

Mismatch Repair Defects and O^6 -Methylguanine-DNA Methyltransferase Expression in Acquired Resistance to Methylating Agents in Human Cells*

(Received for publication, March 27, 1997, and in revised form, July 30, 1997)

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Fifteen variants with ≥ 30 -fold resistance to *N*-methyl-*N*-nitrosourea were isolated from the Burkitt's lymphoma Raji cell line. Eight had received a single treatment with a highly cytotoxic dose. The remainder, including the previously described RajiF12 cell line, arose following multiple exposures to initially moderate but escalating doses. Surprisingly, methylation resistance arose in three clones by reactivation of a previously silent O^6 -methylguanine-DNA methyltransferase gene. Five clones, including RajiF12, displayed the microsatellite instability and increased spontaneous mutation rates at the hypoxanthine-guanine phosphoribosyltransferase locus, consistent with deficiencies in mismatch repair. Defects in either the hMutS α or hMutL α mismatch repair complexes were identified in extracts of these resistant clones by *in vitro* complementation using extracts from colorectal carcinoma cell lines. Defects in hMutL α were confirmed by Western blot analysis. Remarkably, five methylation-resistant clones in which mismatch repair defects were demonstrated by biochemical assays did not exhibit significant microsatellite instability.

The ability to remove altered bases from DNA is central to cellular protection against DNA damage by cytotoxic drugs. Removal can be effected by excision repair, which may involve the replacement of relatively long or short stretches of DNA or by direct reversal of the damage. An example of the former is provided by the excision of cisplatin-DNA adducts by the long-patch nucleotide excision repair pathway. Loss of the nucleotide excision repair pathway in the genetic disorder xeroderma pigmentosum is associated with sensitivity to cisplatin (for review see Ref. 1). The *in situ* demethylation of DNA O^6 -methylguanine (O^6 -meGua)¹ by the O^6 -meGua-DNA methyltransferase (MGMT) provides an example of the latter strategy, and loss of MGMT expression in the Mex⁻ (or Mer⁻) phenotype confers sensitivity to methylating agents. This selective sensitivity of Mex⁻ cells directly implicates persistent DNA O^6 -meGua lesions in cell death following exposure to

methylating agents (for review see Ref. 2).

As an alternative to DNA lesion removal, tolerance mechanisms also provide escape from cytotoxic DNA damage. One known mechanism of DNA damage tolerance in human cells is loss of DNA mismatch repair. The DNA mismatch correction pathway normally corrects replication errors and prevents recombinational exchanges between nonidentical DNA sequences (for review see Ref. 3). The usual substrates for mismatch correction are mispaired or unpaired normal DNA bases. There is increasing evidence that mismatch repair proteins play a significant part in processing diverse types of drug-induced DNA lesions (for review see Ref. 4). Mismatch repair interacts with DNA damage including O^6 -meGua, 6-thioguanine, and as yet undefined alterations introduced by cisplatin and doxorubicin. It contributes directly to the cytotoxicity of these lesions, and mismatch repair-competent cells may be sensitive to their lethal effects, although other mediators of cell death such as the p53 and p21 proteins are sometimes important for cytotoxic manifestations (5). Cells that acquire resistance to prolonged drug exposure are found to have defects in mismatch repair functions (6–10). The phenomenon of cellular resistance acquired through mismatch repair defects is known as tolerance because the DNA lesions persist but, in the absence of mismatch repair, they are not processed into lethal intermediates and are unable to exert their potential cytotoxic effects. Resistance to these kinds of drugs is a significant therapeutic problem, and DNA damage tolerance may be of clinical importance.

The association of the human cancer syndrome Hereditary Non Polyposis Colorectal Cancer (HNPCC) with defective DNA mismatch repair has provided the genetic framework by which to define the human pathway (11, 12). Complementary biochemical studies have implicated five mismatch repair proteins in the early steps of the correction process. These proteins are encoded by the *hMSH2*, *hMLH1*, *hMSH6/GTBP*, *hPMS2*, and *hMSH3* (also known as *DUP1* or *MRP1* (13)) genes (14–19). The initial correction steps involve the interactions of a number of heterodimers formed by these proteins. Thus, current models of mismatch correction suggest that the initial mismatch recognition is likely to be carried out by one of two complexes designated hMutS α and hMutS β (16, 20, 21), which bind to the mismatched DNA segment. The hMutS α heterodimer is composed of hMSH2 and hMSH6/GTBP. In the hMutS β complex, hMSH2 is partnered by hMSH3. The α and β recognition factors have different, but partly overlapping, specificities for mismatch binding that depend on the mismatch itself and perhaps also on the context in which it appears (21). hMutS α preferentially recognizes single-base mispairs (transition/transversion intermediates) and single-base loops (frameshift intermediates) that arise by DNA slippage during replication of tracts of repeated mononucleotides. The preferred substrates of

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‡ Supported by the Human Capital and Mobility program (Contract CHRX-CT94-0518) of the European Union.

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¹ The abbreviations used are: O^6 -meGua, O^6 -methylguanine; MGMT, O^6 -meGua-DNA methyltransferase; MNU, *N*-methyl-*N*-nitrosourea; PCR, polymerase chain reaction; (kbp, (kilo)base pair(s); CMV, cytomegalovirus; TBS, Tris-buffered saline; HPRT, hypoxanthine-guanine phosphoribosyltransferase.

hMutS β are two to four base loops. Although this simple model is compatible with much of the experimental evidence, the properties of the only reported hMSH3-defective human cell line suggest that mismatch recognition by the α and β complexes may be governed by more complex factors than these simple numerical rules (19). After recognition of the mismatch, a second heterodimer, hMutL α , is recruited (17). This complex comprises the hMLH1 and hPMS2 proteins and probably serves to assemble the components necessary for the excision of the mismatched DNA segment.

Deficiencies in the both the hMutS α and hMutL α complexes have been found in drug-resistant cells (reviewed in Ref. 22). To investigate the relative frequencies of defective hMutS α and hMutL α complexes in tolerance to a methylating agent, we isolated and characterized a number of *N*-methyl-*N*-nitrosourea (MNU)-resistant variants of the Raji cell line. We evaluated two different protocols that were designed to mimic different therapeutic regimes. In the first, cells were exposed to a single high dose of the methylating agent. The second involved chronic exposure to escalating MNU doses. Several methylation-tolerant clones were isolated using both treatment regimes. Three independent clones had acquired MNU resistance through reexpression of their silent *MGMT* gene. We examined several phenotypic characteristics and defined the mismatch repair defect in a number of the remaining methylation-tolerant clones. These defects included hMutL α but were predominantly in hMutS α , most likely hMSH6/GTBP. In several resistant clones, a demonstrable defect in mismatch repair was not accompanied by a detectable mutator phenotype.

EXPERIMENTAL PROCEDURES

Materials—Chemicals were obtained from Sigma except where stated otherwise. Formamide (Fluka) was deionized with AG501-X8 resin (Bio-Rad). Recrystallized MNU was a gift from Dr. Peter Swann, Department of Biochemistry, University College London, UK. Antibodies against hMLH1 and hPMS2 were obtained from Pharmingen and against hMSH2 from Santa Cruz Biotechnologies.

Cell Lines and Culture—The TK⁻Mex Raji cell line is routinely maintained in our laboratory. At the start of this study, three cultures of Raji cells were expanded from a small inoculum (100 cells), and a single clone was isolated from each population by dilution and seeding into 96-well plates. These three clones were used to generate MNU-resistant derivatives. The LoVo, DLD-1 and HCT116 colorectal carcinoma cell lines were obtained from C. Dixon, Cancer Genetics Laboratory, Imperial Cancer Research Fund and cultured as described previously (23).

Isolation of MNU-resistant Cells—Multiple cultures of exponentially growing cells, 10^6 – 10^7 cells in 10 ml RPMI medium supplemented with 10% fetal calf serum, were treated with 500 μ M MNU. After a period of 28 days of culture to allow the outgrowth of survivors, surviving cells were cloned by single cell plating in 96-well plates.

Chronically treated cells received 0.01 mM MNU. When exponential growth resumed, they were treated with 0.02 mM MNU. The procedure was repeated using successive treatments with 0.03, 0.04, 0.05, 0.1, 0.32, and 0.5 mM MNU. Clones were isolated by single-cell plating. A single representative of each treated culture was chosen for further characterization.

***O*⁶-Methylguanine-DNA Methyltransferase Measurements**—*O*⁶-Methylguanine-DNA methyltransferase activity in cell extracts was measured using heat-depurinated [³H]MNU-treated DNA as described previously (23).

Microsatellite Instability—Analysis was performed on subclones derived from each resistant clone. Colonies that had undergone approximately 20 cell doublings were lysed *in situ* in 96-well plates, and aliquots were removed for PCR. Dinucleotide repeat microsatellites were amplified using fluorescently-labeled primers. Lengths were determined on an ABI automatic DNA sequencer as described previously (23). Four loci were analyzed: D10S197, D11S904, D13S175, and D17S941. Mononucleotide repeat microsatellites, Bat25, Bat26, and Bat40 were amplified, separated on sequencing gels, and analyzed by Southern blotting using one of the radiolabeled PCR primers as a probe.

Mutation Rate Analysis—Cultures (25 for each clone tested) were initiated using small (100 cell) inocula and expanded to $\sim 10^6$ cells,

which were distributed in 96-well plates (approximately 10^4 cells/well) in medium supplemented with 5 μ g/ml 6-thioguanine. After 28 days, the frequency of positive wells containing 6-thioguanine-resistant cells was determined. Mutation rates were calculated from the equation, $M = -\ln P_0 \cdot C^{-1} \cdot \ln 2$, where P_0 is the number of cultures without mutant clones, and C is the total number of cells placed in selection. In some cases, mutation frequencies were estimated by plating growing cultures in 96-well plates under the same selective conditions.

***O*⁶-Methylguanine Processing Assay**—Cell extracts were prepared as described (24). Plasmid pSVori methylated with 0.48 mM MNU for 30 min at 37 °C was incubated with extract in the presence of [α -³²P]dATP, and incorporation of radioactivity into material adhering to DE81 paper (Whatman) was determined as described (24).

Mismatch Binding Assay—The preparation of cell extracts and details of the bandshift assay for mismatch binding have been described previously (25). The substrates were 34-mer-duplex oligonucleotides containing a single GT mismatch (25) or an unpaired CA dinucleotide (duplex C in Ref. 26).

Mismatch Repair Substrate—The substrate for *in vitro* mismatch correction was constructed from molecules derived by subcloning a 211-bp *PvuII*/*PstI* fragment of the previously described HK7 M13 (27) into the pBK-CMV phagemid (Stratagene). The inserted region contained the heteroduplex cassette sequence that can be used to generate specific mismatches within restriction endonuclease sites that are diagnostic for strand-specific mismatch correction.

For the experiments described in this paper, C-containing viral strands were purified by standard techniques (28). Closed circular duplexes that contained T in the complementary position were purified by banding on CsCl gradients. After ethanol precipitation, duplex circular DNA was linearized by digestion with *NdeI*. After phenol extraction and ethanol precipitation, linear DNA (150–250 μ g) was mixed with a 2-fold excess of single-stranded DNA, and the mixture was adjusted to 50% formamide, 10 mM EDTA, pH 8.0, in a total volume of 2–3 ml. The mixture was dialyzed sequentially against 95% formamide, 10 mM EDTA, pH 8.0, for 2 h; 50% formamide, 200 mM Tris-HCl, 10 mM EDTA, pH 8.0, for 2 h; 100 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM EDTA for 2 h; and finally against 10 mM Tris-HCl, 1 mM EDTA, pH 7.5, for 2 h. Nicked circular molecules were purified by agarose gel electrophoresis and electroelution. The purified 4470-bp molecules contain a unique CT mismatch that inactivates an *MluI* restriction site. The mismatched T is 580-bp 5' of a single nick (see Fig. 1). Small amounts of reannealed matched linear molecules were present in all preparations. These and their *MluI* digestion products could be easily resolved from the diagnostic products and did not detectably affect the correction assay.

In Vitro Mismatch Correction—Cell extracts were prepared from 1 – 5×10^9 cells as described previously (24). Mismatch correction was assayed in 25 μ l of 30 mM Hepes-KOH, pH 8.0, 7 mM MgCl₂, 0.5 mM dithiothreitol, 0.1 mM each dNTP, 4 mM ATP, 40 mM phosphocreatine, 1 μ g of creatine phosphokinase (rabbit muscle-type I), 90 ng of DNA substrate, and up to 200 μ g of cell extract. Mixtures were incubated for 60 min, and the reaction was terminated by the addition of 10 mM EDTA, 0.5% SDS. Samples were freed of protein by proteinase K digestion (1 mg/ml, 15 min) followed by phenol extraction. DNA was ethanol-precipitated, dissolved in buffer, and digested with *MluI*, which is diagnostic for removal of the mismatch. Digestion products were separated on 0.8% agarose gels in 40 mM Tris-acetate, pH 8.0, 1 mM EDTA buffer containing ethidium bromide and visualized under short wavelength ultraviolet light. The mismatched substrate is shown schematically in Fig. 1. An *MluI* site is located at position 463, and digestion of the uncorrected substrate generated unit-length linear 4470-bp molecules. Digestion of molecules that have undergone nick-directed correction (TC to GC) to generate the second *MluI* site produces two fragments of 3.9 kbp and 567 bp (see Fig. 4, fragments A and D). Digestion of the small amount of contaminating matched linear molecules generated during the annealing reaction produces traces of fragments of 3.3 and 1.17 kbp (see Fig. 4, fragments B and C) that are resolved from the products of mismatch correction. Thus, digestion with *MluI* of DNA recovered after incubation with repair-proficient cell extracts generated a mixture of unit-length linear molecules and fragments A–D. The smaller fragments (C and D) were not generally visible. In the products recovered from repair-defective extracts, only unit-length linear DNA was visible together with a small amount of fragment B from contaminating linear molecules. Thus, the presence of fragment A (3.9 kbp) that is resolved from unit-length 4.47-kbp molecules, is diagnostic for mismatch correction.

hMSH2 Sequencing—Cytoplasmic RNA was extracted from parental Raji cells and RajiF12 variant and used to generate hMSH2 cDNA

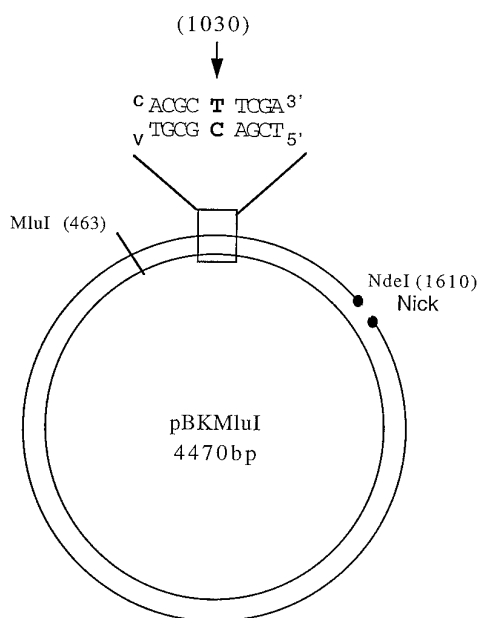


FIG. 1. **Substrate for *in vitro* mismatch repair.** Mismatched heteroduplexes containing a CT mispair were generated from pBK-CMV molecules containing the subcloned heteroduplex cassette region of HK7 (27). Viral DNA containing C at position 1030 was annealed with an excess of linearized duplexes containing an AT pair at position 1030, with the T at the position complementary to the C. The CT mispair is located 580 bp 5' from a unique nick. Correction of the mismatch generates a second *MluI* restriction site that is diagnostic for repair.

using Moloney murine leukemia virus reverse transcriptase (New England Biolabs, Schwalbach/Taunus, Germany). PCR was used to amplify the hMSH2 cDNA in five overlapping fragments. The following primers, designed to introduce *Bam*HI and *Eco*RI recognition sequences, were used: fragment 1, CGGGATCCCAACCAGGAGGTGAGGAGG and CGGAATTCCTGGCCATCAACTGCGGACAT; fragment 2, CGGGATCCAGATCTTCTTGTGTTTCGTC and CGGAATTCGCCAACAATAATTTCTGTG; fragment 3, CGGGATCCTGGATAAGAACAGAATAGAGG and CGGGATCCCCACAATGGACACTTCTGC; fragment 4, CGGGATCCCACCTGTTCCATATGTACG and CGGAATTCAAAATGGGTTGCCAAACATGC; fragment 5, CGGGATCCGTGATAGTACTCATGGCCC and CGAATTCGACAATAGCTTATCAATATTACC.

The sequences of the primers used to amplify fragments 2, 3, 4, and 5 were taken from Ref. 29. The PCR reactions were carried out in 100 μ l of 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 3 mM MgCl₂, 250 μ M each dNTP, 0.15 μ M each primer, and 10 units/ μ l AmpliTaq DNA polymerase (Perkin-Elmer). 30 cycles of 93 °C for 1 min, 60 °C for 2 min, 72 °C for 3 min, followed by 1 cycle of 72 °C for 10 min were performed. Fragments were purified from agarose gels and ligated into M13mp18 or M13mp19 vectors (Life Technologies, Inc., Paisley, UK). After bacterial transformation, individual clones were sequenced using M13 sequencing primer.

Exons 4, 7, 9, 11, and 14 were individually amplified by a two-step PCR procedure using nested primers (30). First step amplifications were performed in 25 μ l containing 75 ng of genomic DNA under the conditions described above. Two μ l of the PCR products were used as templates in further rounds of amplification in 100- μ l reaction volumes using biotinylated primers. In this case, the annealing step was carried out at 65 °C. Biotin-labeled single-stranded DNA was recovered by binding to magnetic beads (Dynabeads, Dynal A. S., Oslo, Norway) and sequenced.

Western Blot Analysis—The cell extracts (50 μ g) that were used in mismatch correction assays and prestained low molecular weight markers (Bio-Rad) were denatured and separated on 8% SDS-polyacrylamide gels. Proteins were transferred to nylon membranes (Zeta-Probe, Bio-Rad) using a semi-dry electrophoretic transfer apparatus (Trans-Blot, Bio-Rad) at room temperature. The membrane was blocked by immersion at room temperature for 1 h in TBS-Tween (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 80) containing 5% skimmed powdered milk. The blocked filter was incubated with purified mouse anti-hPMS2 (1 μ g/ml), anti-hMLH1 (1 μ g/ml), or hMSH2 (0.1 μ g/ml) in TBS/Tween plus 3% bovine serum albumin for 1 h at room temperature. After washing several times with TBS-Tween, the appropriate horseradish

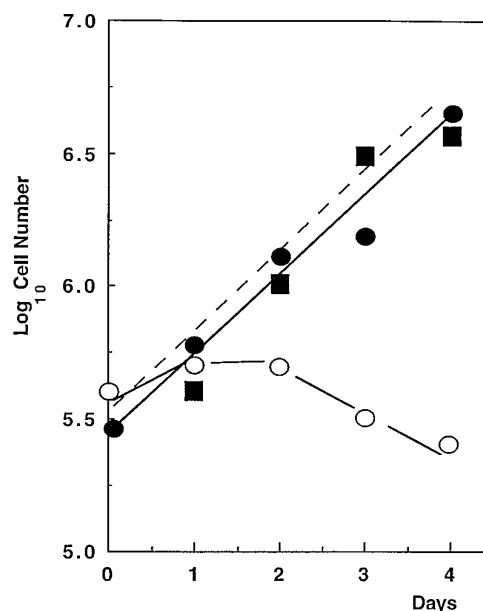


FIG. 2. **MNU-resistance of clones.** Exponentially growing cultures of clones Raji 9 (●) and Raji 102 (■) were treated with 0.3 mM MNU. The parental Raji cells (○) received 0.01 mM MNU in parallel. Cell growth was monitored by daily cell counts by light microscopy. The hatched line represents the growth of untreated Raji cells. The data are presented as cumulative cell number.

peroxidase-conjugated secondary antibody diluted in TBS-Tween plus 3% bovine serum albumin was added for 30 min. After several washes with TBS-Tween, the filter was developed using the ECL detection kit (Amersham International). Membranes were stripped for reprobing by immersion in 2% SDS, 100 mM β -mercaptoethanol, 62.5 mM Tris-HCl, pH 6.8, for 30 min at 50 °C.

RESULTS

Resistance to MNU—Eighteen individual cultures of the Mex⁻ Burkitt's lymphoma cell line Raji were treated with MNU. Nine cultures received a single treatment with 0.5 mM MNU that resulted in an estimated survival of 10⁻⁷. The remainder were treated with an initial dose of 0.01 mM; surviving cells were allowed to recover and were retreated with 0.02 mM MNU. This regime of repeated treatments was continued with escalating doses up to a maximum of 0.5 mM. Individual colonies were isolated from each treated culture by cloning in 96-well plates. To ensure independence, only one MNU-resistant clone from each culture was characterized. Fifteen clones were analyzed further.

Fourteen clones exhibited an increase in MNU resistance of at least 30-fold as measured by the drug concentration required to arrest cell growth. Proliferation of the parental Raji cells was inhibited by treatment with 0.01 mM MNU (Fig. 2). Following treatment, these sensitive cells underwent one cell division during the first 24 h, but thereafter there was no further increase in cell number. In contrast, clones isolated after acute or chronic MNU treatment withstood exposure to \geq 0.3 mM MNU and continued to proliferate at rates closely similar to that of the untreated cells. Two examples are shown in Fig. 2. The extent of MNU resistance in the isolated clones was comparable to that previously reported for the methylation-tolerant RajiF12 cells (6). The effect of higher MNU concentrations was not systematically evaluated for all of the clones, but some, for example Raji10, were resistant up to at least 1 mM MNU. In all, 14 of 15 clones, 8 from acute exposure and 6 from chronic exposure to escalating drug doses, exhibited resistance to 0.3 mM MNU. The clones isolated after acute treatment were given a single number designation: Raji 3, Raji 7, etc. Those derived by chronic exposure were numbered Raji 101, Raji 102, etc. One

TABLE I
Summary of properties of MNU-resistant clones

	Phenotype				
	1	2	3	4	5
Mex status	Mex⁺	Mex ⁻	Mex ⁻	Mex ⁻	Mex ⁻
(A) _n repeats	Stable	Unstable	Unstable	Stable	Stable
(CA) _n repeats	Stable	Unstable	Stable	Stable	Stable
Relative HPRT mutation rate ^b	×1	×4	×3-4	×1	×1
Mismatch binding	Proficient	Proficient	Proficient/ Deficient	Deficient	Proficient
Mismatch repair	Proficient	Deficient	Deficient	Deficient	Proficient/ Deficient
Representative clones	Raji 101 Raji 105 Raji 106	Raji 10	Raji 8 Raji 9 Raji 19 RajiF12	Raji 103 Raji 102	Raji 3 Raji 7 Raji 12 Raji 17 Raji 104

^a Significant changes in phenotype are shown in bold type.

^b HPRT mutation rates in Raji 10, Raji 102, Raji 103, RajiF12, and the parental Raji cell line were determined by fluctuation analysis. The remaining values are estimates based on mutation frequencies of populations maintained in continuous culture.

clone, Raji 107, was not significantly more resistant to MNU than Raji and was not characterized in detail, although it served as a control in some experiments. The RajiF12 cell line, isolated following chronic MNU treatment, has been described previously (6).

Resistance to the unrelated DNA cross-linking agent, mitomycin C, was not significantly increased (<2-fold) in any of the the MNU-resistant clones (data not shown). This indicates that the loss of MNU sensitivity in these cells is not a consequence of a generalized resistance to DNA damaging agents arising, for example, through loss of a common apoptosis pathway.

We investigated MGMT expression, microsatellite stability, and spontaneous mutation at the HPRT locus in the resistant clones. These properties together with mismatch binding by cell extracts, were used to assign the resistant clones to five different but overlapping phenotypes. The ability to carry out mismatch correction and to process DNA O⁶-meGua *in vitro* were also analyzed. The findings are summarized in Table I. Complementation with extracts prepared from colorectal carcinoma cells and Western blot analysis was used to define defective mismatch repair functions in the resistant clones.

Phenotype 1. Increased MGMT Expression—The TK⁻ variant Raji cells are Mex⁻ and cell extracts containing undetectable levels of MGMT (<0.05 units/mg of protein). In contrast, extracts of three of the resistant cell lines, Raji 101, Raji 105, and Raji 106, contained approximately 0.3 units of MGMT/mg of protein. The colorectal carcinoma cell line LoVo included for comparison was also Mex⁺ and expressed 0.7 units of MGMT/mg of protein. These values lie in the normal range for Mex⁺ cell lines, including the Mex⁺ Raji variant (Table II). The remaining clones isolated by the escalating dose regime and all of the clones derived by acute treatment did not contain detectable MGMT activity (Table II and data not shown). Raji 101 and Raji 106 did not exhibit microsatellite instability at either (A)_n or (CA)_n repeats (Table III). The HPRT⁻ mutation frequency in cultures of these three clones was comparable to that of the parental Raji cells, consistent with the absence of a mutator phenotype. Bandshift assays with cell extracts indicated that all were proficient in the recognition of a GT mismatch and a two-base loop in the standard substrates (Table I, data not shown; see Fig. 6). Extracts of Raji 101, Raji 105, and Raji 106 were also able to correct a single CT mismatch to regenerate a *Mlu*I restriction site in the nicked circular DNA duplex (Table I, data not shown). Since Mex⁺ Raji cells exhibit comparable 30–50-fold resistance to MNU and there is no evidence of mismatch repair defects in Raji 101, Raji 105, and Raji 106 cells, it seems likely that the resistance of these three clones is a direct consequence of their expression of MGMT. The phenotype of Raji 101, Raji 105, and Raji 106 was desig-

TABLE II
MGMT activity in MNU-resistant clones

The MGMT values (mean of three separate determinations) for all the MNU-resistant clones that arose after multiple exposures to MNU are presented here. MGMT values for the eight clones isolated after a single MNU treatment were all <0.05 units/mg of protein.

Clone	MGMT activity
	units/mg of protein
Raji	<0.05
Raji Mex ⁺	0.31
LoVo	0.7
Raji 101	0.32
Raji 105	0.33
Raji 106	0.20
Raji 102	<0.05
Raji 103	<0.05
Raji 104	<0.05

nated Phenotype 1 (Table I).

Phenotype 2. Generalized Microsatellite Instability—One clone, Raji 10, exhibited considerable instability at both mono- and dinucleotide repeat microsatellites. Examples are shown in Fig. 3, and the data are summarized in Table III. The rate of mutation at (CA)_n repeats was 5×10^{-3} mutations/cell/generation, an increase of >60-fold over the rate in the parental Raji cells. (A)_n microsatellites were also unstable in Raji 10, and the rate of mutation was increased 20-fold compared with parental Raji cells (80 cf. 4×10^{-4} mutations/cell/generation). The rate of mutation at the HPRT locus in Raji 10 measured by fluctuation analysis was 10^{-6} mutations/cell/generation, approximately 4-fold higher than that of the parental Raji cells (2.7×10^{-7} /cell/generation). Extracts of Raji 10 cells were proficient in binding to both a single GT mismatch and a CA loop in the bandshift assay (data not shown) but, unlike extracts of the parental Raji cells, were unable to correct a CT mismatch in the nicked circular heteroduplex (Fig. 4a). In addition, in comparison to the parental Raji cells, Raji 10 cell extracts carried out little mismatch repair-related DNA synthesis on an MNU-methylated plasmid (Fig. 5). These data indicate that Raji 10 cells are also defective in processing O⁶-meGua by the mismatch repair pathway. Raji 10 was the single representative of Phenotype 2. The microsatellite instability, mismatch binding proficiency, and general properties of Raji 10 are consistent with a mismatch repair defect involving the hMutLα complex.

Phenotype 3. Selective Microsatellite Instability—Three clones (Raji 8, Raji 9, Raji 19) together with the previously described RajiF12 cell line, exhibited selective microsatellite instability at (A)_n microsatellites (Fig. 3; Table III). The (A)_n microsatellite mutation rate in the four clones was between 3×10^{-3} and 10^{-2} mutations/cell/generation, an increase of 7–25-

TABLE III
Microsatellite instability in MNU-resistant clones

The values in the tested column are the product of the number of subclones analyzed and the number of loci. Four dinucleotide loci (D10S197, D11S904, D13S175, and D17S941) and three mononucleotide repeats (Bat 25, Bat 26, and Bat 40) were analyzed in resistant clones. At least 10 subclones were analyzed at each locus. Subclones of Raji and RajiF12 were also examined at other dinucleotide loci including Afm015, Afm028, Afm042 Afm057, Afm073, Afm123, Afm144, Afm200, Afm234, and D12S329. Data for each type of microsatellite have been pooled for each variant.

Clone	Mononucleotides			Dinucleotides		
	Tested	Altered	Rate	Tested	Altered	Rate
Raji	117	1	4×10^{-4}	630	0	$<8 \times 10^{-5}$
Raji 101	43	0	$<10^{-3}$	95	0	$<5 \times 10^{-4}$
Raji 106	40	0	$<10^{-3}$	210	0	$<2 \times 10^{-4}$
Raji 10	30	5	8×10^{-3a}	143	13	5×10^{-3a}
RajiF12	120	9	4×10^{-3a}	630	0	$<8 \times 10^{-5}$
Raji 8	47	3	3×10^{-3a}	110	0	$<4 \times 10^{-4}$
Raji 9	41	7	8×10^{-3a}	103	0	$<5 \times 10^{-4}$
Raji 19	30	7	10^{-2a}	93	0	$<5 \times 10^{-4}$
Raji 102	90	0	$<6 \times 10^{-4}$	108	0	$<5 \times 10^{-4}$
Raji 103	66	1	8×10^{-4}	124	0	$<4 \times 10^{-4}$
Raji 7	33	0	$<10^{-3}$	92	0	$<5 \times 10^{-4}$
Raji 12	31	0	$<10^{-3}$	90	0	$<5 \times 10^{-4}$
Raji 104	43	0	$<10^{-3}$	108	0	$<5 \times 10^{-4}$

^a Significantly unstable compared to Raji ($p < 0.01$ by chi-square).

fold over the parental Raji cells. There was no evidence of instability at four (CA)_n microsatellites (Table III). The spontaneous mutation rate at the HPRT locus in RajiF12 was previously estimated to be 3.5-fold higher than the parental Raji cells (6). This value was confirmed by fluctuation analysis (Raji = 2.7×10^{-7} ; RajiF12 = 10^{-6} mutations/cell/generation). The HPRT⁻ mutation frequencies in cultures of Raji 8, Raji 9, and Raji 19 were also increased between 3- and 4-fold over the parental level (Table I), compatible with a moderate mutator phenotype at this locus. Extracts of Raji 8, Raji 9, and Raji 19 were all proficient in binding to a GT mismatch and to a CA loop in the bandshift assay (data not shown). A GT mismatch binding and single-base mismatch correction defect in RajiF12 have been reported previously (6, 31) and were confirmed in this study (data not shown). All four clones were also unable to correct a CT mismatch in the *in vitro* assay (Fig. 4 and data not shown). Extracts of Raji 9 and RajiF12 were deficient in processing the MNU-treated plasmid substrate. Despite their CT mismatch correction defect, Raji 8 extracts were competent in *O*⁶-meGua processing (Fig. 5b) and incorporated similar levels of dAMP to the parental Raji extracts.

Raji 8, Raji 9, Raji 19, and RajiF12 were assigned to Phenotype 3 on the basis of their shared selective microsatellite instability at mononucleotide repeats, evidence of a mutator phenotype at HPRT, and mismatch correction defect. This phenotype is heterogeneous, however, and of the four clones, only RajiF12 is detectably defective in mismatch binding; Raji 8 retains some ability to process *O*⁶-meGua. Direct sequencing of all 16 exons of the RajiF12 hMSH2 gene did not reveal any mutations. The mismatch binding deficiency of RajiF12 is, therefore, most likely due to a defective hMSH6/GTBP.

Phenotype 4. No Extensive Microsatellite Instability—Extracts of two other clones, Raji 102 and Raji 103, exhibited an impaired mismatch binding similar to that previously reported for RajiF12 (Fig. 6). They did not, however, display a significant mutator phenotype at microsatellites or at HPRT as measured by fluctuation analysis (Fig. 3; Tables 1 and 3). Because of this absence of a significant mutator effect, they were assigned to a separate group, Phenotype 4 (Table I). The phenotype of Raji 103 was consistent with a partial defect. A single alteration was observed in one mononucleotide microsatellite in Raji 103 (Table III), but we did not observe enough changes to conclude that Raji 103 exhibited instability. The HPRT mutation rate was also not significantly different from that of the Raji parental cells (3 versus 2.7×10^{-7}). Raji 103 cell extracts were able to perform limited mismatch correction (Fig. 4b) and retained a

corresponding detectable, but reduced, level of binding to either a GT mismatch or a CA loop (Fig. 6). Binding to an AC mismatch, which is independent of both hMSH2 and hMSH6/GTBP, by extracts of Raji 103 and the parental Raji cells, was indistinguishable (Fig. 6). This serves as a positive control for the extracts. Extracts of Raji 103 were defective in processing plasmid DNA containing *O*⁶-meGua (Fig. 5). Incorporation of dAMP into the methylated substrate by Raji 103 extracts was somewhat higher than repair-defective cell extracts, consistent with the partial nature of the mismatch correction and binding defects in these cells.

Raji 102 cells also showed no evidence of microsatellite instability at either (A)_n or (CA)_n microsatellite loci (Table III), and the spontaneous HPRT mutation rate in Raji 102 was similar to that of the parental Raji cells (1.5×10^{-7} mutations/cell/generation) of 2.7×10^{-7} in the parental cells). Extracts of Raji 102 cells did not, however, detectably correct the CT mismatch (Fig. 4c) or bind to a GT mismatch or to a CA loop (Fig. 6). Raji 102 extracts were also deficient in processing plasmid DNA containing *O*⁶-meGua (Fig. 5b). The DNA mismatch binding and correction defects were directly implicated in the methylation resistance of Raji 102. A spontaneous revertant to MNU sensitivity, Raji 102B, which arose during normal culture, was fully correction-proficient (Fig. 4c). Extracts of Raji 102B had also regained mismatch binding activity (Fig. 6).

Phenotype 5. Resistance Without Additional Phenotype—The remaining five clones, Raji 3, Raji 7, Raji 12, Raji 17, and Raji 104, were assigned to Phenotype 5 (Table I). These clones remained Mex⁻ as determined by direct assay of MGMT in cell extracts. Representative members, Raji 7, Raji 12, and Raji 104, showed no detectable microsatellite instability at either (A)_n or (CA)_n microsatellite loci (Table III). Within the limits of detection of the assay, all were proficient at mismatch binding. Notwithstanding the absence of detectable microsatellite instability or deficiency in mismatch recognition, Raji 7, Raji 12, and Raji 104 were found to be defective in CT mismatch correction *in vitro* (see Fig. 8d). Processing of MNU-treated plasmid DNA was investigated in Raji 12 and Raji 104. Raji 12 extracts did not process the MNU-treated plasmid. Despite the mismatch repair defect, the level of dAMP incorporation by Raji 104 extracts approached that of the repair-competent Raji and Raji 107 extracts (Fig. 5b). The mismatch repair defect in Raji 104 that is associated with methylation tolerance is not, therefore, detectable by this assay.

Identification of Mismatch Repair Defects in Phenotypes 2–5—The CT mismatch correction assay was used to define the

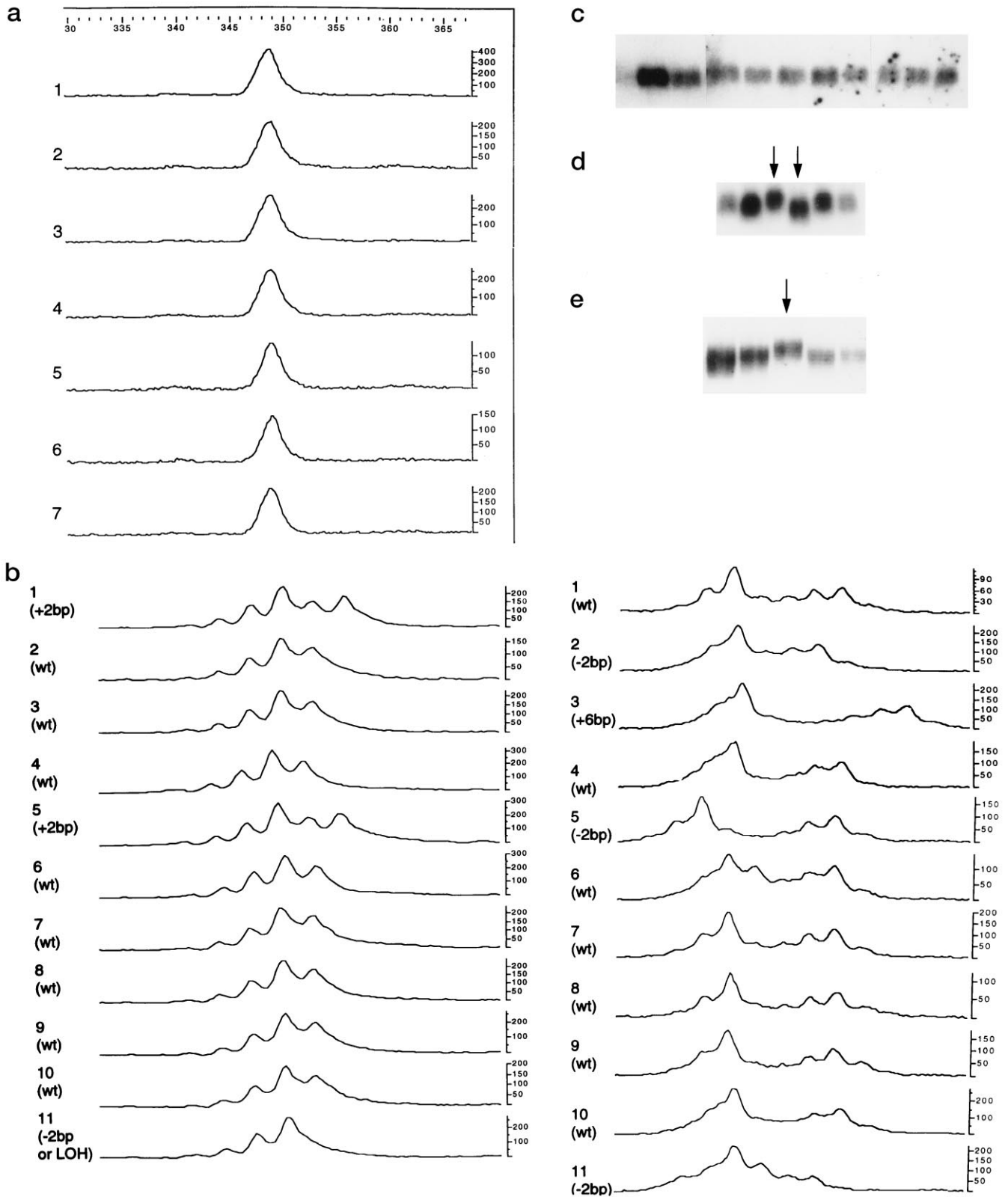


FIG. 3. Examples of microsatellite analysis in MNU-resistant clones. *a*, stability of dinucleotide repeats in Raji 17. CA repeat microsatellite D17S941 was amplified by PCR using fluorescent-labeled primers. Products were separated on DNA sequencing gels and detected by fluorometry. Seven subclones of Raji 17 that had been grown for at least 20 generations are illustrated. Both alleles of this locus appear to be the same length. *b*, instability in dinucleotide repeats in Raji 10. Two loci D13S175 (*left panel*) and D10S197 (*right panel*) were amplified by PCR and fluorescent primers. Products were separated and detected as above. Eleven subclones for each locus are illustrated. Deviations from the wild-type (*wt*) pattern are labeled ($\pm 2bp$). *c-e*, instability at poly(A) microsatellites. Poly(A) microsatellites Bat 25 (*c*) or Bat 40 (*d* and *e*) were amplified by PCR. Products were separated on sequencing gels, transferred by Southern transfer, and analyzed by probing with a radioactively labeled primer. *c*, no instability is apparent in the 10 subclones of Raji 103 illustrated; *d*, examples of variation in subclones of Raji F12. *e*, examples of variation in Raji 19. *Arrow(s)*, altered allele(s). A full summary of these analyses is presented in Table III.

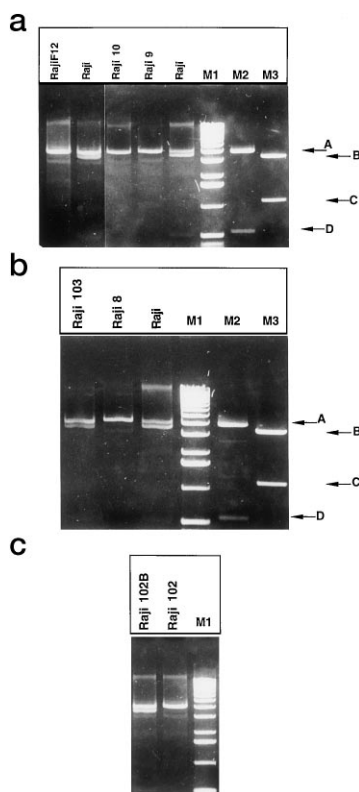


FIG. 4. CT mismatch correction in representatives of phenotypes 2–4. *a*, extracts of RajiF12, Raji 10, or the parental Raji cells (150 μg of protein) were incubated with CT mismatch-containing pBK-*Mlu*I DNA as described under “Experimental Procedures.” DNA was recovered and digested with *Mlu*I, and the products were analyzed by agarose gel electrophoresis. Markers: *M1*, 1-kilobase ladder (Life Technologies); *M2*, *Mlu*I-digested pBK-*Mlu*I DNA containing the diagnostic *Mlu*I site; *M3*, *Mlu*I- and *Nde*I-digested pBK-CMV DNA, which mimics the contaminating fragments derived from linear DNA regenerated during construction of the substrate. *b*, same as *a* with extracts of Raji 103 and Raji 8; *c*, same as *a* with Raji 102 and Raji 102B. Raji 102B arose during routine culture. It exhibited normal sensitivity to MNU.

biochemical defects in representatives of phenotypes 2–5. Extracts of wild-type HeLa (Fig. 7*a*) or Raji (Fig. 4) cells carried out efficient repair of the mismatched substrate. The extent of correction increased with increasing extract concentration up to 200 μg of protein/assay, at which point >50% of the mismatched molecules were repaired (Fig. 7*a*). In contrast, extracts of the colorectal carcinoma cells DLD-1, HCT116, or LoVo, which are known to be defective in mismatch correction, were unable to carry out detectable repair of the mismatched substrate (Fig. 7*b*). Full repair was restored when 100 μg of HCT116 and a similar amount of DLD-1 extract were combined (Fig. 7*b*). Partial complementation was also observed when LoVo and HCT116 extracts were mixed.

Colorectal carcinoma cell extracts were used to complement the mismatch repair deficiencies in extracts of the MNU-tolerant clones. Mixing Raji 10 and Raji 9 extracts complemented their defects and restored wild-type levels of CT mismatch repair (Fig. 8*a*). Wild-type levels of repair were restored to phenotype 2 Raji 10 cells by the addition of DLD-1, but only a very minor increase in correction was achieved by the addition of HCT116 extract (Fig. 8*a*). Conversely, phenotype 3 Raji 9 cell extracts were fully complemented by HCT116 but not by DLD-1 extracts. All these data are consistent with a defective hMutL α complex in Raji 10 and a defective hMutS α complex in Raji 9 cells. Using a similar approach, repair activity was restored to RajiF12 and Raji 102 by HCT116 but not DLD-1 cell

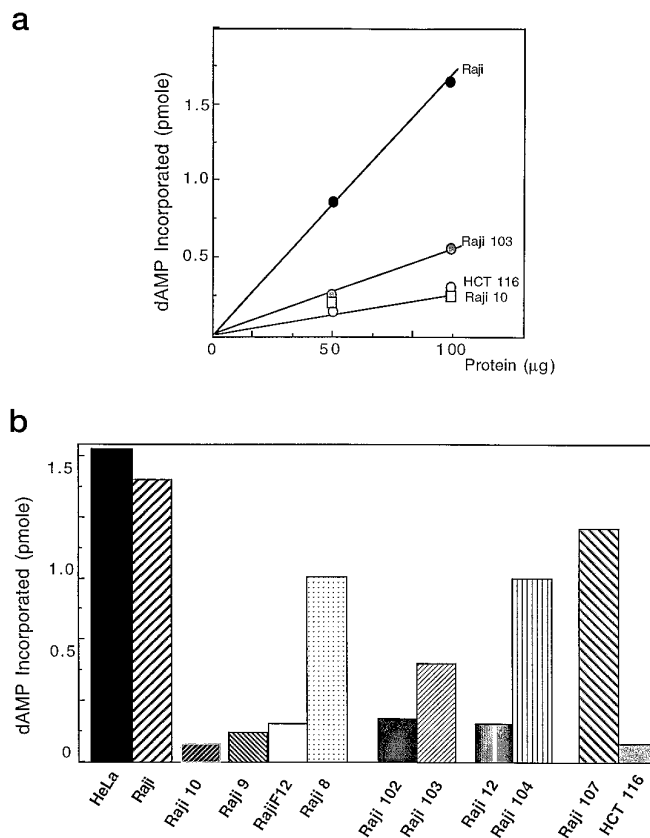


FIG. 5. Mismatch repair-dependent processing of DNA O⁶-meGua. Cell extracts were incubated for 30 min with MNU-treated plasmid DNA in the presence of ³²P-labeled dATP as described under “Experimental Procedures.” Incorporation into material retained by DE81 paper was quantitated. *a*, extracts of Raji (filled circle), Raji 103 (gray circle), Raji 10 (open circle), or HCT116 (open square) were incubated at the concentrations shown. *b*, incorporation by extracts (100 μg of protein) of each of the cell lines shown was quantitated as described. The data presented were taken from an experiment such as the one in Fig. 5*a* in which incorporation increased linearly with extract concentration. Raji 107, which exhibits a normal sensitivity to MNU and no detectable defect in mismatch repair but had survived the selective treatment with escalating MNU doses, is included as a control.

extracts (Fig. 8*b*). The methylation-tolerant phenotype, the absence of detectable mismatch binding, and the correction defects of these clones are therefore also most likely the result of defective hMutS α . Their selective mononucleotide microsatellite instability indicates that a defect in hMSH6/GTBP is more probable. This was confirmed in the case of RajiF12 by the absence of hMSH2 gene mutations. The partial repair activity of Raji 103 was augmented by the addition of extracts of HCT116 but not of DLD-1 (Fig. 8*c*). This observation, together with the partial mismatch binding activity of Raji 103 extracts, is consistent with an impaired hMutS α function in Raji 103 cells. We conclude that the methylation tolerance of members of both phenotypes 3 and 4 is associated with defects in hMutS α , most probably in the hMSH6/GTBP subunit.

The same analysis was performed on two of the members of phenotype 5 that were mismatch correction-defective. Combining extracts of Raji 12 and Raji 104 complemented their CT mismatch repair defects and restored wild-type levels of correction. Correction was also restored to Raji 12 cell extracts by the addition of Raji 9 or DLD-1 extracts. In contrast, Raji 10 and HCT116, but not Raji 9 or DLD-1 extracts, complemented Raji 104 (Fig. 8*d*). Thus, Raji 12 and Raji 104 belong to different biochemical complementation groups. Despite their apparent lack of mutator phenotype, they are defective in hMutL α and hMutS α , respectively.

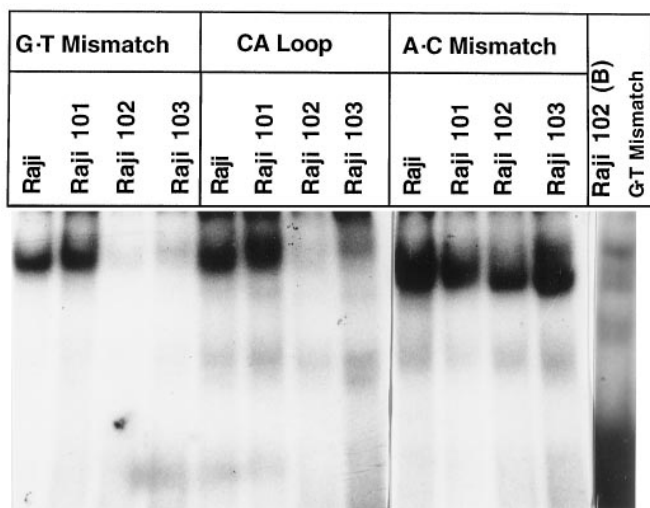


FIG. 6. Mismatch binding by Raji 102 and Raji 103 extracts. Cell extracts (15 μ g protein) of Raji 102, Raji 103, the Mex⁺ revertant Raji 101 or the parental Raji cells as indicated were incubated with radiolabeled duplexes containing a single GT mispair (left panel), AC (right panel), or CA loop (center). The extreme right panel shows GT mispair binding by 15 μ g of Raji 102B, the MNU-sensitive revertant of Raji 102. Bound and free oligonucleotides were separated on 6% polyacrylamide gels as described under "Experimental Procedures."

In an alternative approach, Western blotting was used to investigate the defective mismatch repair component in Raji 10 and Raji 12. Antibody against hPMS2 recognized a band of the expected size (115 kDa) in extracts of the parental Raji cells. A band of similar intensity was observed with extracts of Raji 104 and Raji 9. This material was undetectable in extracts of HCT116 and Raji 10 cells and was present in diminished levels in extracts of Raji 12 cells (Fig. 9). The same blots were probed with antibody against hMLH1. hMLH1 (83 kDa) was present in the parental Raji, Raji 104, and Raji 9 extracts, but the protein was not observed in Raji 10 or HCT116 extracts. hMLH1 expression also appeared to be diminished in three independent extracts of Raji 12. The levels of hMSH2 (Fig. 9) were comparable in wild-type, HCT116, Raji 10, and Raji 12. As expected, hMSH2 expression was somewhat reduced in DLD-1 (20). The unrelated ERCC1 protein (data not shown) was present in comparable amounts in all extracts including Raji 10 and Raji 12 and serves as an internal control. Thus, the mismatch repair defects of Raji 10 and Raji 12 are associated with the absence of detectable hMutL α in Raji10 and a reduced level of this complex in Raji 12.

DISCUSSION

MGMT Reactivation—The development of resistant disease bedevils most chemotherapy regimes, and the level of expression of MGMT is a known factor in resistance to anticancer methylating agents such as dacarbazine and temozolomide (32, 33). Among our *in vitro* derived MNU-resistant cell lines, three clones (Phenotype 1) had gained resistance as a consequence of a stable increase in MGMT expression. The parental Raji cells have retained their Mex⁻ phenotype for many years in our laboratory, and these experiments were initiated from clonal Mex⁻ populations. This makes it unlikely that the altered Mex status is the result of outgrowth of a subpopulation of preexisting Mex⁺ cells. It is more probable that MNU treatment both initiated and selected for the change to a Mex⁺ phenotype. MGMT expression is regulated by an epigenetic mechanism in which the usual relationship between cytosine methylation and gene expression is apparently reversed (34, 35). Thus, the silent MGMT gene in Mex⁻ cells, including the parental Raji cells used in this study, is hypomethylated at CpG sites com-

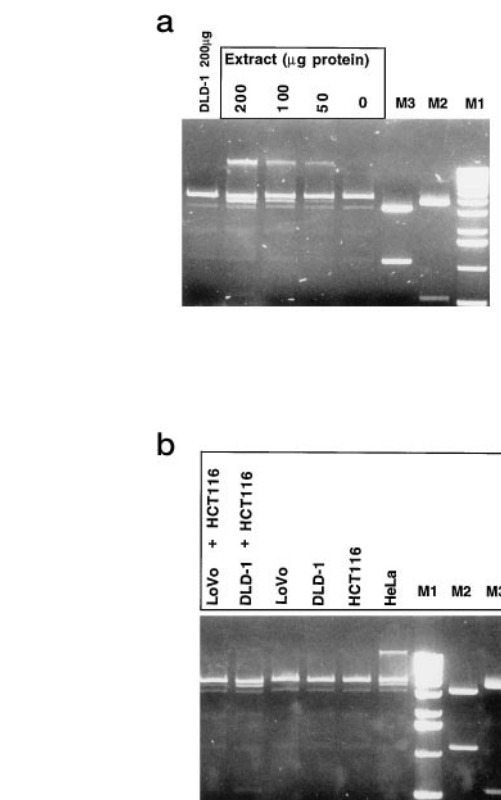


FIG. 7. CT mismatch correction by HeLa and colorectal carcinoma cell extracts. a, HeLa cell extracts (0–200 μ g of protein as shown) were incubated with CT mismatch-containing pBK-*MluI* DNA as described under "Experimental Procedures." An extract of DLD-1 cells (200 μ g of protein) was included as a control. DNA was recovered and digested with *MluI*, and the products were analyzed by agarose gel electrophoresis. b, colorectal carcinoma cell extracts. HeLa, HCT116, DLD-1, or LoVo cell extracts (100 μ g of protein) were incubated with CT mismatch-containing pBK-*MluI* DNA as described under "Experimental Procedures." DLD-1/HCT116 and LoVo/HCT116 were assayed in combination (100 μ g of protein from each extract) as indicated. DNA was recovered and digested with *MluI*, and the products were analyzed by agarose gel electrophoresis.

pared with its expressed counterpart in Mex⁺ cells. Reduction of cytosine methylation by treatment of Mex⁺ cells with azacytidine can reduce MGMT gene expression (34), although the relationship between methylation status and MGMT expression is complex (36). It seems probable that reactivation of the previously silent MGMT gene by altered methylation underlies the MNU resistance of the Raji 101, Raji 105, and Raji 106 clones. There is some evidence that chronic MNU treatment of cells can effect increases in DNA cytosine methylation (37) and, although our sample size is too small for statistical significance, it is interesting that all three revertants arose during the escalating dose regime. Although selection for a Mex⁺ phenotype has not previously been reported after MNU treatment, chronic exposure of hamster cells to the DNA cross-linking chloroethylnitrosourea, mitozolomide, which kills cells by introducing DNA cross-links that MGMT can prevent, is known to increase MGMT expression without apparent MGMT gene amplification (38). Drug-induced reactivation of the silent MGMT gene may be a more common phenomenon than has been suspected to date.

Epigenetic Regulation of Mismatch Repair Genes?—The widespread changes in methylation that are likely to accompany MNU-induced reactivation of MGMT will also tend to alter the expression of other genes. It is possible, therefore, that the high frequency of mismatch repair defects that are commonly observed among methylation-tolerant cell lines

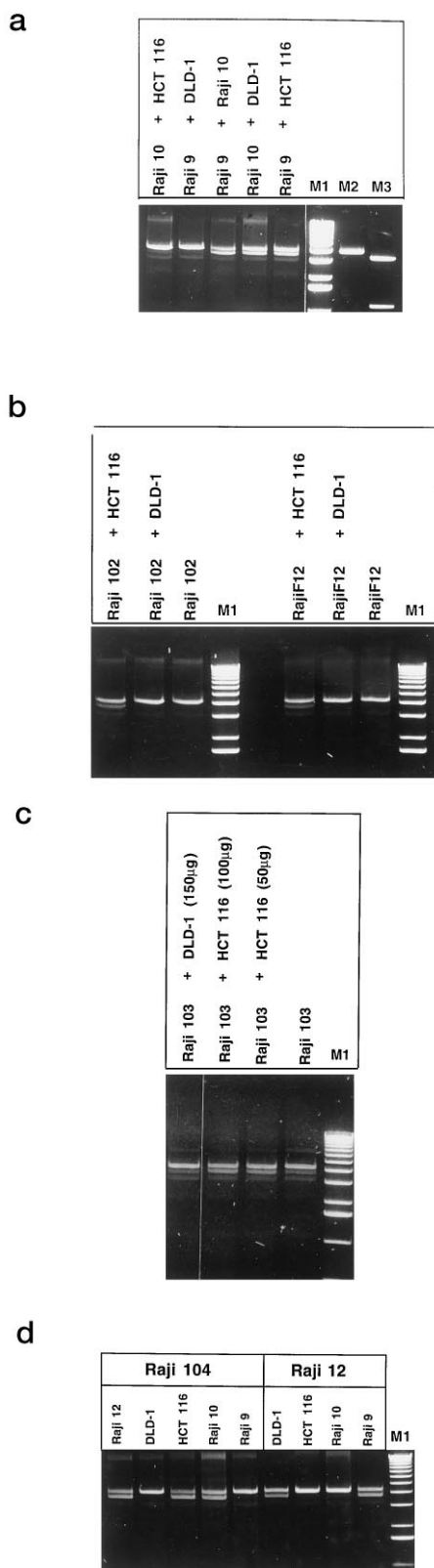


FIG. 8. Complementation of MNU-resistant cell extracts by colorectal carcinoma cell extracts. *a*, Raji 10 and Raji 9. CT mismatch correction by extracts of Raji 10 or Raji 9 (100 μ g of protein) was determined in the presence of 100 μ g of the colorectal carcinoma cell extracts shown. Where indicated, Raji 9 and Raji 10 extracts were combined. DNA was recovered and digested with *Mlu*I, and the products were analyzed by agarose gel electrophoresis. Markers are as in Fig. 4*a*. *b*, RajiF12 and Raji 102, CT mismatch correction by extracts of RajiF12 or Raji 102 (100 μ g of protein) was determined in the presence of 100 μ g of HCT116 or DLD-1 cell extracts as shown. *c*, Raji 103. Extracts of Raji 103 (100 μ g of protein) supplemented by extracts of

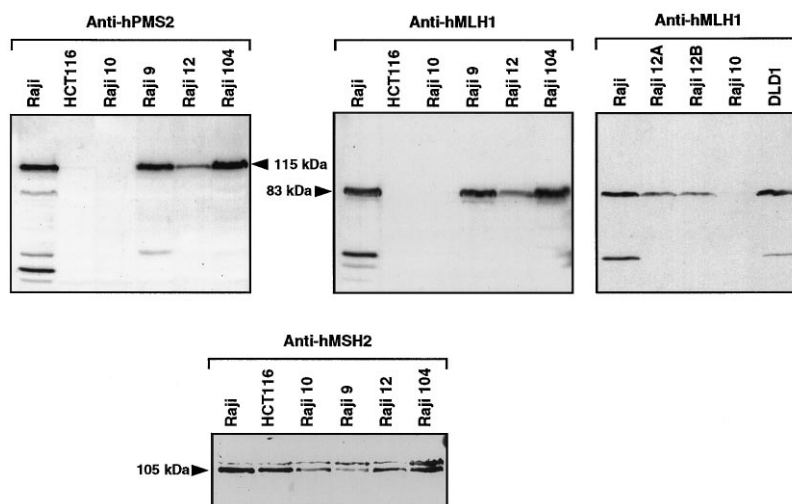
partly reflects a sensitivity of mismatch repair gene expression to changes in DNA cytosine methylation. Indeed, the frequency at which mismatch repair-defective methylation-tolerant cells arise in our and others' experiments is probably too high to be accounted for by the mutational inactivation of two mismatch repair alleles, even taking into account the use of potent mutagens in their selection. The properties of Raji 102 cells in particular are consistent with epigenetic changes. Their resistance was correlated with a loss of mismatch binding and repair as a consequence of a defect in the mismatch recognition protein hMSH6/GTBP. Recovery of mismatch recognition and repair ability in Raji 102B coincided with the cells' spontaneous reversion to normal MNU sensitivity. Similar spontaneous loss of methylation tolerance has been observed in Chinese hamster (6) and HeLa cell lines.² In the former case, reversion to MNU sensitivity was also accompanied by the reappearance of mismatch recognition activity. Phenotypic reversal of this nature could result from spontaneous reversion of MNU-induced mutations or subsequent spontaneous compensatory mutations. It is perhaps more plausible, however, that alterations in cytosine methylation underlie the reversible loss of hMSH6/GTBP function in some tolerant cells, including Raji 102. Widespread changes in DNA cytosine methylation are common in colon carcinogenesis (39). A susceptibility to silencing might contribute to the loss of expression of critical mismatch repair genes during the development of mismatch repair-defective colon tumors. Loss of repair capacity by gene silencing would not be associated with mismatch repair gene mutations. The recent demonstrations that loss of hMLH1 expression is frequently observed in tumors and tumor cell lines that do not have mutations in the *hMLH1* gene and that the absence of expression is correlated with increased cytosine methylation in the *hMLH1* promoter (40) are consistent with this suggestion. There are also indications that exogenously supplied genes are more frequently inactivated by cytosine methylation in mismatch repair defective cells (41).

Mismatch Repair Defects Associated with Known Phenotypes—In Raji 10, the only representative of Phenotype 2, instability at both mono- and dinucleotide microsatellites and complementation of repair in cell extracts suggested that the hMutL α complex was defective. Western analysis confirmed this inference. The phenotype of Raji 10 was similar to hMLH1-defective methylation-tolerant colorectal carcinoma cell lines HCT116 (15, 42) and SW48 (23). Human tumor cell lines with deficient hPMS2 also exhibit a methylation-tolerant phenotype (18), suggesting that the hMutL α complex is involved in processing *O*⁶-meGua into a lethal lesion from which tolerance offers an escape. hMutL α defects are also associated with mismatch repair-related tolerance to methylation damage in HeLa cells (9) and tolerance to cisplatin and doxorubicin DNA damage in ovarian carcinoma cell lines (8). The latter resistant lines do not produce detectable hMLH1 mRNA and provide evidence that the hMLH1 protein is required to stabilize its hPMS2 partner (43). Thus, hPMS2 deficiency can occur as a secondary consequence of hMLH1 defects. Normal amounts of hMLH1 are present in the endometrial carcinoma cell line HEC-1A (8), which is mutated in hPMS2 (18), indicating that hMLH1 may not require hPMS2 for stability. The absence from Raji 10 of detectable levels of either component of hMutL α most

² P. Karran, unpublished data.

HCT116 or DLD-1 as shown were used to assay CT mismatch correction. *d* Raji 104 and Raji 12. CT mismatch correction by extracts of Raji 104 or Raji 12 (100 μ g of protein) combined with other cell extracts as shown was determined.

FIG. 9. Western blot analysis of mismatch repair proteins. Cell extracts were separated on 8% SDS-polyacrylamide gels, and proteins were transferred to a nylon membrane that was probed successively with anti-hPMS2 (left panel), anti-hMLH1 (right panels), or anti-hMSH2 (bottom). Immunoreactive proteins were detected as described under "Experimental Procedures."



likely reflects a primary defect in hMLH1 in these cells. An apparently reduced level of both hMLH1 and hPMS2 in Raji 12 cell extracts is also compatible with a defective hMLH1.

Six resistant clones were assigned, somewhat arbitrarily, to two separate phenotypes, 3 and 4, on the basis of a mild mutator effect and/or a mismatch binding defect. Complementation analysis indicated that despite the heterogeneity in cellular phenotype, members of Phenotypes 3 and 4 were most likely defective in a single gene product, the hMSH6/GTBP component of hMutS α . Inactivation of the *hMSH6/GTBP* gene has been reported in other methylation-tolerant cell lines. Both alleles are mutated in the methylation-tolerant MT1 derivative of the TK6 lymphoblast cell line (44). In addition, chromosome transfer experiments suggest that hMSH6/GTBP variants make up the larger of two genetically defined complementation groups of methylation-tolerant HeLa cells (Ref. 45).³ Neither MT1⁴ nor the HeLa variants⁵ exhibited any detectable defect in mismatch binding in the bandshift assay. Raji 8, Raji 9, and Raji 19 were similar in this regard. These repair-defective cells apparently produce a partially functional hMSH6/GTBP that is able to interact with hMSH2 and mismatched DNA as determined by the bandshift assay. In the case of Raji 8, the defective hMSH6/GTBP also permits considerable processing of DNA *O*⁶-meGua as measured by nonsemiconservative DNA synthesis. Other hMSH6/GTBP defects, such as those in the colorectal carcinoma line DLD-1 (16, 23) or RajiF12 (6), abolish the interaction of the recognition complex with mismatched DNA. In the particular case of RajiF12, the presence of an apparently normal-length hMSH6/GTBP protein in Western blots⁴ and the absence of protein-truncating mutations in RajiF12 hMSH6/GTBP cDNA⁶ indicate that abolition of mismatch recognition by the RajiF12 hMutS α complex is most probably the result of inactivation of hMSH6/GTBP by missense mutation. This presumed mutation might disrupt the RajiF12 hMSH6/GTBP protein interaction with hMSH2, mismatched DNA, or both. Thus, hMSH6/GTBP variants, some of which have impaired mismatch binding, whereas others bind apparently normally and may even allow some mismatch processing, can be readily isolated by selection for methylation tolerance. Detailed characterization of these defective proteins offers a way to define regions of the hMSH6/GTBP gene product that interact with mismatched DNA or with the other proteins involved in the initial steps of mismatch correction.

The reason for the predominance of hMSH6/GTBP defects among our methylation-tolerant cells is not clear. It may reflect the preferential recognition of *O*⁶-meGua-containing base pairs by the hMutS α complex, although analogous hMSH2 variants, which are common among mismatch repair-defective human tumors, appear to be rather rare among methylation-tolerant cells. Alternatively, the preponderance of hMSH6/GTBP variants may have a genetic rather than a biochemical basis. Heterozygosity for hMSH6/GTBP in Raji cells would facilitate the emergence of tolerant cells with hMSH6/GTBP defects. Heterozygosity would not necessarily compromise mismatch repair capacity in these parental cells because, in general, expression of a single copy of the known mismatch repair genes, by chromosome transfer into defective cells (10, 19, 42) or in cells of heterozygous individuals (15, 46, 47), provides sufficient mismatch repair activity. It is also possible that the hMSH6/GTBP gene might be particularly susceptible to epigenetic silencing of the type outlined above.

Mismatch Repair Defects Without Precedented Phenotypes—About a third of the resistant clones did not display a detectable increase in MGMT expression. There was no loss of mismatch binding activity or evidence of a microsatellite instability, two features of mismatch repair-defective cells. Nevertheless, Raji 3, 7, 12, 17, and 104 all exhibited full resistance to the cytotoxic action of MNU. Mismatch repair appeared normal in Raji 3 and Raji 17. We have not characterized these clones in detail, and in particular, we have not determined the extent of formation and persistence of DNA methylation adducts. It is possible that their resistance is a consequence of a reduced load of *O*⁶-meGua in DNA which might come about through a metabolic change that protects the DNA from damage or by an increased *O*⁶-meGua repair that is independent of MGMT. Alternatively, the cells may exhibit a methylation-tolerant phenotype that does not involve loss of the mismatch repair pathway or results from mismatch repair defects that selectively impair recognition of DNA *O*⁶-meGua and escape detection by the correction assays we used.

The most striking observation among Phenotype 5 clones was the mismatch repair deficiency of Raji 7, Raji 12, and Raji 104. Despite the absence of detectable microsatellite instability, they were unable to correct the CT mismatch *in vitro*, and complementation analysis identified defects in hMutS α in Raji 104 and hMutL α in Raji 12. Western blotting indicated a possible reduced expression of hMutL α in Raji 12. It appears that the *in vitro* mismatch correction assay but not the microsatellite assay is sensitive to the alteration in hMutL α expression in Raji 12. Together with Raji 102 (and possibly also Raji 103),

³ G. Aquilina and M. Bignami, unpublished data.

⁴ J. Jiricny and F. Palombo, personal communication.

⁵ M. Bignami, personal communication.

⁶ R. Hampson and P. Karran, unpublished data.

which have mismatch recognition defects that can be detected by *in vitro* assay but display no significant cellular manifestations of compromised mismatch repair, Raji 7, Raji 12, and Raji 104 comprise a group of phenotypically silent mismatch correction-deficient cell strains. Methylation tolerance resulting from a loss of mismatch repair is often accompanied by only moderate increases in spontaneous mutation rate (6, 45, 48). It appears that some mismatch repair defects do not confer dramatic microsatellite instability on tolerant cells. It has also been pointed out that microsatellite instability and an extreme mutator phenotype at HPRT are not necessarily associated in tumor cell lines (49). It is interesting in this regard that heterozygous dominant negative mutations in mismatch correction genes do not confer a significantly more dismal prognosis as measured by age of onset and overall tumor incidence (46). In addition, if there are mismatch repair-defective tumors that do not display microsatellite instability, the frequency of mismatch repair defects among human tumors is likely to have been underestimated.

In summary, we have isolated several methylation-tolerant cell lines in which we have demonstrated mismatch repair defects by a biochemical assay. Three additional clones gained their MNU resistance through reactivation of a previously silent MGMT gene. This observation and the reported hypermethylation of the promoter region of the *hMLH1* gene in some tumor cells that do not express the hMLH1 protein (40), suggests that mismatch repair genes might be susceptible to silencing by cytosine methylation. Together with previous studies of methylation-tolerant cells (6, 7, 9, 45) and human colorectal carcinoma lines (18, 23, 42), our data indicate that both the hMutS α and hMutL α mismatch repair complexes participate in processing DNA O⁶-meGua into a lethal intermediate. The majority of our methylation-tolerant clones were defective in the hMutS α mismatch recognition complex, most probably the hMSH6/GTBP protein, and the considerable phenotypic heterogeneity among hMSH6/GTBP-defective variant cells may reflect different inactivating mutations. More than a third (5 of 12) of methylation-tolerant clones were demonstrably deficient in mismatch correction but did not display detectable mutator effects such as microsatellite instability. These included cells in which either hMutS α or hMutL α appeared to be defective. Thus, microsatellite instability may not be diagnostic for all mismatch repair deficiencies, even those involving known proteins.

Acknowledgments—The assistance of the Cell Production and Oligonucleotide Synthesis Laboratories of the Imperial Cancer Research Fund, Clare Hall is gratefully acknowledged. We thank Drs. Margherita Bignami and Rick Wood for critical comments on the manuscript.

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J. Biol. Chem. 1997, 272:28596-28606.

doi: 10.1074/jbc.272.45.28596

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