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A mismatch recognition defect in colon carcinoma confers DNA microsatellite instability and a mutator phenotype

GABRIELE AQUILINA*, PATRICIA HESS*, PAULINE BRANCH†, CATRIONA MACGEOCH†, IDA CASCIANO†‡, PETER KARRAN†, AND MARGHERITA BIGNAMI*§

*Istituto Superiore di Sanitá, Section of Chemical Carcinogenesis, Viale Regina Elena 299, 00161 Rome, Italy; and †Imperial Cancer Research Fund, Clare Hall Laboratories, South Mimms, Hertfordshire, EN6 3LD, United Kingdom

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ABSTRACT **We have analyzed spontaneous mutations in the adenine phosphoribosyltransferase gene of Chinese hamster clone B cells that exhibit a mutator phenotype because of defective mismatch binding. The mutator phenotype conferred increases in a limited number of mutational classes. The rates of transitions and most transversions were not significantly increased. The rates of A to T transversions and −2 frameshifts were strikingly elevated. These mutations were in repeated elements and 5 of 9 of the frameshifts were dinucleotide deletions in DNA sequences resembling microsatellites. The mismatch binding protein that is defective in the mutator line is a G·T mismatch recognition factor. Band-shift analysis indicated that the preferred substrate for the mismatch recognition protein is duplex DNA containing an extrahelical mono- or dinucleotide within repeated sequences. In agreement with a role in preventing minus frameshifts, a defective binding protein conferred an instability in clone B microsatellite DNA. A mismatch binding defect was also detected in LoVo, a human colorectal carcinoma cell line. Extracts of clone B or a second mismatch binding-deficient line, Raji–F12, did not complement LoVo extracts, indicating that these lines share a common defect. Our data provide a mechanistic explanation for the relation between defective mismatch recognition and the microsatellite instability of human colon cancer.**

DNA mismatch repair corrects replication errors (1), accounts for gene conversion by correcting mispaired bases within heteroduplex DNA (2), and acts as a barrier to illegitimate recombination between species (3). The most thoroughly characterized mismatch repair system is the *Escherichia coli* methylation-directed pathway that is dependent on the *mutS*, *mutL*, *mutH*, and *dam* genes (4, 5). Correction of DNA mismatches is initiated by the MutS protein that recognizes and binds to single base mispairs in duplex DNA (6). Small single-stranded regions of up to 4 bases are also recognized by this protein (7). MutL is then recruited by DNA–MutS and the resultant complex orchestrates incision of the mismatched DNA strand by MutH. Strains defective in any step of this pathway display a mutator phenotype because they accumulate uncorrected DNA replication errors (8).

Mismatch repair in higher eukaryotes has been observed both *in vivo* (9) and in cell extracts (10–13). We have previously demonstrated that loss of a mismatch recognition protein confers a mutator phenotype on mammalian cells, consistent with its involvement in mismatch repair (14). Two variant cell lines—one hamster (clone B) and one human (Raji–F12)—are resistant to DNA methylation damage because of a defective G·T binding protein (15). Relative to their parental lines, clone B and Raji–F12, respectively, exhibit 2.2- and 3.6-fold increases in the rate of mutation at the adenine phosphoribosyltransferase (*aprt*) or the hypoxanthine-guanine phosphoribosyltransferase gene (14).

Much human cancer displays a familial component with an inherited tendency to develop specific types of tumors, often at a relatively early age. Individuals exhibiting hereditary nonpolyposis colorectal cancer (HNPCC) exhibit familial aggregation of tumors. The syndrome is characterized by an early onset of tumors in the colon or other organs, particularly endometrium (16). Family studies have recently localized HNPCC genes to chromosomes 2p and 3p (17, 18). Simultaneously, instability in DNA microsatellites of the type $(CA)_n$ was identified in HNPCC colon tumors (19).

We report here that the mismatch binding defect of clone B cells is associated with increases in mutations at repeated DNA sequences. These mutations are predominantly A to T transversions and minus frameshifts. The frameshifts comprise a high proportion of dinucleotide deletions within reiterated sequences. The defect in clone B cells predisposes them to the microsatellite instability seen in human colon carcinoma (20, 21). Investigation of the properties of the G·T binding activity indicated its probable involvement in prevention of dinucleotide loss by slipped mispairing during replication. The same activity is also defective in the human colorectal carcinoma cell line LoVo, which exhibits microsatellite instability (22).

MATERIALS AND METHODS

Cell Culture. CHOMT$^+$ and clone B cells were cultured as described (23). The human colorectal cells HT29, Colo320DM, and Colo320HSR (American Type Culture Collection) were grown in modified Eagle's medium containing 15% fetal calf serum. LoVo cells were grown in Ham's F-12 medium plus 10% fetal calf serum.

Isolation of *aprt* Mutations and Sequence Analysis. Independent spontaneous *aprt* mutants were isolated from clone B and CHOMT$^+$ cells as described (23). Rates of mutation were determined by fluctuation analysis (24, 25). Briefly, 108 (CHOMT$^+$) or 78 (clone B) independent cultures were grown from 100 to 3×10^6 cells. Each culture was then distributed among five plates containing medium supplemented with 0.4 mM 8-azaadenine. One 8-azaadenine-resistant clone was isolated from each mutant-containing culture. *aprt* sequences were amplified from purified DNA by PCR using primers flanking each of the 5 *aprt* exons (26). The mutated exon was identified by single-strand conformation polymorphism or sequenced directly (27).

Mismatch Binding Assay. Mismatch binding was assayed in cell extracts as described (28). Duplex substrates of the following sequences were used:

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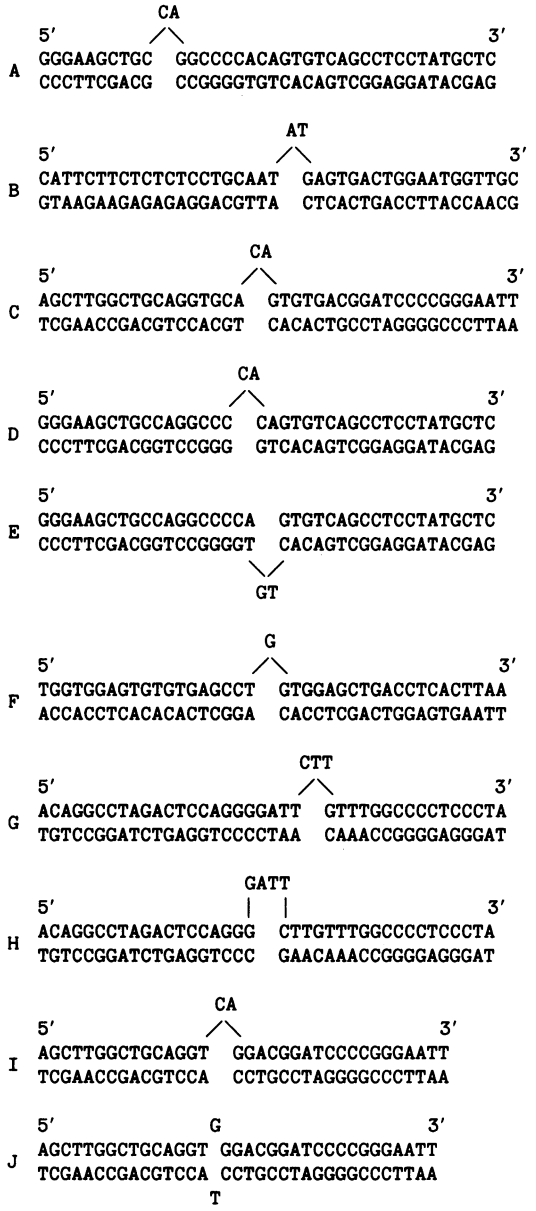
Abbreviation: HNPCC, hereditary nonpolyposis colorectal cancer.

‡Present address: Laboratory of Population Genetics, Istituto Nazionale per la Ricerca sul Cancro (IST), Sassari, Italy.
§To whom reprint requests should be addressed.



Microsatellite Polymorphisms. DNA isolated from independent clones of CHOMT$^+$ and clone B was amplified by PCR with specific primers flanking defined hamster-specific $(CA)_n$ repeated regions. Thirty cycles (1 min at 90°C, 30 sec at 55°C, and 30 sec at 70°C) were performed. Amplification products were separated on 6% denaturing polyacrylamide gels, transferred to Hybond N$^+$ (Amersham), and detected by probing with a terminally ^{32}P-radiolabeled PCR primer. Hybridization was performed overnight at 42°C in 130 mM sodium phosphate, pH 7.0/250 mM NaCl/10% PEG 4000/7% SDS.

Primer	Forward	Reverse
22.1	AAGCTTGACATCCCAACTGG	AGTGTGCGTGTGTACTTGTGTT
11.1	TTTTCCAAGTATGTGCTTCCTG	AAACAAGGTTCAGTGGGATAGC
10.1	GCCTAGGCTCAAACAAGCAC	TATAAGACACAAGTAGTGAGTG
21.1	TTTCCCAAAGAAGTCATATGCC	CCTTCCTGCAATCTCAAGATG

RESULTS

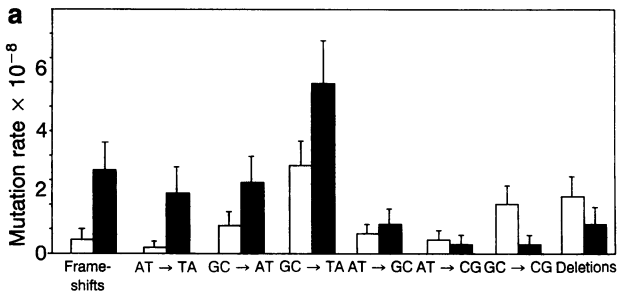
Fifty-four independent spontaneous *aprt* mutants from the mutator cell line clone B were compared to 42 mutants of the parental CHOMT$^+$ cells (23). A to T transversions, frameshifts, G to A transitions, and G to T transversions occurred more frequently in clone B (Table 1). The mutation rate at the *aprt* locus of clone B is 2.2-fold higher than the parental cells. Applying this correction revealed a slight increase in G to T transversions (<3-fold) and G to A transitions (2-fold). In contrast, frameshifts and A to T transversions were, respectively, almost 6- and 8-fold overrepresented in clone B (Fig. 1*a*). Fig. 1*b* shows the sequence contexts of the two major overrepresented mutational classes. The A to T transversions occurred preferentially in short runs of reiterated bases or at direct/inverted repeats. The frameshifts were found exclusively in repeated sequences. Three frameshifts were losses of a single base in monotonic runs. Most striking was a CA deletion in the sequence $(CA)_2(GT)_2$ in three mutants and the loss of AT or CT dinucleotides occurring in the sequences $GCA(AT)_2GAG$ and $CTT(CT)_3CCT$, respectively. The sequence contexts of the frameshifts are consistent with the slipped mispairing model of mutation (30). Their overrepresentation in the defective clone B cells suggested that the G·T binding activity prevents minus frameshifts and that DNA containing extrahelical bases might be a normal substrate for binding.

We investigated whether mismatch binding proteins could recognize substrates containing extrahelical bases. Since normal hamster and human cells contain similar mismatch binding activities these experiments were carried out with extracts of human Raji cells (14). Oligonucleotides based on the sequences surrounding the frameshifts at sites 1783, 2296, and 2230 in the *aprt* gene (Fig. 1*b*) and containing an extrahelical CA, GT, AT, or G were used as substrates for a band-shift assay. Raji extracts bound to a duplex containing an extrahelical CA or GT in the sequence $(CA)_2(GT)_2$ (Fig. 2*a*, substrates D and E). The complex formed (B, arrow) migrated at the same position as that observed with a G·T mispair (substrate J) and competition experiments established that the two complexes were identical (data not shown). Complex B was also formed with substrates with either a single extrahelical G or AT in repetitive sequences (substrates F and B, respectively). Sequence requirements for mismatch recognition were also investigated. Binding was observed only when the extrahelical element was part of a repeated motif. Thus, when a lone CA upstream from the $(CA)_2(GT)_2$ element was extrahelical (substrate A), binding was absent. Similarly, replacement of the G·T mispair in the random sequence with an extrahelical CA (substrate I) abolished binding. However, if the isolated CA in the random sequence was replaced with extrahelical CA within the $(CA)_2(GT)_2$ repeat, binding was restored (substrate C). Complex B was not formed with two substrates, G and H, based on the sequences of 3- or 4-base deletions in the hamster *aprt*

Table 1. Spontaneous mutational spectra of parental and mismatch recognition-defective clone B cells

	No. of mutants	
	CHOMT$^+$	Clone B
Transitions		
GC to AT	4	7
AT to GC	3	3
Transversions		
AT to TA	1	6
AT to CG	3	1
GC to TA	12	24
GC to CG	7	1
Frameshifts	2	9
Deletions	8	3
Complex changes	2	0
Total mutants	42	54

Some spontaneous *aprt* mutations in CHOMT$^+$ are taken from ref. 23. Complex changes include multiple substitutions, insertions, and duplications.



b

MUTANT	MAP SITE	SEQUENCE CONTEXT	TYPE
Frameshifts			
clone 2	455-458	CCCCAT [C] [CCC] GGCGT	-1bp
clone 39	627-630	AAGGA [C] [CCC] GCCTC	+1bp
clones 8, 27, 16	1783- 6	GCCC [CA] [GT][GT] CA	-2bp
clones 14, 36	2230- 1	GAGCC [G] [TG] [TG] GAG	-1bp
clone 58	2284- 9	TTCTT [CT] [CT] CCTGC	-2bp
clone 33	2296- 9	CTGCA [AT] [AT] GAGTG	-2bp
Transversions AT>TA			
clone 18	395	GGCTA T GGCGG	T>A
clone 11	673	ACC TT A AGTCC	A>T
clone 22	1692	CCTAG A CTCCA	A>T
clone 25	1703	GGGGA T TCTTG	T>A
clone 1	1747	GGCTG T G TGCT	T>A
clone 28	1986	TAGAA A TCCAG	A>T

FIG. 1. Rates of different classes of mutation and sequence context of the classes of mutation overrepresented in clone B. (*a*) Rate of mutation of each class of mutation was determined by Luria–Delbrück fluctuation analysis. Open bars, CHOMT$^+$; solid bars, clone B. (*b*) Location of each mutation in the *aprt* gene (map site) is according to the published sequence (29). Wild-type sequences are written with the bases deleted in the mutants shown displaced from the duplex. Repeated elements are shown boxed.

gene (refs. 23 and 26; this work) and containing 3- or 4-base extrahelical elements. Extracts of G·T binding-defective clone B were also unable to bind to substrates with the extrahelical CA in the repeated element (Fig. 2*b*) or that contained an extrahelical G (data not shown). Similar results were obtained with a second defective cell line, Raji–F12 (data not shown). Thus, binding to duplexes containing one or two extrahelical residues is a property of the G·T mismatch binding activity that is absent from two tolerant cell lines. Extrahelical bases are, however, efficiently recognized by this activity only when they occur in reiterated DNA sequences.

The preferred substrates for the mismatch recognition protein(s) resemble slipped mispaired intermediates in microsatellite DNA regions. We examined the stability of microsatellite DNA in the binding-defective hamster cells.

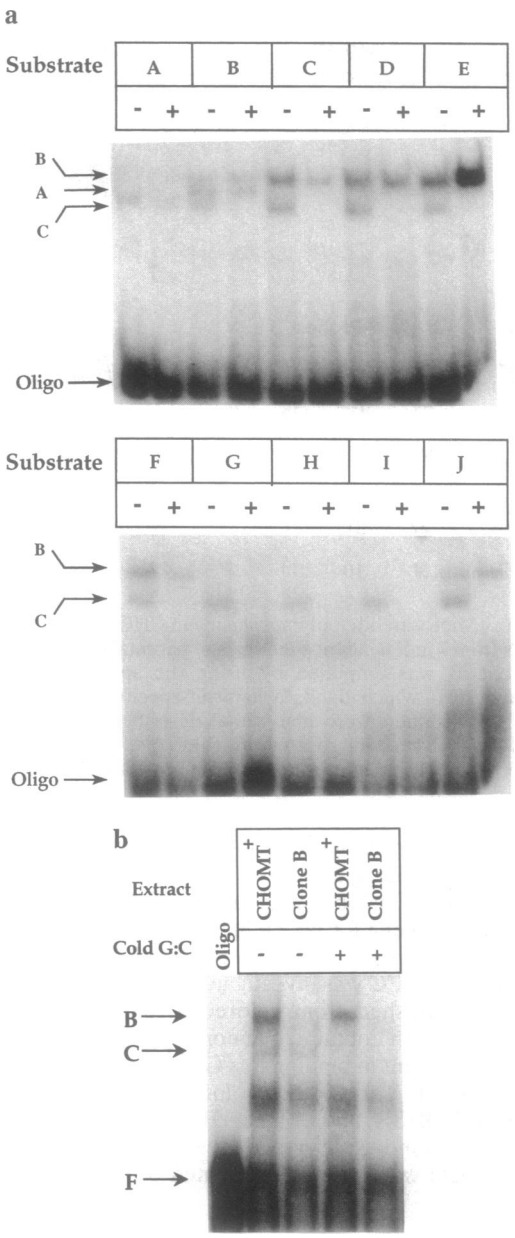
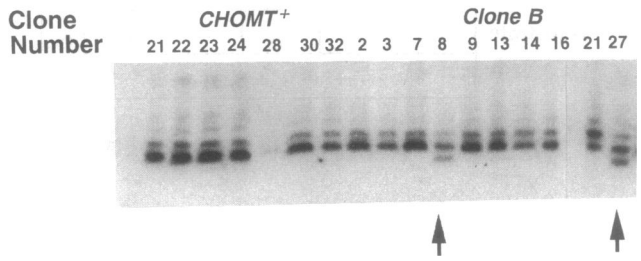


FIG. 2. Mismatch binding at slippage intermediates in repeated sequences and its defect in clone B hamster cell extracts. (*a*) Oligonucleotide binding by extracts of Raji cells (15 μg) was determined by band-shift analysis using heteroduplex oligonucleotide substrates with the sequences described. Assays were performed in the absence (lanes −) or presence (lanes +) of a homoduplex competitor to suppress nonspecific binding as indicated. Arrows A and B indicate positions of the mismatch-specific complexes. Complex C is a nonspecific complex. Position of the uncomplexed oligonucleotide (oligo) is also shown. (*b*) Extracts of either parental CHOMT$^+$ or clone B extracts were assayed for binding to substrate D containing an extrahelical CA dinucleotide as described. Arrows B and C indicate positions of the mismatch-specific and nonspecific complexes, respectively. Position of the uncomplexed oligonucleotide is indicated by F.

DNA from 50 independent clones of the parental CHOMT$^+$ cells and 58 of the clone B cells was amplified by using specific primers flanking four hamster microsatellite regions comprising 22–24 CA repeats (Fig. 3*a*). Twenty-seven of 132 microsatellites were polymorphic in clone B, while no mutations were observed in 121 analyses of CHOMT$^+$ (Fig. 3*b*). Thus, the mutation rate at microsatellites is at least 25-fold elevated in clone B. The clone B polymorphisms are consis-

a



b

	Probes						
CHOMT$^+$	22.1	11.1	10.1	21.1	Total	Mutation rate	Relative rate
No. clones	21	31	45	24	121	< 0.8 %	1
No. mutations	0	0	0	0	0		
Clone B							
No. clones	27	31	47	27	132	20 %	>25
No. mutations	4	10	11	2	27		

FIG. 3. Microsatellite polymorphisms in CHOMT$^+$ and clone B. (*a*) DNA from 7 independent subclones of parental CHOMT$^+$ and 10 from clone B was amplified by using the specific primer pair designated 10.1, which flanks a hamster-specific $(CA)_{22}$ repeated region. PCR products were analyzed as described. Arrows indicate clones with shortened microsatellites. (*b*) Pooled data from analysis of all combinations of microsatellite primers and DNA.

tent with loss of dinucleotide repeats. The G·T binding factor that is absent from clone B is therefore implicated in stabilizing microsatellite sequences.

Microsatellite instability is a characteristic feature of HNPCC (19), a gene for which has been mapped to chromosome 2p (17). The possibility that mismatch binding might be defective in HNPCC was investigated by assaying extracts of cell lines established from colorectal tumors. Extracts of LoVo cells, which contain an abnormality of chromosome 2 (31), were deficient in binding to a G·T mismatched substrate (Fig. 4, lane 4). Three other colorectal carcinoma lines—HT29, Colo320HSR, and Colo320DM—contained significant G·T mismatch binding activity (lanes 1–3). The deficiency of LoVo extracts was not complemented by extracts of Raji–F12 (lane 10) or clone B (data not shown) cells, indicating that the three lines share a common defect. Other binding activities were unimpaired in LoVo cell extracts. Thus, when LoVo extracts were added to limiting amounts of Raji or HT29 cell extracts, a more than additive increase in binding was observed (lanes 8 and 13). A similar synergism is seen when extracts of wild-type Raji and binding-deficient F12 are mixed (ref. 14; Fig. 4, lane 14). A possible explanation is the involvement of two proteins in formation of the complex. LoVo cells are therefore deficient in a protein involved in binding to G·T mismatches and extrahelical bases, and this protein normally functions to stabilize microsatellite DNA.

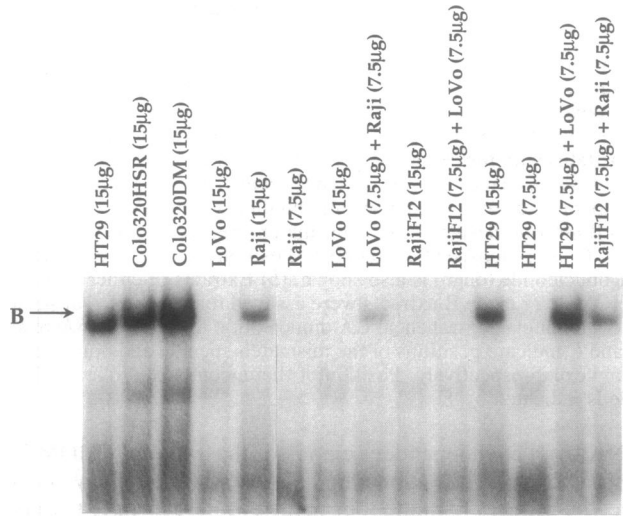


FIG. 4. Mismatch binding in extracts of human colorectal carcinoma cells. Binding to the G·T mismatched heteroduplex J was determined by using 7.5 or 15 μg of cell extract, either alone or in the combinations indicated. Position of migration of the mismatch-specific complex B is indicated.

DISCUSSION

Two methylation-tolerant cell lines—clone B and Raji–F12—are mutators with a common defect in a mismatch recognition protein (14). The increased mutation rate in clone B affects a limited range of mutations in the *aprt* gene. This does not reflect an intrinsic property of the *aprt* gene since inactivation of the APRT protein can result from all possible mutational events and mutations are distributed evenly without hot spots (32). Loss of the MutS mismatch binding protein from *E. coli* increases the incidence of both types of transitions and frameshifts, indicating that mismatch correction selectively prevents these kinds of mutation (33). The classes of mutations increased by the mismatch binding defect of clone B are mainly A to T transversions and −2 frameshifts.

Spontaneous A to T transversions are rare in cultured mammalian cells (<2%) (26, 34). They can arise through A·A or T·T mispairs, which are the least well repaired mismatches in transfected monkey cells (9). Increases in reversion of specific alleles by A to T transversion in *mutS E. coli* indicate that this type of mutation can be affected by mismatch correction only in a limited range of sequence contexts (35). Extrahelical structures could be intermediates in A to T transversion by dislocation mutagenesis (36) since these mutations occur within short repeats in mammalian cells (26, 37). Alternatively, transversions might arise via insertion of A opposite apurinic sites generated by loss of A and displaced into an extrahelical conformation (38).

Mismatch correction prevents frameshifts in transfected heteroduplex DNA, and purified *E. coli* MutS protein binds to DNA containing up to 4 extrahelical bases (7). Repeated dinucleotides are unstable in mismatch recognition-defective *E. coli* (39) or *Saccharomyces cerevisiae* (40). Extrahelical dinucleotides were recognized by the mammalian G·T binding protein only within regions of repeated dinucleotides. These observations, and the striking increase in −2 frameshifts among clone B *aprt* mutations, indicate that mammalian G·T binding activity promotes the repair of extrahelical bases in misaligned strands at reiterated sequences. The increase in ΔCA, ΔAT, and ΔCT frameshifts in the *aprt* gene is paralleled by the elevated instability in clone B microsatellites. The mutator phenotype in microsatellites is therefore more pronounced than in the *aprt* gene. This difference may reflect the multiple mechanisms by which mutations of different classes arise, as some will be unaffected by the defect in clone B cells.

The association between microsatellite instability and colon cancer prompted us to investigate G·T binding activity in colorectal carcinoma cell lines. LoVo cells are defective in mismatch binding. It is striking that deletion of a CT in the sequence $(CT)_3$, which is identical to one of the mutations we observed in the *aprt* gene of clone B, is also present in the LoVo β_2-microglobulin gene (45). LoVo cells have a cytogenetically detectable abnormality of chromosome 2, where one HNPCC gene that predisposes cells to microsatellite instability is located. Recently, LoVo cells have been shown to exhibit microsatellite instability (22). An identical mis-

match binding defect is therefore associated with microsatellite instability in a colorectal carcinoma cell line and a methylation-tolerant hamster line. These observations provide direct evidence that defective mismatch binding underlies the microsatellite instability in colon carcinoma. The substrate preferences for binding provide a mechanistic explanation for the phenomenon.

After this work was completed, one HNPCC gene was identified as a human homologue of the *S. cerevisiae MSH2* gene (41) that encodes a mismatch recognition factor hMSH2 (42, 43, 46). The human homolog is mutated in individuals from HNPCC families (42, 43). Microsatellite instability was also demonstrated in a human colon carcinoma cell line that was defective in generalized mismatch repair *in vitro* (44). Our data indicate that clone B and LoVo cells share a defined defect in mismatch recognition. This impairment confers microsatellite instability but not necessarily a generalized mutator phenotype, as only a limited number of mutational classes in the *aprt* gene are affected.

Note Added in Proof. While this paper was in press, a deletion in the hMSH2 gene of LoVo cells was described together with an observed defect in mismatch repair by LoVo cell extracts (47).

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