. coli* Ada protein in 50 μL of reaction buffer containing 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 1 mM DTT at 37 °C for 1 h, followed by analysis by *Hpa*II digestion as described in Experimental Procedures. All DNAs were 5′-labeled in the bottom strand.

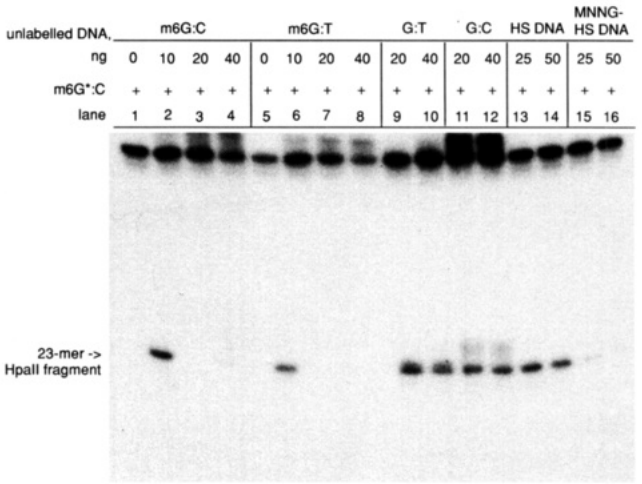


FIGURE 8: Inhibition of human MGMT by DNA containing m6G:T base pairs. Aliquots containing 2 ng of m6G:C substrate DNA (5′-labeled in the top strand) were mixed with the unlabeled DNAs as indicated in 50 mM Tris, pH 7.5, 1 mM EDTA, and 1 mM DTT in 50-μL volumes. Repair of m6G was initiated by adding 10 μg of HT29 extract (as a source of human MGMT) and incubating for 1 h at 37 °C. Products were subjected to *Hpa*II digestion, followed by electrophoretic analysis as before. (Due to field inhomogeneities, the leftmost 23-mer band did not migrate, as it appears, from the sample in lane 2, but from that in lane 1. The amount of skew to the right decreases with increasing lane number.)

deduce that the reported conversion of m6G to G during synthesis of m6G-containing substrates (Borowy-Borowski & Chambers, 1987) must have minimal in our m6G oligonucleotide preparations.] That the *E. coli* Ada protein was capable of removing methyl groups is shown in lanes 3 and 4, in which a DNA containing an m6G:C pair (synthesized totally from phosphoramidites) was cleaved by *Hpa*II only after treatment with *E. coli* Ada protein to convert the m6G:C pair to a G:C pair.

Recognition of DNA Containing m6G:T Pairs by MGMT. We questioned whether MGMT might provide the reason that the DNAs containing m6G:T and G:T pairs were similarly incised by our extracts. The DNA repair protein MGMT might convert the m6G:T pair to a G:T pair, and this latter might serve as a substrate for the known G:T incision process. MGMT, present in extracts from Mer⁺ cells, reacts stoichiometrically with substrates containing m6G:C pairs, inactivating itself by relocating the m6G methyl group onto one of its own cysteine residues. To examine whether MGMT recognizes m6G:T pairs, we determined whether unlabeled m6G:T-containing DNA could compete with labeled m6G:C-containing DNA substrate for MGMT. The 45-bp m6G:C DNA, 5′-labeled in the top strand (Figure 1), served as the MGMT substrate. Human MGMT (HT29 cell-free extract) was assayed in the presence of several unlabeled competitive substrates (Figure 8; the leftmost migrating bands are skewed 1 lane to the right; see legend). Upon reaction with MGMT, the sequence CC^{m6}GG in the substrate (which is not cleavable by *Hpa*II) is converted to CCGG (and becomes cleavable by *Hpa*II). Thus the amount of MGMT reaction is indicated by the amount of *Hpa*II 23-mer in Figure 8. The unlabeled m6G:C (lanes 2–4) and m6G:T (lanes 6–8) DNAs competed with the labeled substrate approximately equally well in the reactions. As expected, competition by DNAs containing G:T or G:C pairs was not observed (lanes 9–12). As a control, herring sperm (HS) DNA treated with MNNG to produce m6G:C pairs (lanes 15 and 16), but not nontreated DNA (lanes 13 and 14), showed competition with the substrate as anticipated.

Lack of Contribution of Human MGMT to Incision of DNAs Containing m6G:T Pairs. Because the human DNA repair protein MGMT interacted with DNAs containing m6G:T base pairs in DNA, the possibility that the m6G:T substrate was incised in a two-step reaction (as in the preceding) was viable. Three lines of experimental evidence all show that MGMT did not influence the activity that incised the 45-bp DNA containing m6G:T base pairs. First, MGMT is reported to be low or undetectable in Mer⁻ cell lines such as A1235 and A172 (Yarosh et al., 1983; 1984) from which extracts that incise the DNA containing m6G:T pairs have been prepared. In the second and third experiments we inactivated any residual MGMT that might nevertheless be present in these extracts. In the second experiment, we took advantage of the fact that MGMT is inactivated irreversibly during the repair of m6G in MNNG-treated cells (Yarosh et al., 1984). We incubated HT29 cells (1.5 pmol of MGMT/mg of protein) with 7 μM MNNG for 1 and 4 h prior to preparing cell-free extracts. Figure 9A depicts the amounts of the 23-mer *Hpa*II-cleavable fragment from the 5′-labeled top strand of the DNA substrate containing the m6G:C pair as a function of amount of extracts prepared from the nontreated (first three lanes) and treated cells (last six lanes). The MNNG treatment reduced to undetectable the amount of active MGMT extractable from the cells. When assayed for incision activity with DNA substrates containing either m6G:T pairs (Figure 9B) or G:T pairs (Figure 9C), the amounts of 20-mer product produced

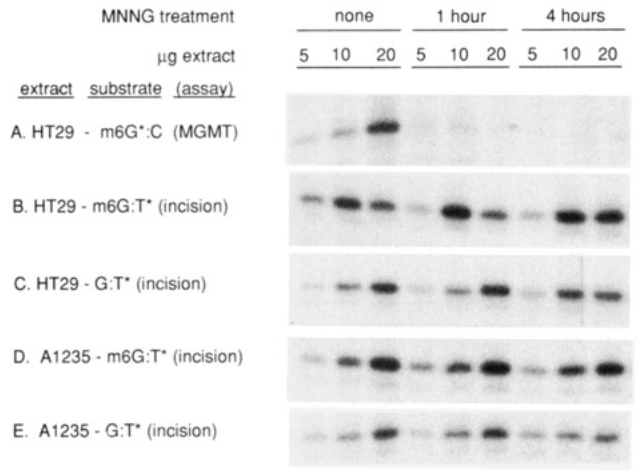


FIGURE 9: Effect on MGMT and incision activities of a 1- or 4-h MNNG treatment prior to the preparation of cell-free extract. Cell lines and substrates shown next to (A–E) indicate the cells used for extract preparation and the substrate analyzed. (A) MGMT activity in HT29 extracts: HT29 cell-free extract (prepared from non-MNNG-treated or 1 and 4 h, 7 μM, MNNG-treated cells as indicated) were incubated with 8 ng of m6G:C substrate (2 ng of 5′-labeled in the top strand plus 6 ng of unlabeled) in nicking buffer for 16 h at 30 °C. The DNA was analyzed for MGMT as described under Experimental Procedures. Incision reactions were similarly done with 8 ng of substrate. (B) Incision activity in HT29 extracts assayed with the m6G:T substrate 5′-labeled in the bottom strand. (C) Incision activity in HT29 extracts assayed with the G:T substrate 5′-labeled in the bottom strand. (D) Incision activity in A1235 extracts assaying the m6G:T substrate 5′-labeled in its bottom strand. (E) Incision activity in A1235 extracts assayed with the G:T substrate 5′-labeled in the bottom strand. Incubation was at 30 °C for 16 h throughout.

by extracts from the treated and the nontreated cells were qualitatively similar at 5 or 10 μg of extract. However, an increase in the incision product from the m6G:T substrate was (and has consistently been) observed at high extract concentration (20 μg/50 μL) in the extract prepared from 4-h MNNG-treated HT29 cells. A similar experiment was done with the Mer⁻ A1235 cells. This line lacks detectable MGMT [Yarosh et al. (1984); see also Figure 10, lane 7]. A1235 was treated with MNNG (as for HT29 cells) to inactivate any "residual" MGMT prior to extract preparation. The extracts were tested for ability to incise DNAs containing m6G:T and G:T pairs, labeled on the 5′ terminus of the bottom strand (Figure 1). Panels D (m6G:T) and E (G:T) of Figure 9 show that incision activity was observed in extracts from the MNNG-treated Mer⁻ cells and that the activity was unaffected by MNNG treatment of the cells. The results are interpreted to mean that the DNA containing the m6G:T pair is recognized and incised by the cell-free extract incision activity independent of the amount of MGMT. (See Discussion.) The decrease in m6G:T incision signal at high HT29 extract concentrations (no MNNG treatment, Figure 9B), and its reversal by MNNG treatment, were unexpected, particularly in view of the absence of such effects in A1235 cells (Figure 9D). The observation leads us to believe that some factor in HT29 extracts may bind the DNA with m6G:T pairs and thereby block incision activity. (See next section.)

The third experiment showing that MGMT does not stimulate incision of DNAs with m6G:T pairs by demethylation takes advantage of the fact that MGMT activity in extracts is inactivated by adding MNNG-treated DNA (containing m6G residues). We did experiments in which MNNG-treated or nontreated DNA was added to HT29 or A1235 extracts prior to performing both an MGMT assay (Figure 10, lanes 1–3 and 7–9) on DNAs with m6G:C pairs and 5′-labeled in the top strand, Figure 1, and an incision

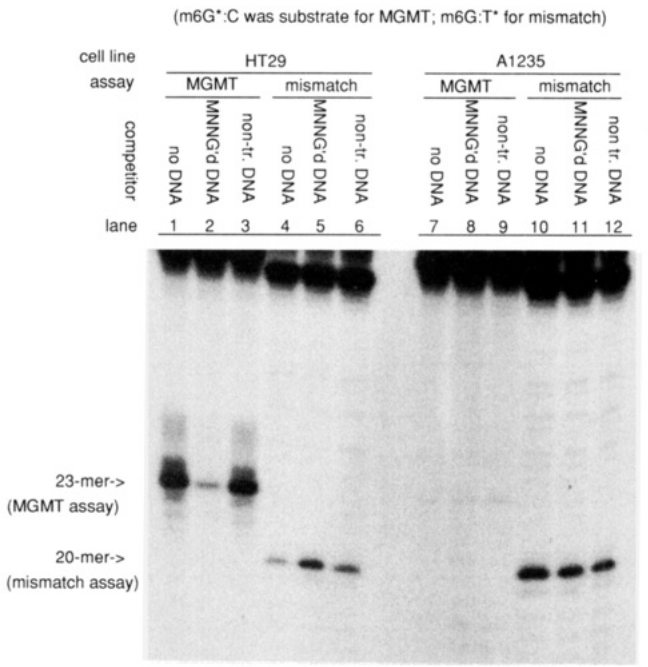


FIGURE 10: Effect of in vitro addition of MNNG-treated herring sperm (HS) DNA on MGMT and incision activities. To assay the effect of MNNG-treated DNA on MGMT activity, aliquots of cell-free extract from HT29 or A1235 cells (20 μg of protein) were incubated with 2 ng of m6G:C substrate (5′-labeled in the bottom strand) and either no DNA (lanes 1 and 7), 100 ng of MNNG-treated HS DNA (lanes 2 and 8), or 100 ng of nontreated HS DNA (lanes 3 and 9) and assayed for MGMT activity. To evaluate m6G:T mismatch activity, reactions with cell-free extract (20 μg) and 8 ng of m6G:T DNA with either no HS DNA (lanes 4 and 10), 100 ng of MNNG-treated HS DNA (lanes 5 and 11), or 100 ng of non-treated HS DNA (lanes 6 and 12) were assayed.

assay (lanes 4–6 and 10–12) with DNAs containing m6G:T pairs and 5′-labeled on the bottom strand. Figure 10 shows that the addition of MNNG-treated DNA (lane 2), but not nontreated DNA (lane 3) or no DNA (lane 1), to HT29 extracts considerably reduced MGMT activity. By contrast, the addition of DNAs to the HT29 extract selectively increased incision activity (compare lanes 5 and 6 to lane 4). Such an increase, which we observe consistently to a greater or lesser (as here) extent, may be due to the removal of an inhibitory factor (possibly the same as in the previous experiment) which would otherwise block incision activity by binding the m6G:T-containing substrate. A similar experiment in which no DNA, MNNG-treated DNA, or nontreated DNA was added to A1235 extracts gave the results in lanes 7–12. No MGMT activity was detected in nontreated cells (lane 7) as previously reported for this line (Yarosh et al., 1984), and the addition of DNA, MNNG-treated (lane 8) or not (lane 9), did not change this. In addition, MNNG-treated DNA did not reduce the incision activity of the A1235 extract any more than non-treated DNA did (compare lanes 10–12).

We conclude (see Discussion) that the extract activity that incises DNAs containing m6G:T pairs does not require MGMT and is independent from MGMT and that the activity recognizes the m6G:T base pair directly without its prior conversion to a G:T pair.

An Inhibitor of m6G:T Incision in HT29 Extracts. We tested whether the putative inhibitor in HT29 cells, alluded to above, might be a DNA-binding factor and whether it would show specificity for one or the other of the substrates. In order to observe the effect of the inhibitor in blocking the incision of m6G:T DNA more definitively, the conditions for the incision reaction were altered from those used in the

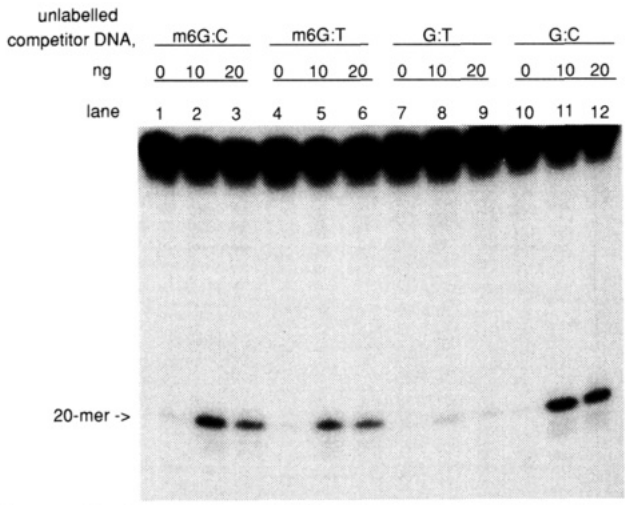


FIGURE 11: Increase in m6G:T incising activity of HT29 cell-free extract with added DNA. Aliquots of HT29 cell-free extract (20 μg; from HT29 cells treated for 1 h with MNNG to inactivate MGMT) were incubated in DNA nicking buffer with 2 ng of m6G:T substrate (5′-labeled on the bottom strand), 40 ng 1 kb DNA ladder, and the indicated amounts of unlabeled DNAs. "20-mer→" designates the size of the incision products generated.

FIGURE 12: Inhibition of m6G:T substrate incision by A1235 cell-free extract with addition of unlabeled G:T DNA. (Added unlabeled m6G:T DNA also inhibits incision of G:T substrate.) (A) Aliquots containing 10 μg of A1235 extract protein were added to mixtures of 2 ng of m6G:T DNA substrate (5′-labeled on the bottom strand), 40 ng of 1-kb ladder, and, as competitor DNAs, the indicated amounts of unlabeled m6G:T (lanes 1–5) or G:T (lanes 6–10) DNAs. (B) Aliquots containing 10 μg of A1235 extract protein were added to mixtures of 2 ng of G:T DNA substrate (5′-labeled on the bottom strand), 40 ng of 1-kb ladder, and, as competitor DNAs, the indicated amounts of unlabeled m6G:T (lanes 1–5) or G:T (lanes 6–10) DNAs. Reaction mixtures were incubated for 16 h at 30 °C and the amounts of 20-mer incision product assayed as in previous figures. Labeled substrates had the sequences shown in Figure 1. Unlabeled competitor DNAs had sequences based on the DNA used by Wiebauer and Jiricny (1989, 1990) and are shown in Experimental Procedures.

previous experiments by elevating the inhibitor (extract) to substrate DNA ratio. This was achieved by replacing the 6 ng of unlabeled 45-mer duplex DNA, which was used to minimize nonspecific breakdown of the 2 μg of ^{32}P-labeled 45-mer substrate DNA during the reaction, with 40 ng of unlabeled 1-kb ladder DNA, which apparently has relatively low affinity for the inhibitor. (See Experimental Procedures.) Reactions were carried out in which the ability of 20 μg of HT29 extract protein to incise 2 μg of labeled m6G:T substrate was measured in the presence of increasing amounts of unlabeled m6G:T, G:T, m6G:C, and G:C DNAs. Figure 11 shows that addition of 10–20 ng of three of the four DNAs increased incision of the substrate which was only detectable with difficulty in the absence of added DNA (lanes 1, 4, 7, and 10) but that addition of 10 or 20 ng of the G:T substrate had no effect (lanes 7–9), probably because this unlabeled DNA competed directly and well for the activity that incises the labeled m6G:T substrate. Thus, there is a DNA-binding factor in HT29 extracts that inhibits m6G:T incision activity, the effects of which can be reversed by added DNA. The fact that unlabeled m6G:T duplex restored activity, whereas added G:T duplex did not, can be explained (i) if the m6G:T duplex binds the incision activity (which would act on both of these substrates) less well than the G:T duplex binds and/or (ii) if the inhibitor has much greater affinity for the m6G:T duplex than it does for the G:T duplex.

Possible Identity of the Activities That Incise the m6G:T and G:T Substrates. We wanted to know whether the activity that incises the m6G:T substrate is indeed the same activity that incises the G:T substrate. To take a first step toward resolving this question, incision reactions on the labeled substrates were performed in the presence of increasing amounts of competitor sequences. Constant amounts of A1235 extract were incubated with the m6G:T substrate (labeled on the 5′ terminus of the bottom strand) in the presence of increasing amounts of unlabeled G:T competitor (Figure 12A) and, conversely, with the G:T substrate (labeled on the 5′ terminus of the bottom strand) in the presence of increasing amounts of unlabeled m6G:T competitor (Figure 12B). [In this experiment, the competitor DNAs, 45-mers with sequences based on that of Wiebauer and Jiricny (1989, 1990; see Experimental Procedures), are different from the substrate 45-mers that had the structures shown in Figure 1.] Figure 12 shows the results of one of three experiments with similar outcomes. Both substrates compete for the activity that incises the other. Added G:T DNA is a stronger competitor than added m6G:T DNA as assayed by incising activity with both the m6G:T (Figure 12A) and the G:T (Figure 12B) DNA substrates. In addition, added unlabeled G:T DNA is more effective at inhibiting the m6G:T activity than it is the G:T activity (compare right sides of Figure 12A and B.) Although competition experiments cannot prove that two proteins (associated with the two activities) do not exist, the result is consistent with the interpretation that one activity incises both substrates and that the affinity of the activity for the G:T substrate is greater than that for the m6G:T substrate.

DISCUSSION

We have found and partly characterized an activity in human tumor cell extracts, prepared according to Manley et al. (1980), that produces incisions both 5′ and 3′ (Figure 4) to the T involved in a m6G:T base mismatch in a synthetic oligodeoxynucleotide (Figure 1), presumably leaving a one-nucleotide gap. Under the conditions used, the activity varied in proportion to protein concentration, but acted relatively slowly, with the amount of incised product still increasing even after 12 h of incubation. Competition experiments showed the activity to be very similar to, and possibly identical to, the activity from human cells that incises G:T base mismatches (Wiebauer & Jiricny, 1989, 1990). The stoichiometrically acting m6G-DNA repair protein MGMT, which normally demethylates m6G in m6G:T-type substrates and is inactivated by doing so, was found to be inactivated (and presumably demethylated) by the m6G:T substrate. However, MGMT is not required for the generation of products of the incision reaction because extracts, demonstrably

diminished in (or lacking) MGMT, incised the m6G:T substrate.

This last conclusion is most clearly perceived as follows. First, we estimate that in the three lanes designated by "4 hours" in panels B and D of Figure 9 there was almost certainly no detectable G:T DNA present to be converted to incised products: the radioactivity in any G:T DNA produced by MGMT demethylation of the labeled m6G:T DNA in Figure 9B (not measurable directly in our experiments) would be approximately equal to the radioactivity in the 23-mer G:C product of MGMT action on the labeled m6G:C substrate (Figure 9A), given that the labeling protocols for the two 45-mer duplexes were the same. Thus, if m6G:T were not a substrate, no incision product bands would be apparent in the "4 hour" lanes, Figure 9, panels B and D (or in Figure 10, lanes 10–12). But these bands are easily detectable, so the m6G:T duplex must be a substrate for the incision activity. Second, by kinetic analysis of a bimolecular reaction limited by the rate of collision of substrate with enzyme, a decrease in the amount of any G:T DNA generated by MGMT acting on m6G:T DNA would be expected to result in a corresponding decrease in the amount of incised product if G:T mismatched duplexes constitute the only authentic substrate. For example, in Figure 10, the band in lane 5 should be 10–20-fold less intense than the bands in lanes 4 and 6, reflecting the 10-20-fold difference seen between the bands in lane 2 and those in lanes 1 and 3. Our reaction is indeed limited both by enzyme (Figure 5) and by substrate (unpublished data), but our data are inconsistent with these expectations. Therefore, to account for the intensity of the bands seen in Figure 10, lane 5 (as well as in the "4 hour" lanes in Figure 9B and D and in Figure 10, lanes 10–12), we conclude that the m6G:T DNA must be as proximal a substrate for the incision activity as is the G:T substrate.

We conclude that extracts prepared from Mer$^+$ HT29 cells contain a DNA-binding factor, possibly sequence-specific (because adding 40 ng of 1-kb ladder had no activating effect on incision by HT29 extracts), that inhibits incision of the m6G:T but not of the G:T substrate. The amount of inhibitory factor appeared to be reduced when a 4-h, 7 μM, MNNG treatment was delivered to the HT29 cells prior to extract preparation. While the incorporation of m6G into synthetic oligonucleotides has been problematic, we have used synthetic procedures reported to ensure the stability and deprotection of m6G-containing moieties during the procedure. Further, we have shown by polymerase incorporation experiments that the base at position 25 of the top strand of our m6G-containing DNAs is not G and behaves as if it were m6G.

We previously presented a hypothesis that accounted for the elevated incorporation of [^{3}H]-dThd into repaired regions of MNNG-treated Mer$^-$ cells (Day et al., 1987): the m6G:C base pair produced in DNA by MNNG would be incised on the strand containing the poorly matched C, and incorporation of nucleotides would occur, including a T opposite the m6G. This incision–synthesis reaction sequence was hypothesized to cycle because the m6G:T base pair generated was itself supposed to act as a substrate for incision. Our current work shows that the details of this incision scheme are likely incorrect because m6G:C is not a substrate for the in vitro reaction. In addition, our initial studies of the capacity of the extracts to perform DNA synthesis on incised substrates (not presented here) indicate that while dCMP is incorporated opposite G as observed by Wiebauer and Jiricny (1989, 1990), very little incorporation of any nucleotide occurs across from m6G. The protein that incises the m6G:T substrate may be a true mismatch endonuclease as is the *E. coli* Vsr protein described by Hennecke et al. (1991). However, if the m6G:T incision activity represents a protein similar to (or identical with) G:T mismatch repair activity, then by analogy to the results of Wiebauer and Jiricny (1989, 1990), the one-base gap produced across from m6G would be due to glycolytic removal of the mismatched T. This would be followed by AP incision into the resultant AP site by class I and class II AP endonucleases. [Class I AP endonucleases, termed lysases, operate by β elimination and produce 5′-PO_4-terminated and blocked 3′ ends, while class II are hydrolytic and produce 3′-OH-terminated and blocked 5′ ends (Levin & Demple, 1990).] Blocked ends can be removed by enzymes such as class II AP endonucleases themselves (3′ ends), or 5′-deoxyribophosphodiesterase [5′ ends; Price and Lindahl (1991)]. Complete repair of incised m6G:T substates would be achieved by (or attempted by) DNA polymerase β followed by ligation. In agreement with this scenario, DNA polymerase β has been reported not to incorporate nucleotides across from m6G (Abbotts et al., 1988).

To accommodate our findings in explaining the observations that m6G is a lethal, mutagenic, and SCE-inducing lesion and, further, that m6G:C base pairs are not incised by the activity, DNA replication across template m6G must be invoked. We believe that semiconservative incorporation of a T across from m6G, followed by gap production removing the poorly paired T, would initiate the biological effects specific to m6G. We propose that lethality and SCE would be favored outcomes if the incision were long-lived. [A long-lived gap is believed to induce SCE (Painter, 1980).] Mutation would result from the cases in which either no incision occurred or a non-dCMP deoxynucleotide was incorporated into the gap and ligated into position. In the latter case, we would envision that DNA polymerase β (following findings implicating this polymerase in G:T mismatch repair) would infrequently insert dTMP across from m6G, both in agreement with in vitro data showing that polymerases preferentially incorporate dTMP across from m6G and to account for the fact that m6G produces G → A transitions in mammalian cells (Ellison et al., 1989). Such insertion would also regenerate the poorly matched m6G:T substrate, which presumably would have a certain probability of being replicated past before the poorly matched pair was again recognized by the incision activity.

An activity that incises m6G:T mismatches has not been reported to our knowledge. Indeed, Leonard et al. (1990) stated the absence of such activity. Three possible explanations for the discrepancy occur: (i) the neighborhood of the mismatch may be important; our substrate is highly rich in GC in the area of the mismatch; (ii) our preparation is a *whole-cell* extract (Manley et al. (1980) whereas others, such as Wiebauer and Jiricny (1989, 1990), used *nuclear* extracts (Dignam et al., 1983); and (iii) the inhibitor of m6G:T incision present in HT29 may have been present in the extracts used by previous workers.

The activity that we have observed is very similar in its incision pattern to the G:T (Wiebauer & Jiricny, 1989, 1990; Yeh et al., 1991) and G:A (Yeh et al., 1991) mismatch-incision activities reported previously, in that it produces one nick in the non-G strand on either side of the mismatched base. To our knowledge there is no evidence linking the analogous activities of *E. coli*, either the MutL–MutS–Vsr, and CC-(A/T)GG-dependent, very short-patch G:T mismatch endonuclease activity (Grilley et al., 1990; Hennecke, et al., 1991) or the mut Y-associated G:A mismatch activity (Au et al., 1989) to the recognition of m6G:T mismatched bases.

There is a report (Karran & Marinus, 1982) implicating the *dam*-dependent mismatch-repair system (Modrich, 1989) in the recognition of m6G in DNA however.

In *E. coli*, the deoxycytidine methylation (Dcm) protein methylates the same sequence—CC(A/T)GG (May & Hattman, 1975)—that the Vsr (very short-patch repair) protein, together with the short-patch polymerase (Pol I), acts to restore during G:T mismatch repair (Lieb, 1985; Sohail et al., 1990; Hennecke et al., 1991). This fact suggested to us that mammalian cells may perform the Wiebauer–Jiricny type G:T mismatch repair, also involving a short-patch polymerase, DNA polymerase β, in order to restore the CG sequences at which cytosine is methylated by mammalian systems. In this regard, it is interesting to note that the mismatched G in our substrate, and those used previously (Wiebauer & Jiricny, 1989, 1990; Yeh et al., 1991) in which Wiebauer–Jiricny type G:T mismatch repair was observed, was contained in a CpG sequence. Studies in which human cell extracts have not been reported to contain a Wiebauer–Jiricny type of G:T mismatch repair activity, but in which G:T base mismatches were repaired, have employed substrates in which the mismatched G is not preceded by a C. The conclusions of these studies were that the nicked G-containing strand of a plasmid containing a G:T mismatch is preferentially repaired (Holmes et al., 1990) or that G:T mismatches are repaired by a long-patch repair system blocked by inhibitors of DNA polymerase α (Thomas et al., 1991). Thus it is quite possible that the repair of m6G:T mismatches, as well as of G:T mismatches, by the Wiebauer–Jiricny activity may show sequence specificity.

The physical basis for the specificity of an activity that recognizes both G:T and m6G:T mismatches is an interesting question. m6G is different from G at two pairing positions: in m6G, the guanine N1 proton is lost and the O6 is methylated, not double-bonded to C6. Thus specificity may be based on the enzyme's detection of both thymine and the 2-amino group of the purine opposite the thymine. The prediction would be that 2-aminopurine:T and 2,6-diaminopurine:T pairs would both be substrates for the m6G:T/G:T mismatch incision reaction.

The observation in MGMT-containing HT29 cells of an inhibitor of the m6G:T (but not of the G:T) incision reaction that is possibly a DNA-binding protein is interesting. The function of such a protein might be to prevent the disruptive incision at m6G:T until MGMT could repair the m6G. MGMT is a very rapidly, but stoichiometrically, acting protein (Olsson & Lindahl, 1980; Harris et al., 1983) that is greatly depleted by MNNG concentrations [1 μM; Yarosh et al. (1984)] considerably less than those leading to appreciable toxicity [5 μM; Scudiero et al. (1984)] in HT29 cells. Thus, by binding the m6G:T pairs more strongly than the incision protein does, the inhibitor may protect (from incision activity) the m6G that is both left after MNNG-induced depletion of MGMT and driven into m6G:T base pairs by semiconservative DNA synthesis. The relative deficiency of such a protein in the MGMT-lacking line A1235 indicates the possibility that the intracellular amounts of the inhibitor and of MGMT may be under coordinate control, but more data are required to make this conclusion.

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