DNA Mismatch Binding and Incision at Modified Guanine Bases by Extracts of Mammalian Cells: Implications for Tolerance to DNA Methylation Damage

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ABSTRACT: Two activities involved in separate pathways for correcting G·T mispairs in DNA have been assayed on duplex substrates containing modified guanine bases. The first, the G·T mismatch incision activity, is specifically involved in short-patch repair of mispairs arising *via* deamination of 5-methylcytosine. The second activity can be detected by its ability to bind to G·T mispairs and may initiate correction by a long-patch mechanism. 6-Thioguanine and O^6-methylguanine paired with thymine were efficiently incised by cell extracts if the modified guanine was in a CpG dinucleotide. Incision was not observed when either purine was paired with cytosine. Extracts of cells that are tolerant both to methylation damage and to 6-thioguanine in DNA also incised 6-thioguanine·T and O^6-methylguanine·T base pairs. The data suggest that this activity is unlikely to contribute significantly to the biological effects of O^6-methylguanine in DNA. A defect in this pathway is therefore unlikely to explain the cross-resistance of tolerant cells to the two base analogs in DNA. In binding assays, 6-thioguanine·T base pairs were recognized efficiently and to an equivalent extent by the same protein complex as G·T mispairs. O^6-Methylguanine·T base pairs were also recognized but with reduced efficiency. No binding was observed to 6-thioguanine·C or O^6-methylguanine·C base pairs. Recognition by the binding complex was essentially independent of the base immediately 5′ to the mismatched guanine but was somewhat more efficient if O^6-methylguanine was preceded by a purine. Extracts of two tolerant lines with a known defect in G·T mismatch binding failed to form complexes with substrates containing the modified bases. The ability of the G·T binding factor to recognize both O^6-methylguanine·T and 6-thioguanine·T pairs indicates that the long-patch repair pathway is more likely to be involved in mediating the cytotoxicity of the two-base analogs.

Single-base mispairs that arise during DNA replication in mammalian cells and escape proofreading can be corrected by a postreplicative excision repair pathway. This pathway involves excision and resynthesis of a relatively long stretch of DNA ("long-patch repair") and efficiently corrects DNA mismatches *in vivo* (Brown & Jiricny, 1988) and *in vitro* (Holmes et al., 1990; Thomas et al., 1991). In addition to formation by replication errors, G·T mismatches also arise in resting DNA *via* deamination of 5-methylcytosine (5-meC). A dedicated pathway ("short-patch repair") corrects the mispairs that arise from these deamination events. Short-patch repair is initiated in mammalian cells by a specific thymine-DNA glycosylase that removes the mismatched base. The subsequent incision is performed by an endonuclease specific for abasic sites (AP endonuclease), and repair is completed by the replacement of the correct base as a repair patch of one nucleotide (or very few nucleotides) (Wiebauer & Jiricny, 1989, 1990). We have previously shown that mammalian cells partly overcome the problem of recognition of such ectopic thymine residues among the mass of DNA thymines by limiting incision to sites of deaminated 5-meC (Griffin & Karran, 1993). Since 5-meC is effectively confined to CpG sequences in mammalian DNA, the first two steps of the short-patch pathway can be assayed by measuring incision adjacent to the thymine base in oligonucleotides containing a CpG·T or a 5-meCpG·T mismatch.

In addition to the G·T-specific DNA glycosylase activity, cell extracts also contain proteins that bind to single-base mispairs in DNA. These proteins, which are assayed by band-shift analysis, form different complexes with DNA fragments containing various single-base mismatches. One complex is formed with A·C, T·T, or T·C mismatches (Stephenson & Karran, 1989) whereas a different complex is formed preferentially with G·T mispairs (Jiricny et al., 1988). Binding to G·T mismatches is independent of the immediate sequence context of the mismatch (Jiricny et al., 1988; Griffin & Karran, 1993). This is an important requirement for an activity that is involved in correcting replication errors and is consistent with its involvement in the long-patch correction pathway.

DNA mismatch correction has been indirectly implicated in the lethality of simple methylating agents. The sensitivity of *Escherichia coli dam* mutants to *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) is reversed by a second mutation in either *mutS* or *mutL* (Jones & Wagner, 1981; Karran & Marinus, 1982). The *E. coli* MutS and MutL proteins are involved in recognition of DNA mismatches (Modrich, 1991). Mammalian cells can acquire resistance to MNNG or the related methylating agent *N*-methyl-*N*-nitrosourea (MNU) in an apparently similar fashion [for review, see Karran and Bignami (1992)]. The cytotoxicity of both of these alkylating drugs is dependent on replication of damaged DNA and is partly a consequence of their ability to methylate guanine at the O-6 position. Cells lacking a specific repair enzyme, O^6-meG-DNA methyltransferase (MGMT), are hypersensitive to both MNNG and MNU. This hypersensitivity can be corrected by expression of a transfected MGMT. O^6-meG is also mutagenic because DNA polymerases preferentially

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incorporate thymine opposite the methylated base to generate G:C to A:T transitions. Thus, O^6-meG·T mispairs are likely intermediates in cell killing and mutagenesis [for reviews, see Pegg (1990) and Karran and Bignami (1992)]. The acquired resistance of tolerant cells to alkylating agents is a consequence neither of detoxification or exclusion of drug from the cells nor of an increased DNA repair capacity. Instead, these cells are able to grow despite the presence of O^6-methylguanine (O^6-meG) in their DNA. On becoming tolerant to methylating agents, cells simultaneously acquire a mutator phenotype (Goldmacher et al., 1986). In two tolerant lines this phenotype is conferred by a defect in the mismatch binding activity that preferentially recognizes G·T mismatches in DNA (Branch et al., 1993). In a third line, tolerance is associated with the loss of the long-patch repair pathway and results in failure to correct all single-base DNA mispairs in a cell-free extract (Kat et al., 1993). Thus, mammalian cells as well as *E. coli* can acquire resistance to methylating agents through a compromised DNA mismatch correction pathway.

An interesting feature of tolerant cells is their frequent cross-resistance to 6-thioguanine (6-thioG) in DNA (Green et al., 1989; Aquilina et al., 1990). This base analog shares a number of properties with O^6-meG including the capacity to code ambiguously during replication and the inability to form a perfect base pair (Rappaport, 1993). Cells are able to salvage 6-thioG *via* hypoxanthine/guanine phosphoribosyltransferase (HPRT) and to incorporate the thiolated base into DNA. Subsequent replication of the substituted DNA is aberrant, and discontinuities accumulate in daughter strands (Pan & Nelson, 1990). Tolerant cells have acquired the ability to perform efficient replication of DNA highly substituted with 6-thioG (Aquilina et al., 1990).

The cross-resistance of tolerant cells to O^6-meG and 6-thioG in DNA, together with the absence of cross-resistance to O^6-ethylguanine and other DNA damage, suggests that an important requirement for tolerance is a similarity of the base analog to guanine. It has been proposed [reviewed in Karran and Bignami (1992)] that this similarity allows recognition of O^6-meG- and 6-thioG-containing base pairs by a mismatch correction system. We have assayed the "short-patch" G·T mismatch incision and the "long-patch" G·T mismatch binding activities with substrates containing O^6-meG and 6-thioG in order to investigate whether either of these pathways might operate on DNA containing the modified purines. Our observations suggest that the short-patch mismatch repair pathway does not contribute to the lethality of the methylated base and that long-patch correction is the more likely candidate for the lethal pathway that is altered to confer simultaneous resistance to both 6-thioG and O^6-meG on tolerant cells.

MATERIALS AND METHODS

Cells and Cell Culture. The Mex$^-$ HeLa cell line (HeLaMR) and its MNU/6TG tolerant derivatives 5A1 were maintained in Dulbecco's modified Eagle's medium containing 10% calf serum. The TK$^-$ Mex$^-$ variant of the Burkitt's lymphoma line Raji, its MNU/6TG tolerant derivative RajiF12, and the parental TK$^+$ Mex$^+$ Raji cells were maintained in RPMI 1640 containing 5% calf serum.

Synthesis and Purification of Unmodified Oligonucleotides. 34mer oligonucleotides synthesized as described previously (Griffin & Karran, 1993) were 5′ end-labeled by T4 polynucleotide kinase with [γ-^{32}P]ATP and purified by elution from 12% denaturing polyacrylamide gels. Duplex substrates were prepared by annealing a 5-fold molar excess of the unlabeled strand to the appropriate labeled strand.

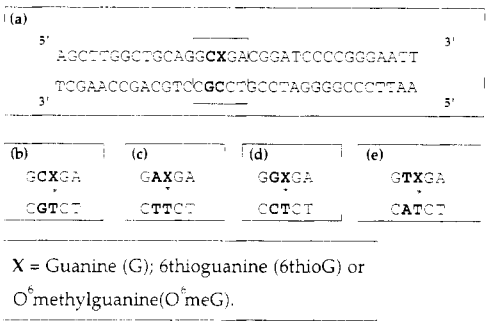

FIGURE 1: Sequence of 34mer oligonucleotides used in this study. Both strands of the perfectly paired duplex are shown in frame a. At the mismatch site (position 16 of the upper strand as written), the upper strand contained either a guanine, 6-thioguanine, or O^6-methylguanine (denoted X) opposite a thymine or cytosine. The mismatch was preceded on the 5′ side by the bases shown in frames a–e.

Synthesis and Purification of Modified Oligonucleotides. 34mer oligonucleotides containing O^6-meG and 6-thioG (Figure 1) were prepared as previously described (Xu et al., 1992). In brief, oligonucleotides (1-μmol scale) were synthesized on a 391-DNA automatic synthesizer (Applied Biosystems) using PAC amidites of the normal bases (Pharmacia). The portion of the oligonucleotide 3′ to the modified guanine was synthesized on the machine, and then a versatile monomer, 5′-*O*-(4,4′-dimethoxytriphenylmethyl)-*N*^{2}-(phenylacetyl)-2′-deoxy-6-(2,4-dinitrophenyl)thioguanosine 3′-*O*-(2-cyanoethyl *N*,*N*-diisopropylphosphoramidite) (Glen Research), was added manually. Ten milligrams of the monomer was dissolved in 0.1 mL of anhydrous CH_3CN, and 0.1 mL of 0.5 M tetrazole in anhydrous CH_3CN was added. The bottom end of the cartridge containing the CPG-support and partially synthesized oligomer was disconnected from the machine, and the mixture of the monomer and tetrazole was injected from a gas-tight syringe. The syringe was used to draw the solution into and out of the cartridge several times over a period of 3 min to increase coupling yield; then the cartridge was immediately reconnected to the synthesizer to complete the synthesis.

After synthesis, the CPG-support bearing the synthetic oligomer was divided into two parts and transferred into two Eppendorf tubes. To one of the tubes were added 100 μL of mercaptoethanol and 900 μL of concentrated ammonia, and the mixture was left for 2 days at 25 °C. The product of this reaction was a substituted and deprotected oligomer containing 6-thioguanine. The deprotected oligomer containing O^6-meG was generated by adding 1 mL of 10% (v/v) 1,8-diazabicyclo-[5.4.0]undec-7-ene/methanol to the other tube and incubating for 2 days at 25 °C.

The products were first purified on a Nensorb nucleic acid purification cartridge (DuPont) according to the manufacturer's instructions. The oligomers were further purified by FPLC (Xu & Swann, 1992). Oligonucleotides of identical lengths containing guanine, O^6-meG, or 6-thioG are well separated under these conditions. The FPLC-purified oligomers were desalted with Sep-Pak columns (Waters). Heteroduplex oligonucleotides were prepared by annealing single strands one of which was radioactively labeled at the 5′ end by T4 polynucleotide kinase.

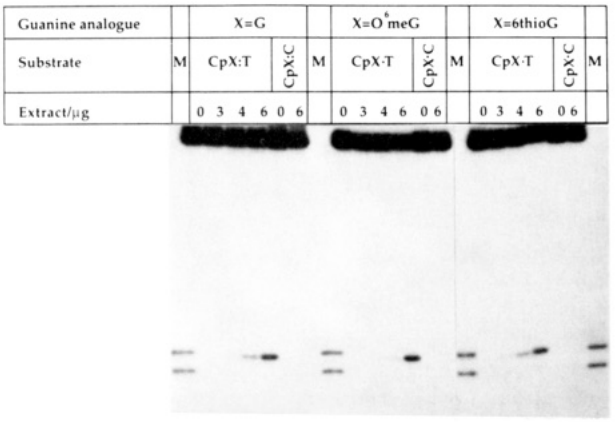

FIGURE 2: Incision at G·T or guanine analog·T base mispairs by extracts of HeLaMR cells. Duplex 34mer oligonucleotides containing a CpG·T, Cp-O^6-meG·T, or Cp-6-thioG·T mispair or controls containing a CpG:C, Cp-O^6-meG·C, or Cp-6-thioG·C were radioactively labeled in the strand containing the mismatched pyrimidine and incubated with HeLaMR cell extract as indicated. After 16 h at 30 °C, reaction products were isolated and analyzed as described in Materials and Methods. Radioactively labeled 17mer and 18mer oligonucleotides were included as size markers (M).

Cell Extracts and Assays

Incision Assay. Cell extracts for the G·T mismatch nicking assay were prepared essentially by the procedure of Li and Kelly (1984). Assays were carried out as previously described (Griffin & Karran, 1993). In the standard assay, 40 fmol of end-labeled duplex substrate was incubated with cell extract in 20 μL of 50 mM Pipes·NaOH pH 6.7, 10 μM $ZnCl_2$, 0.5 mM EDTA, 0.5 mM DTT for 16 h at 30 °C. Reaction products were separated on 12% denaturing polyacrylamide gels and detected by autoradiography.

Binding Assay. Mismatch binding was detected by band-shift assays as previously described (Griffin & Karran, 1993). Briefly, end-labeled heteroduplex oligonucleotides (20 fmol) were added to cell extracts prepared as described in Jiricny et al. (1988) that had been preincubated (5 min at 20 °C) with a 2–4-fold excess of unlabeled matched competitor duplex. After a further 20 min at 20 °C in the presence of the labeled substrate, free and bound oligonucleotides were separated by electrophoresis on 6% nondenaturing polyacrylamide gels and detected by autoradiography.

Where necessary, quantitation was carried out by scanning densitometry.

RESULTS

Incision at O^6-meG·T and 6-thioG·T Base Pairs. The substrates used to assay incision activity are shown in Figure 1. HeLa cell extracts specifically incised the T-containing strand of a standard 34mer oligonucleotide in which a G·T mismatch was in a CpG dinucleotide (Figure 2). The extent of reaction increased with extract up to 6 μg, and 20–30% of substrate was cleaved under optimal conditions. Incision was confined to the T-containing strand and occurred immediately 5′ to the mismatched T. Comparison of cleavage at G·T, 6-thioG·T, and O^6-meG·T mismatches in the CpG sequences by HeLa cell extracts is shown in Figure 2. The extent of nicking as a function of the immediate sequence context of the target base pair is presented in Figure 3 (in this figure, only the products of the reaction are shown). Cell extracts efficiently incise G·T mispairs when the mispaired G is in a CpG dinucleotide. The T-containing strands of substrates containing Cp-O^6-meG·T and Cp-6-thioG·T base pairs were

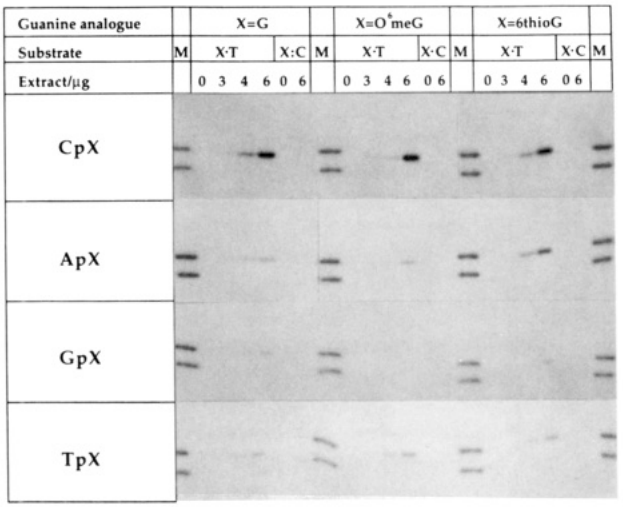

FIGURE 3: Dependence of incision at guanine analog·T base mispairs on local DNA sequence context. Duplex 34mers in which the mismatch position was occupied by base pairs of the general designation NpX·T or NpX·C (where N = C, A, G, or T and X = G, O^6-meG, or 6-thioG) and labeled in the mismatched T- or C-containing strand were incubated with the indicated amounts of HeLaMR cell extract for 16 h at 30 °C. Reaction products were isolated and analyzed as described in Materials and Methods. Radioactively labeled 17mer and 18mer oligonucleotides were included as size markers (M).

each cleaved to similar extents at the phosphodiester bond immediately 5′ to the mismatched T. In contrast, ApG·T, TpG·T, Ap-O^6-meG·T, Tp-O^6-G·T, and Tp-6-thioG·T base pairs were poorly incised and essentially no nicking was observed at GpN·T mismatches (where N = G, O^6-meG, or 6-thioG). Incision at Ap-6-thioG·T pairs was reproducibly more efficient than at ApG·T. No cleavage was observed when G or either of the modified purines was paired with C in any sequence context. When the duplex substrates were radiolabeled in the strand that contained the purine analog, no cleavage products were observed, indicating that incision is confined to the T-containing strand (data not shown). Thus, these results strongly suggest that both O^6-meG·T and 6-thioG·T base pairs are incised by the G·T mismatch incision activity. The cleavage displays approximately the same preference for local sequence context as nicking at G·T mismatches although Ap-6-thioG·T pairs are somewhat more susceptible to incision than a G·T mismatch in the same context. Incision at O^6-meG and 6-thioG bases paired to pyrimidines is therefore effectively confined to O^6-meG·T and 6-thioG·T pairs that occur in CpG sequences in DNA.

Incision by Tolerant Cell Extracts. The cell lines HeLa5A1 and RajiF12 are DNA methylation tolerant variants of HeLaMR and Raji cells, respectively. Both variants display the characteristic cross-resistance to the toxicity of O^6-meG and 6-thioG (G. Aquilina, P.B., unpublished data). Extracts of the tolerant cells were unimpaired in their ability to incise O^6-meG·T and 6-thioG·T base pairs located in Cp-O^6-meG or Cp-6-thioG sequences (Figure 4).

Recognition by Mismatch Binding Proteins. Two mismatch binding activities are present in HeLa, Raji, and CHO cells. One recognizes A·C, T·C, and T·T mispairs whereas the other exhibits a preference for G·T mismatches. The tolerant variant RajiF12 is deficient in G·T binding *in vitro*. In order to investigate the relation between tolerance and mismatch recognition, binding to O^6-meG- and 6-thioG-containing base pairs was assayed in extracts of RajiF12 and its parental line. When the binding reaction was carried out with Raji cell

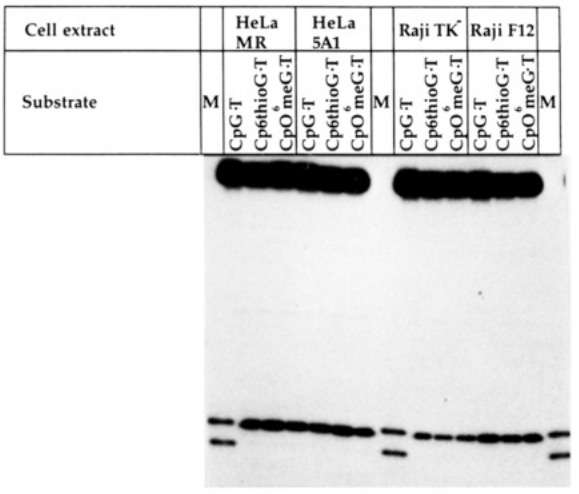

FIGURE 4: Incision at guanine analog·T base mispairs by extracts of two alkylation tolerant cell lines. Duplex 34mer oligonucleotides containing a CpG·T, Cp-6-thioG·T, or Cp-O^6-meG·T mispair as shown were radioactively labeled in the strand containing the mismatched T and incubated with 10 μg of cell extract prepared from the alkylation tolerant cell lines RajiF12 and HeLa5A1 or their respective parental lines, RajiTK$^-$ and HeLaMR. Reaction conditions and analysis of products were as described in Materials and Methods. Radioactively labeled 17mer and 18mer oligonucleotides were included as size markers (M).

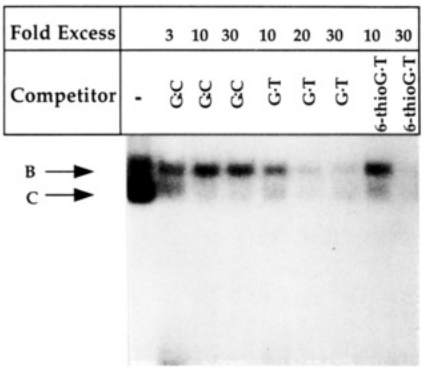

FIGURE 5: Recognition of 6-thioguanine·T base pairs by a G·T mismatch binding activity. Extracts of Raji cells (15 μg) were combined with 60, 200, or 600 fmol of nonradioactive matched duplex 34mer oligonucleotide (3-, 10-, and 30-fold excess as shown) or equivalent amounts of heteroduplex containing a G·T or 6-thioG·T base pair as shown and incubated for 5 min at 20 °C. Duplex 34mer oligonucleotide (20 fmol) containing a 6-thioG·T base pair at the mismatch position and radioactively labeled in the T-containing strand was then added and incubation continued for a further 20 min at 20 °C. The sample in the far left-hand lane was not preincubated with nonradiolabeled oligonucleotide. Formation of protein:oligonucleotide complexes was analyzed as described in Materials and Methods. The unbound oligonucleotide was allowed to migrate out of the gel in order to improve the resolution of bands B and C.

extracts and an oligonucleotide that contained a 6-thioG·T pair, two delayed migrating complexes were observed by gel electrophoresis (Figure 5). The more rapidly migrating complex C was also formed with substrates containing 6-thioG·C base pairs, all perfectly matched and mismatched substrates (data not shown). Its formation was suppressed by the inclusion of a moderate excess of perfectly matched competitor oligonucleotide (G:C). Complex B, which migrated at the position of the complex formed with an oligonucleotide containing a G·T mismatch, was formed when the 6-thioG·T substrate was used. It was not formed with a 6-thioG·C oligonucleotide (data not shown). The extent of specific binding to the 6-thioG·T substrate was unaffected by the matched competitor, but the inclusion of a 10–30-fold excess of nonradioactive oligonucleotide containing a G·T mismatch abolished complex B. Inclusion of similar concentrations of unlabeled 6-thioG·T oligonucleotide also abolished specific binding. These data indicate that the protein complex that recognizes G·T mismatches in DNA fragments recognizes 6-thioG·T (but not 6-thioG·C) base pairs. The similar susceptibility to competition with nonradioactive oligonucleotides suggests that G·T and 6-thioG·T base pairs are recognized with approximately equal affinity.

Less binding was observed with duplexes containing O^6-meG. Complex B was formed with O^6-meG·T base pairs but not with O^6-meG·C pairs. Substrates containing the O^6-meG·T base pair were bound poorly compared to those containing 6-thioG·T or G·T (Figure 6a). Extracts of the methylation tolerant line, RajiF12, which is defective in G·T mismatch binding, did not bind to either the 6-thioG·T duplex or the O^6-meG·T duplex. This observation provides a separate confirmation that recognition of 6-thioG·T and O^6-meG·T base pairs is most probably by the previously described G·T binding activity. The preferential binding to G·T over O^6-meG·T base pairs was investigated further by competition experiments, an example of which is shown in Figure 6b. Formation of the delayed migrating complex on a G·T duplex was effectively abolished by inclusion of a 10–30-fold excess of unlabeled G·T duplex. A similar excess of O^6-meG·T duplex was inefficient as a competitor, and significant reduction in binding was only observed when the competitor duplex was included at a 100-fold excess. Reduction in binding following inclusion of the O^6-meG·C duplex did not exceed that seen with the G·C competitor. These data confirm that O^6-meG·T base pairs are a substrate for the previously described G·T binding activity and that they are recognized somewhat less efficiently than G·T mismatches under the conditions of our experiments.

In the experiments shown in Figure 6a,b, the guanine or guanine analog of the mispair was preceded by thymine at the 5′ side. We have previously demonstrated that G·T mismatch binding does not exhibit a substantial sequence dependence, and this observation holds for 6-thioG·T base pairs (Figure 6c). Throughout the course of this work, however, we observed a moderate but consistent preferential binding to O^6-meG·T base pairs in which the O^6-meG was 3′ to a purine over those in which it was preceded by a pyrimidine. Gp-O^6-meG·T pairs were bound somewhat better than Ap-O^6-meG·T pairs. Examples are shown in Figure 6c (compare to Figure 6a and Figure 7).

Mismatch Binding and Demethylation of O^6-meG. We investigated the relationship between mismatch binding and demethylation at O^6-meG-containing base pairs. RajiTK$^+$ cells are of the Mex$^+$ phenotype and express active O^6-meG-DNA methyltransferase (MGMT). This enzyme, which is specific for double-stranded DNA, demethylates O^6-meG *in situ*. It is active on O^6-meG·T pairs, and the product of the reaction is DNA containing G·T mispairs. Figure 7 shows that G·T mispairs in two different sequence contexts were complexed to similar extents by extracts of Mex$^+$ and Mex$^-$ Raji cells. Binding by the Mex$^-$ Raji cell extract to Gp-O^6-meG·T was more extensive than to Tp-O^6-meG·T, as expected. Extracts of the Mex$^+$ Raji cells contained 0.5 pmol of MGMT/(mg of protein). Following incubation with the extracts of Mex$^+$ Raji cells, the amount of complex formed with both Tp-O^6-meG·T and Gp-O^6-meG·T substrates increased. The 15-μg extract contained 7.5 fmol of MGMT, which is sufficient to demethylate approximately one-third of the O^6-meG·T

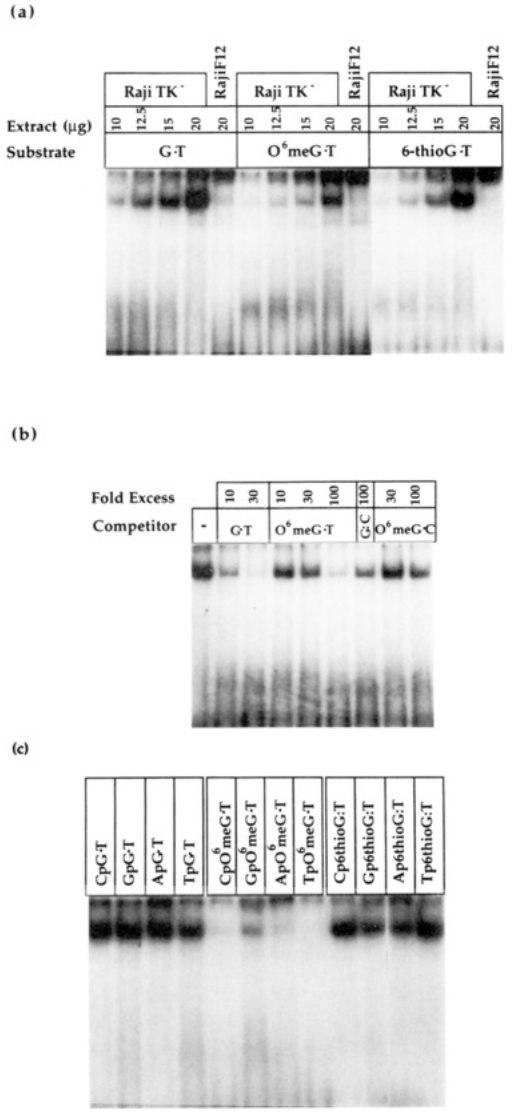

FIGURE 6: Affinity of the G·T binding activity for substrates containing guanine analogs. (a, top frame) Comparative binding to G·T, O^6-meG·T, and 6-thioG·T substrates. Extracts of RajiTK⁻ or alkylation tolerant RajiF12 cells as shown were preincubated for 5 min at 20 °C with 80 fmol of a matched nonradioactive 34mer duplex. Radiolabeled duplexes (20 fmol) containing GpG·T, Gp-O^6-meG·T, or Gp-6-thioG·T at the mismatch position were then added as indicated and incubation continued for a further 20 min. Binding was analyzed by gel electrophoresis as described in Materials and Methods. (b, middle frame) Competition for binding with nonradiolabeled G·T, O^6-meG·T, G:C, and O^6-meG·C duplexes. RajiTK⁻ cell extract (15 μg) was preincubated for 5 min at 20 °C with 80 fmol of matched duplex (lane 1) or with an additional 10–100-fold excess of nonradioactive homo- or heteroduplexes (200–2000 fmol) that contained GpG·T, Gp-O^6-meG·T, G:C, or Gp-O^6-meG·C at the mismatch position. Radiolabeled G·T duplex (20 fmol) was then added, and after a further 20-min incubation, the extent of binding was determined as described in Materials and Methods. (c, bottom frame) Dependence of binding at G·T and guanine analog·T base mispairs on local DNA sequence context. RajiTK⁻ cell extract (20 μg) was preincubated with matched duplex for 5 min at 20 °C. Radiolabeled duplex 34mers (20 fmol) in which the mismatch position was occupied by base mismatches of the general designation NpX·T (where N = C, G, A, or T and X = G, O^6-meG, or 6-thioG as shown). were then added, and the extent of binding was determined as above.

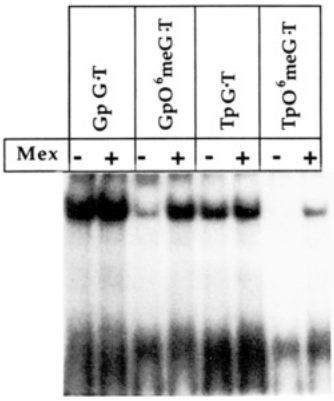

FIGURE 7: Mismatch binding by extracts of Mex⁺ and Mex⁻ Raji cells. Extracts (15 μg) of the Mex⁻ RajiTK⁻ or the MGMT-expressing variant RajiMex⁺ cells as shown were preincubated for 5 min at 20 °C with 40 fmol of a matched nonradioactive 34mer duplex. Radiolabeled duplexes (20 fmol) containing, at the mismatch position, G·T or O^6-meG·T in which the purine was preceded on the 5′ side by either G or T as shown were then added as indicated. Incubation was continued for a further 20 min, and binding was analyzed by gel electrophoresis as described in Materials and Methods.

substrate. The observed increase in binding by the Mex⁺ extract with the O^6-meG·T substrate is consistent with this level of MGMT activity converting part of the O^6-meG·T substrate to a G·T mismatch. This observation confirms that O^6-meG is present in a double-stranded form which allows its demethylation by MGMT in both of the substrates. It further indicates that recognition of O^6-meG-containing base pairs by the mismatch binding activity probably does not prevent recognition of the methylated base by its specific repair enzyme.

DISCUSSION

Acquired resistance to chemotherapeutic agents is a common clinical phenomenon. A number of agents such as the methyltriazenes and procarbazine kill cells by introducing O^6-meG into DNA (Hayward & Parsons, 1984; Schold et al., 1989). The clinical effectiveness of these drugs is compromised by the rapid appearance of tumor resistance. The base analog 6-thioguanine is another therapeutic agent to which resistance is also frequently encountered. It is likely that the phenomenon of methylation tolerance with its associated cross-resistance to 6-thioguanine in DNA is a laboratory model for some aspects of this clinical situation.

It has been postulated that tolerance to guanine analogs in DNA may arise by loss of a mismatch repair function. The recent reports of methylation tolerant cell lines that are selectively defective in the G·T mismatch binding activity (Branch et al., 1993) or are unable to correct single-base mismatches in cell-free extracts (Kat et al., 1993) support this possibility. The work presented here was undertaken to test the hypothesis in mammalian cells by investigating the possible contributions of two known mismatch correction activities. The G·T incision and binding functions operate in different pathways. The former is probably involved in a short-patch mismatch repair targeted to sites of cytosine deamination whereas the latter most likely participates in long-patch correction and binding is essentially sequence-independent (Griffin & Karran, 1993).

The observed nicking of the T-containing strand of O^6-meG·T and 6-thioG·T base pairs suggests that the short-patch pathway might contribute to the lethality of guanine base analogs. Incision at O^6-meG·T base pairs has previously been reported by Sibghat-Ullah and Day (1992), who hypothesize that this reaction underlies the biological effects of O^6-meG in DNA. In our experiments, however, efficient incision was confined to Cp-O^6-meG·T and Cp-6-thioG·T (or Ap-6-thioG·T) sequences. This observation is in agreement with our previously determined sequence preferences for incision at G·T mispairs (Griffin & Karran, 1993). It therefore appears

likely that the same activity is responsible for the incision of both G·T and modified G·T mispairs. We consider it unlikely that the short-patch pathway is involved in the lethal effects of O^6-meG for two reasons. Firstly, the production of O^6-meG in DNA by methylating carcinogens is not random and guanine bases preceded 5′ by a pyrimidine are, on average, 5–6 times less readily methylated at the 6 position than G residues preceded on the 5′ side by a purine (Richardson et al., 1989; Mironov et al., 1993). Secondly, CpG sequences are considerably underrepresented in mammalian genomes and the CpG:GpC ratio is about 0.2 in human DNA (Russell et al., 1976). When these two figures are combined, they indicate that the probability of O^6-meG being produced in the recognition sequence for short-patch repair is about 0.05 times that of the most frequent target for methylation.

The G·T binding activity, which is probably involved in the long-patch pathway, most likely also recognizes O^6-meG·T and 6-thioG·T base pairs. Binding to the former is less efficient, but the latter are recognized to about the same extent as G·T mismatches. Both modified base pairs are bound in all sequence contexts, but there is a slight preference for those contexts in which O^6-meG is preceded by a purine. Thus, there is an equal or better likelihood of long-patch repair acting at sites of formation of O^6-meG throughout DNA. Long-patch correction therefore represents a more probable candidate for the potentially lethal repair pathway that is defective in tolerant human cells. While we cannot be certain that the same protein recognizes G·T mispairs containing modified guanine, this possibility is supported by our observation that the MNU/6-thioG tolerant line that exhibits defective G·T binding is also deficient in binding to O^6-meG·T and 6-thioG·T base pairs.

In extracts of mammalian cells, initiation of long-patch mismatch correction requires that one strand of the DNA heteroduplex is nicked. Repair is directed to the strand that contains the nick (Holmes et al., 1990; Thomas et al., 1991). MNU and 6-thioG share the ability to introduce into DNA random nicks together with the base analog substrates for long-patch mismatch repair. In the case of MNU, collateral damage in the form of other methylated purines, 3-methyladenine or 7-methylguanine, can undergo spontaneous or enzyme-catalyzed depurination to generate AP sites susceptible to cleavage by ubiquitous endonucleases (Friedberg, 1985). Cytotoxicity of 6-thioG is associated with its widespread incorporation into DNA (Herbert et al., 1982). Therefore, even though our data indicate that action of the mismatched thymine-DNA glycosylase is essentially confined to Cp-6-thioG·T (or Ap-6-thoG·T) mispairs, numerous AP sites will nevertheless be generated by this activity. The possibility of concurrent nicking introduces a random directionality into long-patch mismatch repair that might allow correction attempts to be targeted to the pyrimidine opposite the guanine analog.

The relatively weak binding to the O^6-meG·T substrates together with the observed preference for binding to Gp-O^6-meG·T over Tp-O^6-meG·T explains our previous inability to detect binding to substrates of the latter type (Karran & Stephenson, 1990). O^6-meG induces G to A transitions selectively in the 3′ guanine of Purine.p.G sequences in mammalian cells (DuBridge et al., 1987; Richardson et al., 1989). Two possible explanations for this have been considered: the selective methylation of the 3′ guanine and the less efficient demethylation of O^6-meG in this position. Selective methylation at the O^6 position of the 3′ guanine in GpG sequences has been reported (Richardson et al., 1989), and recognition by an *E. coli* methyltransferase of O^6-meG is much less efficient in Gp-O^6-meG·C than Tp-O^6-meG·C in model substrates based on the sequence adjacent to the GpGpA codon 12 of the rodent H-*ras* oncogene (Topal et al., 1986; Georgiadis et al., 1991). Preferential recognition of Tp-O^6-meG·C base pairs over Gp-O^6-meG·C base pairs by antibodies to O^6-methyldeoxyguanosine was also observed in the same H-*ras* sequences (Georgiadis et al., 1991), suggesting that recognition by these proteins is affected by local structural features. A third possibility, that a mismatch recognition protein might compete with and inhibit the repair methyltransferase, has not previously been considered. Analogous to this is the suggested interaction of O^6-meG with the UvrA protein in *E. coli* (Chambers et al., 1985) and the recognition of platinum adducts by HMG proteins of mammalian cells (Pil & Lippard, 1992). Our observation that the G·T binding protein preferentially recognizes Gp-O^6-meG·T pairs supports this possibility. However, under the conditions of our experiments, the binding activity did not form the stable complexes with O^6-meG·C base pairs in any context and MGMT action at its normal substrate is therefore unlikely to be impaired by competing mismatch binding proteins. Furthermore, the more extensive binding observed with the O^6-meG·T substrate in Mex^+ Raji extracts is almost certainly a consequence of MGMT converting the weakly bound O^6-meG·T pair into the more strongly bound G·T mismatch. It therefore appears that the relative instability of the complex formed with O^6-meG·T-containing DNA still allows the highly selective and efficient MGMT to demethylate the modified purine, and competition between these two repair activities is unlikely to underlie the observed sequence bias of MNU-induced transitions.

In summary, our data do not support a role for the mismatched thymine-DNA glycosylase-initiated short-patch mismatch repair pathway in the cytotoxicity of methylating agents. Recognition of mispairs containing either of the two guanine analogs by the mammalian G·T mismatch binding factor which is absent from tolerant cells is fully consistent with a contribution by the long-patch mismatch correction pathway to the lethal effects of these analogs. After completion of this work, the G·T mismatch binding activity was identified as the product of the HNPCC gene (Fishel et al., 1993; Leach et al., 1993; Palombo et al., 1994; Aquilina et al., 1994). A mutated HNPCC gene in colon tumors abolishes long-patch mismatch repair in an *in vitro* assay (Parsons et al., 1993). Our observation that this protein recognizes base pairs containing guanine analogs clearly has important implications for tumor chemotherapy.

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