Cytotoxic Mechanism of 6-Thioguanine: hMutSα, the Human Mismatch Binding Heterodimer, Binds to DNA Containing S^6-Methylthioguanine†

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Received August 26, 1996; Revised Manuscript Received January 7, 1997⊗

ABSTRACT: It has been suggested that the cytotoxicity of 6-thioguanine depends upon (1) incorporation of 6-thioguanine into DNA, (2) methylation by *S*-adenosylmethionine (SAM) of the thio group to give S^6-methylthioguanine, (3) miscoding during DNA replication to give [^{SMe}G]·T base pairs, and (4) recognition of these base pairs by proteins of the postreplicative mismatch repair system. Here we have investigated systematically the ability of proteins present in human cell extracts to bind to DNA containing S^6-methylthioguanine. We found that [^{SMe}G]·T base mismatches were bound by the mismatch binding complex, hMutSα, and that the level of binding was dependent upon the base 5′ to the S^6-methylthioguanine in the order G > C = A > T. Extracts from cells that lack either hMSH2 (LoVo cells) or GTBP (HCT-15 cells), two components of the hMutSα complex, were unable to bind the [^{SMe}G]·T base pair. We also found that hMutSα was able to bind to [^{SMe}G]·C base pairs when the S^6-methylthioguanine was in the sequence 5′-Cp[^{SMe}G]. This suggests that miscoding by S^6-methylthioguanine residues in DNA during DNA synthesis may not be an absolutely required step in the mechanism of cytotoxicity. Also, since CpG sequences are so important in gene regulation, this result may be of considerable significance.

6-Thioguanine is an important drug used for the treatment of acute leukemia (Elion, 1989). It produces a delayed cytotoxicity which has been associated with the incorporation of 6-thioguanine into DNA (LePage, 1963), but the mechanism of this toxicity was unknown until recently when it was shown to involve the postreplicative mismatch repair system (Swann et al., 1996). This system in animals is closely related to the MutHLS repair pathway of *Escherichia coli*, which is perhaps the best understood DNA repair system for correcting DNA mismatches (Modrich, 1991, 1994). In this system, the MutS protein binds to DNA mismatches, where it recruits the MutL protein to the complex. MutL is then thought to activate the MutH protein which nicks the incorrect DNA strand at a GATC sequence to one side of the mismatch. A similar system for postreplicative DNA repair exists in eukaryotes (Modrich, 1994; Kolodner, 1995). In human cells, the MutS homologs, hMSH2 (Fishel et al., 1993; Leach et al., 1993) and GTBP (Drummond et al., 1995; Palombo et al., 1995), have been identified and shown to form a heterodimer (designated hMutSα) that binds to G·T mismatches and to 1−3 base extrahelical loops.

Recent work has shown that in eukaryotic cells the postreplicative mismatch repair system plays an essential role in the cytotoxic action of S_N1 methylating agents such as *N*-methyl-*N*-nitrosourea (MNU)[1] and *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) and that cells that lack a component of this system are resistant to the cytotoxic effects of these agents (Branch et al., 1993, 1995; Hawn et al., 1995; de Wind et al., 1995). Some of these cells are also resistant to the toxic effects of 6-thioguanine (Green et al., 1989; Aquilina et al., 1989, 1995; Hawn et al., 1995). There are some similarities in the toxic effects of 6-thioguanine and MNU; they both show a delayed cytotoxicity [i.e. cell death and chromosomal damage occur in the second round of cell replication after administration of the drug (LePage, 1963; Plant & Roberts, 1971)], and they both produce sister chromatid exchanges (Bodell, 1991; Rasouli-Nia et al., 1994). The toxicity of MNU involves methylation of the 6-position of guanine in DNA to form O^6-methylguanine, misincorporation of thymine opposite the O^6-methylguanine during DNA replication, and recognition of the resultant [^{OMe}G]·T base pair by the postreplicative mismatch repair system [reviewed in Karran and Bignami (1994)]. 6-Thioguanine becomes incorporated into DNA after metabolic conversion to 2′-deoxy-6-thioguanosine triphosphate, and it is known that this incorporation is important in the delayed cytotoxicity of this drug (LePage, 1963). We have recently shown that (1) 6-thioguanine residues in DNA are methylated *in vivo* by SAM to form S^6-methylthioguanine, (2) S^6-methylthioguanine can miscode during DNA replication which leads to incorporation of thymine or cytosine with roughly equal probability, and (3) hMutSα binds to the resultant [^{SMe}G]·T mismatches (Swann et al., 1996). The similarities between the cytotoxic action of 6-thioguanine and MNU, and the close parallel between the metabolic events following the formation of O^6-methylguanine and the formation of S^6-methylthioguanine, lead to the inescapable conclusion that the methylation, miscoding, and recognition of the resultant mismatches by hMutSα are important steps in the mechanism of the cytotoxicity of 6-thioguanine. Recently, hMutSα was shown to bind to cisplatin adducts in DNA, and it has been

† This work was supported by the Cancer Research Campaign, U.K., and by the Medical Research Council, U.K.

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⊗ Abstract published in *Advance ACS Abstracts,* February 15, 1997.

[1] Abbreviations: SAM, *S*-adenosylmethionine; [^{SMe}G], S^6-methylthioguanine; MNU, *N*-methyl-*N*-nitrosourea; MNNG, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine; [^{OMe}G], O^6-methylguanine; [^{6S}G], 6-thioguanine; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; BSA, bovine serum albumin.

S0006-2960(96)02157-5 CCC: $14.00

suggested that this binding is involved in the cytotoxic action of cisplatin also (Duckett et al., 1996).

We now report systematic studies of the binding of hMutSα to S^6-methylthioguanine in DNA. We have measured semiquantitatively the effect of the base 5′ to guanine or S^6-methylthioguanine in G·T and [^{SMe}G]·T mismatches upon the level of hMutSα binding. hMutSα binds to [^{SMe}G]·T in all four sequence contexts but generally less tightly than to G·T mismatches. The identity of the 5′ flanking base appears to have no influence on the binding to G·T mismatches but significantly influences binding to [^{SMe}G]·T base pairs. Surprisingly, it was found that hMutSα can bind to [^{SMe}G]·C base pairs when the S^6-methylthioguanine is in the sequence 5′-Cp[^{SMe}G]. A recent report has shown that hMutSα can also bind to the structurally similar O^6-methylguanine·cytosine base pair, but in this case, the O^6-methylguanine was preceded by a thymine (Duckett et al., 1996).

EXPERIMENTAL PROCEDURES

Synthesis and Purification of Oligodeoxynucleotides. Oligodeoxynucleotides with the general sequence of the 34-base pair DNA duplex used by Griffin et al. (1994) (i.e. AGC TTG GCT GCA GGN XGA CGG ATC CCC GGG AAT T, where N is A, G, C, or T and X is G or S^6-methylthioguanine) were synthesized on an Applied Biosystems 391 DNA synthesizer. Oligodeoxynucleotides containing S^6-methylthioguanine at position X were synthesized using the method of Xu et al. (1995) in which an oligodeoxynucleotide is synthesized with 6-thioguanine in the X-position and the 6-thioguanine then converted into S^6-methylthioguanine by reaction with methyl iodide. Oligomers were separated from failure sequences using Nensorb columns (DuPont), further purified by ion exchange chromatography at pH 12 (Xu & Swann, 1992) using a Mono-Q column (Pharmacia), and finally desalted. Oligodeoxynucleotides prepared in this way were >95% pure as judged by 254 nm absorbance of their chromatography traces.

Preparation of Cell Extracts. Whole cell extracts of HeLa cells were prepared essentially as described in Jiricny et al. (1988). However, the extract was buffer exchanged by gel filtration into 25 mM Hepes (pH 7.6), 400 mM KCl, 1 mM EDTA, 1 mM benzamidine, 20% glycerol, and 5 mM DTT and then concentrated by spin filtration using a Centricon-30 device (Amicon). The total protein concentration was determined using a dye binding-based assay (Bradford, 1976). The extract was aliquoted out, frozen in liquid nitrogen, and stored at −70 °C. Aliquots were used only once in these experiments and were not subjected to further freeze/thaw cycles.

RajiTK$^-$, LoVo, and HCT-15 cell extracts were generously provided by P. Karran (Imperial Cancer Research Fund, U.K.). To be consistent with the HeLa experiments, these extracts were also buffer exchanged and concentrated as above.

Binding Assay. Cell extract at a final concentration of 0.26 mg of protein/mL was preincubated at 0 °C with 12 μg/mL of poly(dI-dC)·poly(dI-dC) in 20 mM Hepes (pH 7.6), 50 mM KCl, 5 mM $MgCl_2$, 2 mM DTT, and 4% Ficoll 400. After 15 min, ^{32}P-labeled 34-base pair DNA duplex (sequence as indicated in the text) was added to a final concentration of 0.5 nM, and the solutions were incubated for a further 20 min at room temperature. Competitor oligodeoxynucleotides, when used, were added to a 40-fold excess (i.e. 20 nM) at the same time as the labeled DNA. Incubated samples were electrophoresed in nondenaturing 6% polyacrylamide gels (8 V/cm for 2 h at 10−12 °C). The dried gels were autoradiographed, and the amount of DNA in each band was measured by scintillation counting.

Assay of O^6-Alkylguanine−DNA Alkyltransferase Activity in Cell Extracts. Cell extract, at a final concentration of 0.26 mg/mL, was preincubated at 0 °C for 15 min with 12 μg/mL poly(dI-dC)·poly(dI-dC) in 50 mM Tris (pH 7.4), 10 mM DTT, 1 mM EDTA, and 0.2 mg/mL BSA. O^6-Alkylguanine−DNA alkyltransferase was then assayed by incubation at room temperature with 0.8 nM ^{32}P-labeled GGCGCT[^{OMe}G]GAGGCGTC (annealed to its fully complementary strand). Samples were removed at various times, and DNA strands in which the methyl group had been removed from the O^6-methylguanine were separated from the original strand by ion exchange chromatography at pH 12 on a NucleoPac PA-100 (Dionex) column. The amount of conversion was assessed by scintillation counting of the eluted fractions.

To determine whether any of the S^6-methylthioguanine in the DNA was converted to 6-thioguanine by the O^6-alkylguanine−DNA alkyltransferase present in the extract, a 34-base pair DNA duplex containing an [^{SMe}G]·T base pair was 5′-labeled with ^{32}P on the S^6-methylthioguanine-containing strand and the DNA incubated with HeLa cell extract under the conditions used for the binding assay. After 20 min of incubation, the 34-base pair DNA duplex was analyzed by ion exchange chromatography as described above except that radiolabeled DNA in the eluate was detected by Cerenkov counting using a Berthold LB 506 C-1 radioactivity monitor.

RESULTS

Protein(s) in the HeLa Cell Extract Bind to DNA Containing G·T and [^{SMe}G]·T Base Pairs. Figure 1A shows that protein(s) in the HeLa extract bound to an oligodeoxynucleotide containing a G·T mismatch in the sequence GpG·T (i.e. with a guanine 5′ to the guanine of the mismatch). The amount of this ^{32}P complex was reduced when excess unlabeled oligodeoxynucleotide of the same sequence containing a G·T mismatch was present but was not reduced by excess oligodeoxynucleotide containing a G·C base pair. The complex was also effectively competed for by excess unlabeled oligodeoxynucleotide of the same sequence containing an [^{SMe}G]·T mispair (i.e. Gp[^{SMe}G]·T), whereas a duplex in which the S^6-methylthioguanine was base paired with cytosine (i.e. Gp[^{SMe}G]·C) competed poorly.

A complex was also formed with a ^{32}P-labeled oligodeoxynucleotide containing S^6-methylthioguanine base paired with thymine (Figure 1B). This complex was competed for effectively by excess unlabeled 34-base pair DNA duplex containing either a G·T or an [^{SMe}G]·T mismatch, but oligodeoxynucleotides in which the guanine or S^6-methylthioguanine were base paired with cytosine (i.e. GpG·C and Gp[^{SMe}G]·C, respectively) had little effect on the level of binding.

Experiments were done to ensure that the protein(s) was not binding to [^{6S}G]·T base pairs produced by enzymatic demethylation of the S^6-methylthioguanine by O^6-alkylguanine−DNA alkyltransferase present in the extract. Using

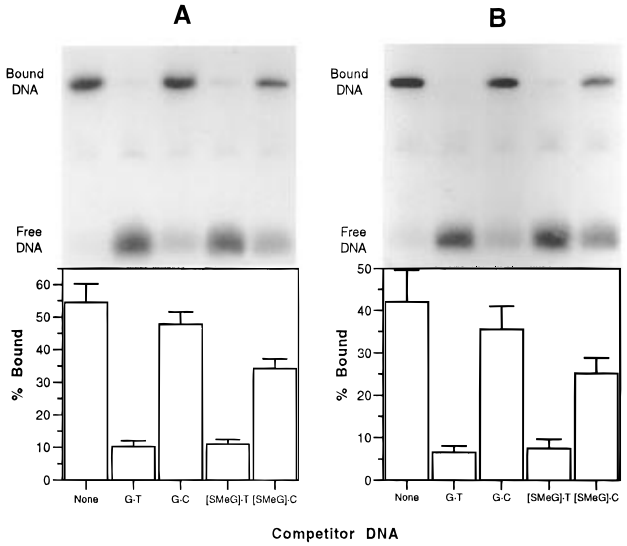

FIGURE 1: Recognition of DNA duplexes containing G·T and [^{SMe}G]·T base pairs by protein(s) in HeLa cell extract. Part A shows the formation of a protein/DNA complex when HeLa cell extract was incubated with a ^{32}P-labeled 34-base pair DNA duplex containing a GpG·T mismatch and the effect of 40-fold excess competitor DNA duplexes containing the base pairs indicated in the figure. Part B shows the same experiment as in part A but with a ^{32}P-labeled 34-base pair DNA duplex containing a Gp[^{SMe}G]·T base pair. Top panels show autoradiograms of polyacrylamide gels with bound and free DNA bands as indicated. Bottom panels show graphs of the level of binding with error bars representing standard deviations for at least three different experiments.

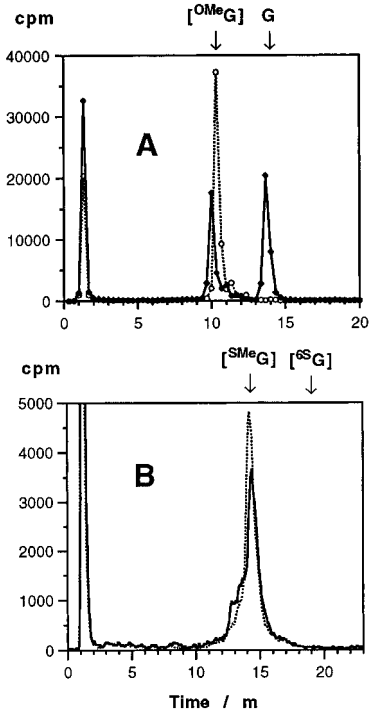

FIGURE 2: (A) Analysis of the amount of O^6-alkylguanine−DNA alkyltransferase present in the HeLa cell extract. A ^{32}P-labeled oligodeoxynucleotide containing [^{OMe}G]·C was incubated with HeLa extract and analyzed by high-performance liquid chromatography as described in Experimental Procedures. Eluted fractions were collected and scintillation counted. The dotted line and open symbols (○) represent the elution profile of the sample before addition of extract. The solid line and closed symbols (◆) represent the trace of the sample after incubation with HeLa cell extract. The [^{OMe}G] shows the elution position of DNA containing O^6-methylguanine, and the G shows the position of DNA containing guanine produced by the action of O^6-alkylguanine−DNA alkyltransferase upon O^6-methylguanine. Part B shows that the HeLa cell extract does not remove the methyl group from S^6-methylthioguanine under the conditions used for the binding assay. ^{32}P-labeled oligodeoxynucleotide containing an [^{SMe}G]·T base pair was incubated with cell extract and analyzed as in part A except that radioactivity was monitored by Cerenkov counting of the eluant (solid line). [^{SMe}G] and [^{6S}G] indicate the elution positions of DNA containing S^6-methylthioguanine and 6-thioguanine, respectively. One can see that none of the S^6-methylthioguanine was converted to 6-thioguanine. The dotted line shows the elution profile of the same oligodeoxynucleotide before incubation with cell extract. The peaks at around 1 min in both graphs are due to residual [^{32}P]ATP from the labeling of the oligodeoxynucleotides.

the assay described in Experimental Procedures, the HeLa cell extract was found to contain 1.7 μmol of O^6-alkylguanine−DNA alkyltransferase per milligram of protein (Figure 2A). Chromatography showed that incubation of a DNA duplex containing an [^{SMe}G]·T base pair with HeLa cell extract under the conditions used for the binding assay produced no oligodeoxynucleotide containing 6-thioguanine (Figure 2B, detection limit of approximately 2%). Comparison of this trace with that for the same DNA sample in the absence of extract shows that incubation with extract has reduced the main peak by about one-tenth and produced new radioactive material that elutes as a shoulder to the main peak. This indicated that the DNA was undergoing some kind of reaction. To investigate this, DNA duplexes containing either an [^{SMe}G]·T, a G·T or a G·C base pair were incubated with HeLa cell extract and the products analyzed on a DNA-sequencing gel. In all cases, the analysis showed that exonucleolytic cleavage of one to three nucleotides from the 3′ end of the parent 34-base pair oligodeoxynucleotide had occurred. Under the conditions used for the band shift assay, only around 10% of the probe had even one nucleotide removed. Since in some cases more than 60% of the DNA was bound, this level of digestion should have little effect on the interpretation of the results.

Influence of the Local DNA Sequence upon the Extent of Binding. The effect of the base 5′ to the guanine and S^6-methylthioguanine in G·T and [^{SMe}G]·T mismatches upon the amount of complex formed is shown in Figure 3. The level of binding to oligodeoxynucleotides containing G·T mismatches was essentially independent of the 5′ base, in agreement with previously published data (Griffin & Karran, 1993; Griffin et al., 1994). By contrast, binding to [^{SMe}G]·T base pairs was strongly influenced by the base 5′ to the S^6-methylthioguanine. The protein(s) bound to the Gp[^{SMe}G]·T oligodeoxynucleotide almost as well as it did to the G·T-containing oligodeoxynucleotides; however, the amount of complex formed with Ap[^{SMe}G]·T and Cp[^{SMe}G]·T was about one-third of that seen with the G·T oligomers, and the amount of complex formed with Tp[^{SMe}G]·T was only about one-sixth of that level. A similar effect of the 5′ base has been reported in studies of the binding of [^{OMe}G]·T mismatches by extracts of Raji cells (Griffin et al., 1994).

When binding of each of these oligodeoxynucleotides was tested in the presence of excess unlabeled oligodeoxynucleotides of the same sequence containing either G·T, G·C, [^{SMe}G]·T, or [^{SMe}G]·C base pairs, essentially the same pattern of competition as that seen in Figure 1 was observed, except when the 5′ base was a cytosine (discussed further below). That is, 34-base pair DNA duplexes containing G·T or [^{SMe}G]·T mismatches could compete for binding, whereas duplexes containing G·C or [^{SMe}G]·C could not.

The ability of each of the mismatch oligodeoxynucleotides to cross compete for the binding of one of the labeled DNA

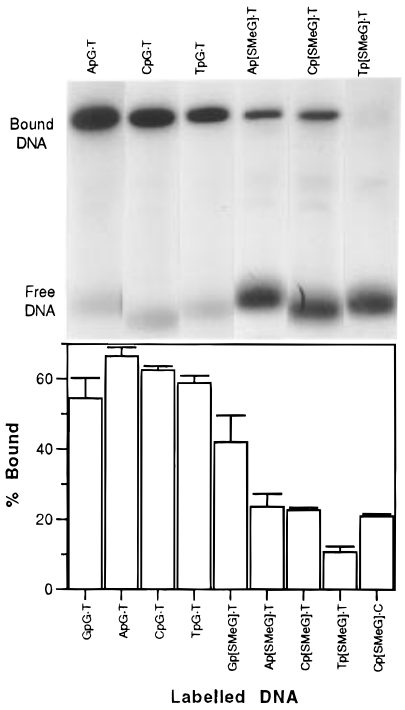

FIGURE 3: Effect of the base 5′ to the mismatched guanine or S^6-methylthioguanine on the extent of binding by protein(s) in HeLa cell extract to DNA duplexes containing G·T and [^{SMe}G]·T mismatches. ^{32}P-labeled 34-base pair DNA duplexes, with the sequence shown, were incubated with HeLa cell extract as described in Experimental Procedure, and free DNA was separated from protein/DNA complexes by gel electrophoresis. The upper panel shows typical autoradiograms for the oligomers, and the lower panel shows a graph of the level of binding in each case with error bars showing the standard deviations for at least three separate experiments.

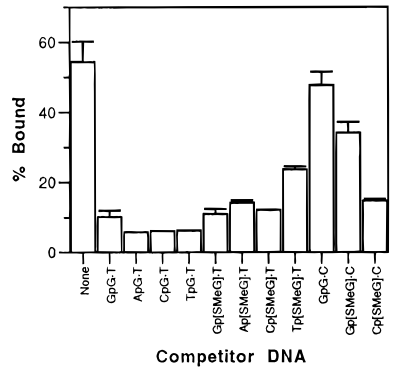

FIGURE 4: Graph showing the ability of 34-base pair DNA duplexes containing various mismatches to compete with a 34-base pair DNA duplex containing a G·T mismatch for binding by protein(s) in HeLa cell extract. HeLa cell extract was incubated with a ^{32}P-labeled 34-base pair DNA duplex containing GpG·T together with a 40-fold excess of the competitor DNA duplex indicated, as described in Experimental Procedures. Error bars represent standard deviations for at least three separate experiments.

duplexes, GpG·T, was also tested. The results shown in Figure 4 show essentially the trend expected from the results given in Figure 3. Those oligodeoxynucleotides that were bound well by the protein(s) were also good competitors for GpG·T binding, whereas oligodeoxynucleotides that were bound less strongly were poorer competitors. These results indicate that it is the same protein species that binds all of the G·T and [^{SMe}G]·T mismatches.

The Same Protein(s) Also Binds to an [^{SMe}G]·C-Containing Oligodeoxynucleotide. In Figure 1, it can be seen that the oligodeoxynucleotide containing an [^{SMe}G]·C base pair (with a guanine 5′ to the S^6-methylthioguanine) competes for binding of the labeled G·T 34-base pair DNA duplex better than the perfectly matched G·C oligodeoxynucleotide (compare lane 5 with lane 3). This effect, although small, was also observed when adenine was 5′ to the S^6-methylthioguanine. However, when cytosine was 5′ to the S^6-methylthioguanine, the effect was much greater, and the Cp[^{SMe}G]·C 34-base pair DNA duplex was as effective a competitor as Cp[^{SMe}G]·T. This led us to suspect that the protein(s) that bound G·T and [^{SMe}G]·T mismatches would also be able to bind to [^{SMe}G]·C base pairs, particularly if the S^6-methylthioguanine had a cytosine on the 5′ side. When labeled Cp[^{SMe}G]·C oligodeoxynucleotide was incubated with HeLa cell extract, a protein/DNA complex was detected (Figure 5), and this complex had the same mobility as that formed with the 34-base pair DNA duplexes containing a G·T mismatch. The extent of binding of the Cp[^{SMe}G]·C 34-base pair DNA duplex was roughly as great as that seen with the oligodeoxynucleotides Cp[^{SMe}G]·T and Ap[^{SMe}G]·T, in which the S^6-methylthioguanine is base paired with thymine. The formation of the complex with Cp[^{SMe}G]·C could be competed for by the CpG·T, Cp[^{SMe}G]·T, or Cp[^{SMe}G]·C oligodeoxynucleotides but not by the perfectly matched CpG·C oligodeoxynucleotide (Figure 5). Cp[^{SMe}G]·C could also compete reasonably effectively for binding of GpG·T (Figure 4).

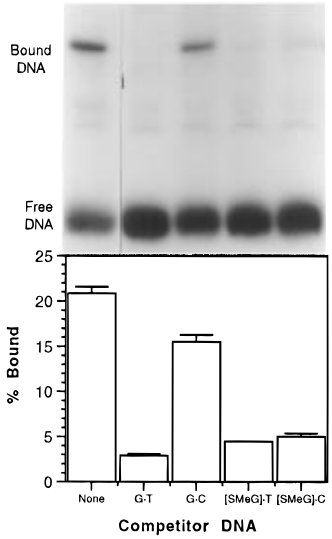

FIGURE 5: Protein(s) in HeLa cell extract binds to a 34-base pair DNA duplex containing an [^{SMe}G]·C base pair. HeLa cell extract was incubated with a ^{32}P-labeled 34-base pair DNA duplex containing Cp[^{SMe}G]·C together with a 40-fold excess of the unlabeled competitor DNA duplex, as described in Experimental Procedures. Error bars represent standard deviations for three separate experiments.

Protein(s) Which Binds [^{SMe}G]·T Base Pairs Is Also Present in Extracts of RajiTK$^-$ Cells but Not in Extracts of LoVo or HCT-15 Cells. Extracts of the O^6-alkylguanine−DNA alkyltransferase deficient cell line, RajiTK$^-$ (less than 0.03 μmol of enzyme per milligram of protein in our assay), contained protein(s) which bound specifically to DNA containing G·T and to DNA containing [^{SMe}G]·T mismatches. The binding with Gp[^{SMe}G]·T showed essentially the same pattern of competition by other oligodeoxynucleotides as that seen with the HeLa cell extract shown in Figure 1B (Figure 6). However, extracts of LoVo and HCT-15 cells did not form a specific complex with DNA containing either G·T or [^{SMe}G]·T base pairs. The same experimental conditions

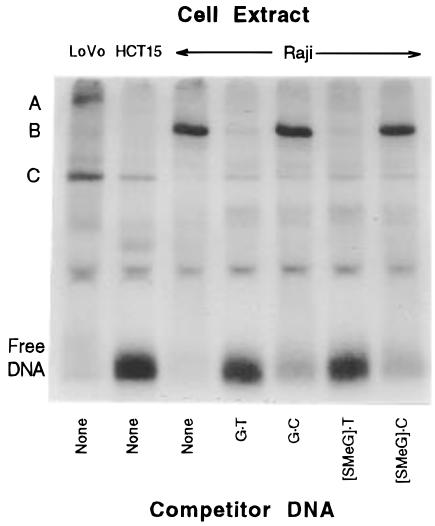

FIGURE 6: Protein(s) which binds [^{SMe}G]·T base pairs is present in extracts of RajiTK$^-$ cells but not in extracts of LoVo or HCT-15 cells. Cell extracts were incubated with a ^{32}P-labeled 34-base pair DNA duplex containing Gp[^{SMe}G]·T together with a 40-fold excess of the competitor DNA duplex, as described in Experimental Procedures. Bands A and C indicate nonspecific complexes, and band B is the G·T specific complex. Bands A and C are competed for by perfectly matched DNA duplexes. Also, they are produced when a perfectly matched DNA duplex is incubated with LoVo cell extract and so do not represent the specific recognition of mismatches in DNA.

[i.e. same concentration of protein, same level of poly(dI-dC)·poly(dI-dC), and same buffer composition] as for the HeLa experiments were used, and under these conditions, the LoVo cell extract gave two complexes (Figure 6; bands A and C) that had different mobilities to the G·T complex formed with HeLa cell extract. These complexes appeared to be due to nonspecific DNA binding proteins as their formation was competed for as effectively by the perfectly matched G·C and [^{SMe}G]·C oligodeoxynucleotides as by the mismatched G·T and [^{SMe}G]·T oligodeoxynucleotides. In addition, these two complexes were also detected when a radiolabeled perfectly matched DNA duplex was incubated with LoVo cell extract. With the HCT-15 cell extract, only a small amount of the non-specific band C could be detected. Band C is presumably the same nonspecific complex that has previously been reported to be present in LoVo, HCT-15, and other cell extracts (Branch et al., 1995).

DISCUSSION

6-Thioguanine shares several similarities with MNU and other S_N1 methylating agents that generate O^6-methylguanine in DNA. Firstly, both MNU and 6-thioguanine display a delayed cytotoxic effect (LePage, 1963; Plant & Roberts, 1971); secondly, they are both able to produce sister chromatid exchanges (Bodell, 1991; Rasouli-Nia et al., 1994), and thirdly, it is known that certain eukaryotic cells that are resistant to the effects of MNU are also resistant to 6-thioguanine (Aquilina et al., 1989, 1995; Green et al., 1989; Hawn et al., 1995). The essential steps in the cytotoxicity of MNU are methylation of the O^6-position of guanine, misincorporation of thymine opposite O^6-methylguanine during DNA replication, and recognition of the [^{OMe}G]·T base pairs by mismatch repair proteins. Recently, we have reported that the similarity between 6-thioguanine and MNU exists because 6-thioguanine follows an analogous pathway (Swann et al., 1996). After incorporation into DNA, the 6-thioguanine is methylated *in vivo* by cellular SAM to give S^6-methylthioguanine. During replication, either thymine or cytosine can be incorporated opposite S^6-methylthioguanine with roughly equal efficiency and resultant [^{SMe}G]·T base pairs are bound by the hMutSα heterodimer.

In this paper, we have studied this binding in more detail. We have shown that protein(s) in HeLa cell extract that bind to oligodeoxynucleotides containing G·T mismatches also bind to oligodeoxynucleotides containing [^{SMe}G]·T base pairs. The complex formed with the G·T-containing oligodeoxynucleotide presumably involves binding by the heterodimer, hMutSα (Drummond et al., 1995; Palombo et al., 1995). There are three reasons for believing that the complex with the [^{SMe}G]·T-containing oligodeoxynucleotides also involves hMutSα. (1) The complexes have the same electrophoretic mobility as the complexes formed with oligodeoxynucleotides containing G·T mismatches. (2) Binding of oligodeoxynucleotides containing a G·T mismatch can be competed for by [^{SMe}G]·T-containing oligodeoxynucleotides, and conversely, complexes with [^{SMe}G]·T-containing oligomers can be competed out by G·T-containing oligodeoxynucleotides. (3) Neither extracts of LoVo cells, which have defective hMSH2 genes (Umar et al., 1994), nor HCT-15 cells, which lack GTBP (Papadopoulos et al., 1995), gave detectable specific binding of [^{SMe}G]·T-containing oligodeoxynucleotides under the same experimental conditions.

Since it is known that hMutSα will bind to DNA containing [^{6S}G]·T mismatches approximately as well as it binds G·T mismatches (Griffin et al., 1994), it was necessary to confirm that the S^6-methylthioguanine in the oligodeoxynucleotides was not being converted to 6-thioguanine by O^6-alkylguanine−DNA alkyltransferase present in the extract and that the complexes we were observing were indeed with [^{SMe}G]·T base pairs and not due to binding of [^{6S}G]·T base pairs formed by such demethylation. The HeLa extract contained 1.7 μmol of O^6-alkylguanine−DNA alkyltransferase per milligram of protein. However, since the reaction between O^6-alkylguanine−DNA alkyltransferase and S^6-methylthioguanine is very slow ($k = 79$ M^{-1} s^{-1}; Swann et al., 1996), one would calculate that less than 0.005% of the S^6-methylthioguanine would be converted to 6-thioguanine during the incubation with HeLa extract in the binding assay. Chromatography of DNA containing S^6-methylthioguanine after incubation with HeLa extract under conditions used for binding showed that no 6-thioguanine had been formed (Figure 2B). Furthermore, specific complexes with [^{SMe}G]·T containing oligodeoxynucleotides were observed using extracts of RajiTK$^-$ cells that lack O^6-alkylguanine−DNA alkyltransferase (Figure 6). These two results show that the complexes seen with [^{SMe}G]·T oligodeoxynucleotides are indeed due to binding of [^{SMe}G]·T base pairs and not of [^{6S}G]·T base pairs produced by demethylation of the S^6-methylthioguanine.

While binding of oligodeoxynucleotides containing a G·T mismatch was essentially independent of the base 5′ to the mismatched guanine, binding of oligodeoxynucleotides containing [^{SMe}G]·T base pairs was strongly influenced by the 5′ base. The pattern of binding, Gp[^{SMe}G]·T > Cp-[^{SMe}G]·T = Ap[^{SMe}G]·T > Tp[^{SMe}G]·T, was essentially the same as that which has been reported for similar oligodeoxynucleotides containing [^{OMe}G]·T (Griffin et al., 1994). This illustrates another similarity between the effects of 6-thioguanine and MNU and suggests that [^{OMe}G]·T base pairs and [^{SMe}G]·T base pairs have a similar structure. In

fact, molecular modeling shows that, although sulfur is bigger than oxygen and carbon−sulfur bonds are longer than carbon−oxygen bonds, because the C−S−C bond has a more acute angle than the C−O−C bond the overall shapes of O^6-methylguanine and S^6-methylthioguanine are expected to be very similar. Although we have not looked at the effect of the base 3′ to the S^6-methylthioguanine, it seems likely that this too will influence hMutSα binding.

The results in Figure 5 show that, in at least one sequence context (that is, with cytosine 5′ to the S^6-methylthioguanine), a complex is formed with oligodeoxynucleotides containing [^{SMe}G]·C base pairs. This complex migrated at the same position as the complex formed between hMutSα and 34-base pair DNA duplexes containing G·T mismatches, and it could be competed for by excess unlabeled CpG·T and Cp-[^{SMe}G]·T DNA duplexes but not by the CpG·C DNA duplex. Also, Cp[^{SMe}G]·C was an effective competitor for the binding of hMutSα to an oligodeoxynucleotide containing a G·T mismatch (Figure 4). Extracts of the G·T mismatch binding defective cell lines LoVo and HCT-15 did not form specific complexes with the Cp[^{SMe}G]·C oligomer (unpublished observations). Taken together, these results lead to the unexpected conclusion that hMutSα can form a complex with an oligodeoxynucleotide that does not contain a mismatched thymine. Although we have only tested the binding of one [^{SMe}G]·C containing oligodeoxynucleotide, the fact that Ap-[^{SMe}G]·C and Gp[^{SMe}G]·C are more effective competitors for hMutSα binding than their respective G·C-containing oligodeoxynucleotides suggests that they too may be bound by hMutSα, albeit with a reduced affinity. These results show that the misincorporation of thymine opposite S^6-methylthioguanine during DNA replication to form [^{SMe}G]·T base pairs may not be an essential step in the recognition by the mismatch repair system. Duckett et al. (1996) have reported binding of purified hMutSα to [^{OMe}G]·T and to [^{OMe}G]·C base pairs. In addition, they show that hMutSα can bind to cisplatin adducts of DNA. Therefore, hMutSα recognizes a variety of DNA substrates in addition to G·T mismatches and 1−3 base extrahelical loops.

In view of the importance of CpG sequences in gene regulation [reviewed in Cross and Bird (1995)], the binding to Cp[^{SMe}G]·C may have important biological consequences. The similarity of mismatch recognition between O^6-methylguanine and S^6-methylthioguanine raises the possibility that hMutSα may also be able to recognize Cp[^{OMe}G]·C base pairs produced by methylation of CpG by the carcinogenic *N*-nitroso compounds. Previous results with oligodeoxynucleotides containing O^6-methylguanine showed that a 34-base pair DNA duplex containing an [^{OMe}G]·C base pair was not an effective competitor for binding to a 34-base pair DNA duplex containing a G·T mismatch (Griffin et al., 1994). However, only data for an oligodeoxynucleotide in which guanine was 5′ to the O^6-methylguanine was given, and it is unclear whether [^{OMe}G]·C in other sequence contexts produced the same result. More recently, purified hMutSα has been shown to bind to an oligodeoxynucleotide containing an [^{OMe}G]·C base pair in the sequence Tp[^{OMe}G] (Duckett et al., 1996). The level of binding was comparable to that seen with an oligodeoxynucleotide of the same sequence containing an [^{OMe}G]·T base pair but was one order of magnitude lower than that seen with the equivalent oligodeoxynucleotide containing a G·T base pair.

ACKNOWLEDGMENT

We are very grateful to Dr. M. Edwards for preparing the HeLa cells and to Dr. P. Karran for giving us the RajiTK$^-$, LoVo, and HCT-15 cell extracts. We also thank Dr. Y.-Z. Xu for the 6-thioguanine-containing oligodeoxynucleotides.

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BI9621573