Mutations of *GTBP* in Genetically Unstable Cells

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The molecular defects responsible for tumor cell hypermutability in humans have not yet been fully identified. Here the gene encoding a G/T mismatch-binding protein (GTBP) was localized to within 1 megabase of the related *hMSH2* gene on chromosome 2 and was found to be inactivated in three hypermutable cell lines. Unlike cells defective in other mismatch repair genes, which display widespread alterations in mononucleotide, dinucleotide, and other simple repeated sequences, the GTBP-deficient cells showed alterations primarily in mononucleotide tracts. These results suggest that *GTBP* is important for maintaining the integrity of the human genome and document molecular defects accounting for variation in mutator phenotype.

Defects in any of four mismatch repair genes (*hMSH2*, *hMLH1*, *hPMS1*, and *hPMS2*) can cause the hypermutability found in many hereditary colorectal cancers (CRCs) and in a subset of sporadic CRC [reviewed in (*1*)]. The newest member of the mismatch repair (MMR) family is *GTBP*, which encodes an hMSH2-related protein that binds to DNA containing G/T mismatches (*2*). GTBP exists as a heterodimer with hMSH2, and the purified heterodimer can correct the MMR deficiency observed in extracts from several hypermutable cell lines in vitro (*2*, *3*). One of these cell lines (LoVo) carries a homozygous mutation in the *hMSH2* gene (*4*, *5*) and is therefore devoid of functional hMSH2 protein, whereas another (HCT-15) contains a full-length hMSH2 protein with a wild-type sequence (*6*, *7*). To determine if HCT-15 cells carry a *GTBP* mutation, we amplified the GTBP coding sequences by reverse transcriptase (RT)–polymerase chain reaction (PCR) (*8*). Upon transcription and translation [in vitro synthesized protein assay (IVSP) (*9*], two of the PCR products generated truncated GTBP polypeptides (Fig. 1). Sequencing of these PCR products (*10*) revealed that the truncations were due to frameshift mutations: A 1–base pair (bp) deletion at codon 222 changed a leucine to a termination codon (Fig. 2A), and a 5-bp deletion/substitution at codon 1103 (TTGATAGAGT to TTTGT) created a new termination codon 9 bp downstream.

MT1 is an alkylation-resistant lymphoblastoid cell line with a biochemical deficiency similar to that of HCT-15 (*3*, *11*). We detected no alterations in GTBP in MT1 cells by IVSP analysis but found two missense mutations in the coding region of *GTBP* by DNA sequencing (*10*). The first was an A-to-T transversion at codon 1145, resulting in a substitution of valine for aspartic acid (Fig. 2C). This asparatic acid is in the highly conserved domain of GTBP, and the same residue is present at the analogous position in human, yeast, and bacterial MutS proteins. The second mutation was a G-to-A transition at codon 1192, resulting in a substitution of isoleucine for valine (Fig. 2D). Cloning of the RT-PCR products revealed that the two mutations were on separate alleles. Neither of these mutations was present in the MMR-proficient and alkylation-sensitive TK6 cells from which the MT1 cells were derived (Fig. 2).

To determine whether *GTBP* alterations occur in other hypermutable colorectal cancers, we analyzed four tumor cell lines that showed microsatellite instability but no evidence of MMR gene mutations (*4*). In each case, the region encompassing codons 38 to 1292 was sequenced and examined by IVSP. One cell line, from patient 543X, was found to contain a 1-bp insertion at codon

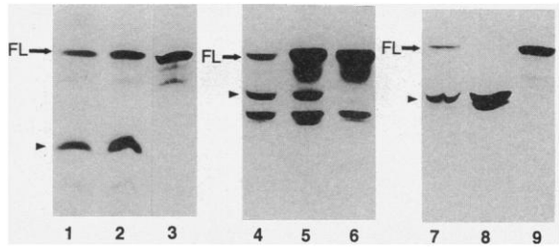

Fig. 1. Generation of altered GTBP polypeptides by *GTBP* sequences from human colon cancer cell lines and tumors. In vitro transcription and translation of RT-PCR products was carried out as in (*9*), and the resultant ^{35}S-labeled proteins were separated by SDS–polyacrylamide gel electrophoresis. The full-length (FL) products correspond to amino acids 32 to 453 (lanes 1 to 3), 692 to 292 (lanes 4 to 6), and 219 to 858 (lanes 7 to 9). The products in lanes 3 and 6 are from healthy control individuals, those in lanes 1 and 4 are from HCT-15 cells, and those in lanes 2 and 5 are from DLD1 cells. The HCT-15 and DLD1 cell lines were derived from the same tumor (*6*). Products in lanes 7, 8, and 9 were derived from the primary tumor, tumor xenograft, and normal colon of patient 543X, respectively. The FL band in lane 7 was likely derived from nonneoplastic cells in the primary tumor. Abnormal polypeptides are marked with an arrowhead. Other truncated polypeptides represent products derived from internal initiation sites (*9*).

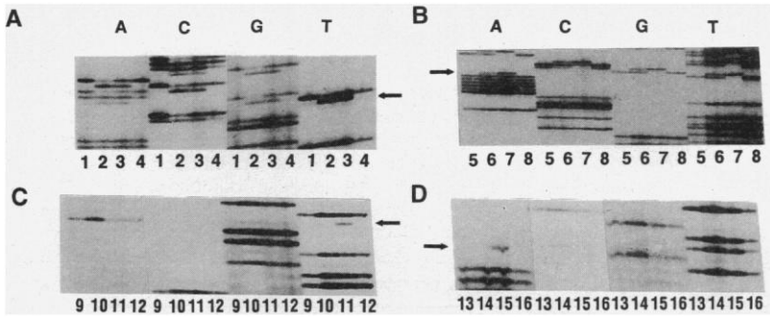

Fig. 2. *GTBP* mutations in genetically unstable cell lines. RT-PCR products were used as templates for direct sequencing as in (*10*). Mutations are indicated by arrows. (**A**) A 1-bp deletion at codon 222 in HCT-15 and DLD1 cells (lanes 2 and 3, respectively). (**B**) A 1-bp insertion at codon 626 in the 543X primary tumor (lane 6) and xenograft (lane 7). (**C**) An A-to-T transversion at codon 1145 in MT1 cells (lane 11). (**D**) G-to-A transition at codon 1192 in MT1 cells (lane 15). Lanes, 1, 4, 8, 9, 12, 13, and 16 contain DNA from normal cells of healthy individuals. Lane 5 contains DNA from the normal colon of patient 543X and lanes 10 and 14 contain DNA from the TK6 cell line, the MMR-proficient parent of MT1.

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626, with the frameshift creating a new termination codon (Fig. 1, lane 8, and Fig. 2B, lane 7). This mutation appeared to be hemi- or homozygous as no wild-type gene product was detectable by IVSP or sequence analysis. The mutation was somatic in origin, as it was not found in RNA from the patient's normal colon (Fig. 1, lane 9, and Fig. 2B, lane 5). The hemizygosity resulted from a somatic loss of a large region of chromosome 2p, demonstrated with polymorphic markers from the region (*7*). Analysis of the primary CRC from which the 543X cell line was derived showed that the mutation occurred before establishment of the line (Fig. 1, lane 7, and Fig. 2B, lane 6).

Mutations in previously studied MMR genes can cause hereditary nonpolyposis colorectal cancer (HNPCC) (*1*). To determine whether germline *GTBP* mutations occur in HNPCC, we analyzed *GTBP* in 20 HNPCC kindreds that did not carry mutations in *hMSH2*, *hMLH1*, *hPMS1*, or *hPMS2* as judged by the IVSP assay (*12*).

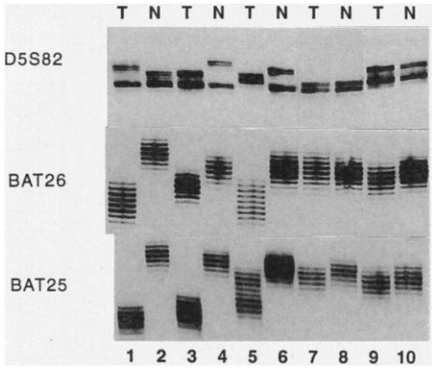

Fig. 3. Alterations in simple repeated sequences in GTBP-defective cells. The $(CA)_n$ marker D5S82 and the $(A)_n$ markers BAT26 and BAT25 were studied in cells defective for *hMSH2* (RB, lane 1), *hMLH1* (595X, lane 3), *hPMS2* (GC, lane 5), or *GTBP* (543X, lane 7, and MT1, lane 9). In each case, the PCR products of normal (N) cells (lanes 2, 4, 6, 8, and 10) of the same individual are shown next to the PCR products from the tumor (T) cells (see Table 1).

We found no germline mutations in *GTBP* in these kindreds by IVSP analysis or by sequence analysis of codons 38 to 1292 (*2*).

The differences in repair capability of HCT-15 and MT1 cell extracts, as compared with extracts from cells with other MMR gene defects (*3*), suggested that the cell lines might also show differences in their mutational spectra. To address this possibility, we analyzed eight markers representing simple repeated sequences. Five of them were $(CA)_n$ microsatellites and three were $(A)_n$ tracts (*13*). Cell lines with *hMSH2*, *hMLH1*, or *hPMS2* mutations showed alterations in all eight markers (Fig. 3 and Table 1). In contrast, cells with *GTBP* mutations showed alterations primarily in the $(A)_n$ tracts, with few mutations in the $(CA)_n$ repeats ($P < 0.01$, χ^2 test, Table 1). Even in the $(A)_n$ tracts, the deviations were usually less severe than those in tumors with other MMR gene defects (Fig. 3) (*14*).

We localized the *GTBP* gene to human chromosome 2p16 by PCR evaluation of a panel of somatic cell hybrids containing segments of chromosome 2 (*15*). Much of chromosome 2p16 had been previously cloned in YAC vectors during a previous search for the *hMSH2* gene (*16*), and *GTBP* was found to reside in YAC 5A11, a clone that also contained *hMSH2*. On the basis of the size of this YAC, *GTBP* was inferred to reside within 1 megabase (Mb) of *hMSH2*. This localization was confirmed by fluorescence in situ hybridization (FISH) with the use of genomic clones of hMSH2 and GTBP. From the co-localization of fluorescent signals, the maximum distance separating GTBP and hMSH2 was estimated at 0.5 Mb (*17*).

Thus, *hMSH2* and *GTBP* may have been produced by duplication of a primordial *mutS* repair gene. Adjacency of duplicated genes is not uncommon in the mammalian genome. Although hMSH2 and GTBP exist in the cell as a heterodimer, the functions of these two proteins seem distinguishable (*2*, *3*) (Table 1). Additionally, germline mutations of *hMSH2* account for about 50% of total HNPCC cases (*18*), whereas our data indicated that germline *GTBP* mutations in HNPCC, if they occur at all, are rare. *GTBP* mutations may therefore not cause sufficient genetic instability to result in predisposition to tumor formation. It is interesting that one of the lines with *GTBP* mutations also carries a *pol* δ mutation in a proofreading domain (*6*), which suggests that its hypermutability (*19*) might be due to the combined effect of defects in *GTBP* and *pol* δ. Alternatively, GTBP may participate in processes other than MMR, and the haplo-insufficiency that would be associated with its germline mutation might be incompatible with normal embryogenesis or development.

The genomic instability observed in cells with GTBP deficiency is less severe than in cells with other MMR gene defects. Many tumors have been shown to manifest a low degree of microsatellite instability compared with that observed in CRC (*20*). It remains to be determined whether GTBP defects play a role in instability in these other tumor types. If so, it would raise the possibility that the molecular defects responsible for genetic instability could be predicted from the spectra of mutations in the tumors. Such predictive power would have important implications for cancer diagnosis and treatment.

Table 1. Mutational spectra of tumor cell lines with various MMR defects. Polymorphic marker sequences containing either $(CA)_n$ or $(A)_n$ repeats, as indicated, were amplified and analyzed by denaturing polyacrylamide gel electrophoresis as in (*13*). The mutations in patients' RB, 595X, and GC cells are described in (*4*), (*18*), and (*21*), respectively. All DNA samples except that from MT1 cells were derived from colorectal tumor xenografts grown in nude mice and were compared with DNA from normal lymphocytic or colon epithelial cells from the same patient. The MT1 lymphoblastoid cell line was compared with its MMR-proficient lymphoblastoid parent, TK6.

Sample	Mutated gene	$(CA)_n$ alterations					$(A)_n$ alterations		
		YH5	CA7	D2S123	D5S82	D18S58	BAT25	BAT26	BAT40
RB	*MSH2*	+	+	+	+	+	+	+	+
595X	*MLH1*	+	+	+	+	+	+	+	+
GC	*PMS2*	+	+	+	+	+	+	+	+
543X	*GTBP*	−	+	−	−	−	+	+	+
MT1	*GTBP*	−	−	−	−	−	+	+	+

REFERENCES AND NOTES

1. P. Modrich. *Science* **266**, 1959 (1994); G. Marro and C. R. Boland, *J. Nat. Cancer Inst.*, in press.
2. F. Palombo *et al.*, *Science* **268**, 1912 (1995).
3. J. T. Drummond, G. Li, M. J. Longley, P. Modrich, *ibid.*, p. 1909.
4. B. Liu et al., *Nat. Genet.* **9**, 48, (1995).
5. A. Umar *et al.*, *J. Biol. Chem* **269**, 14367 (1994).
6. L. da Costa *et al.*, *Nat. Genet.* **9**, 10 (1995).
7. N. Papadopoulos *et al.*, unpublished data.
8. Complementary DNA (cDNA) was generated with the use of random hexamers and reverse transcriptase as described in (*22*). GTBP coding sequences were amplified by PCR, with the use of the cDNA as template and the primers 5′-PGAGGGTTACCCCTGG-3′ and 5′-ACACTGTAAGTCTGTGTACC-3′ for codons 32 to 458, primers 5′-PGGTGAAGGCCTGAACAGCC-3′ and 5′-AAGTCCAGTCTTTCGAGCC-3′-for codons 219 to 858, and primers 5′-PGAGAGGGTTGATACTTGCC-3′ and 5′-AGAAGTCAACTCAAAGCTTCC-3′ for codons 692 to 1292. P denotes the sequence 5′-GGATCCTAATACGACTCACTATAGGGAGACCACCATG-3′, which institutes a T7 promoter and a consensus for translation initiation. Codons are numbered according to (*2*).
9. PCR products were used as templates in coupled transcription-translation reactions (Promega), and the resultant proteins were analyzed by polyacrylamide gel electrophoresis as described in (*22*).
10. The RT-PCR products were sequenced with SequiTherm Polymerase (Epicentre Technologies, Madison, WI) and end-labeled primers chosen from the cDNA sequence (*2*). Primer sequences are available from the authors on request. In HCT-15, the two mutations were far apart, precluding amplification of a RT-PCR product that contained both. We therefore could not determine whether each was in a different allele.

11. V. S. Goldmacher, R. A. Cuzick, W. G. Thilly, *J. Biol. Chem.* **261**, 12462 (1986); A. Kat *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 6424 (1993).
12. The IVSP assay detected 15 of 24 previously identified mutations in *hMSH2*, *hMLH1*, *hPMS1*, or *hPMS2*.
13. The primers and PCR conditions are detailed in B. Liu *et al.* [*Nat. Med.* **1**, 348 (1995)], G. Gyapay *et al.* [*Nat. Genet.* **7**, 246 (1995)], and C. Breukel *et al.* [*Nucleic Acids Res.* **19**, 5804 (1991)].
14. The HCT-15 cell line could not be studied with the techniques in Fig. 3 because normal cells from the patient were not available. However, previous studies have shown that there is less clonal variation in repeated sequences in the HCT-15 line than in other lines with microsatellite instability [D. Shibata *et al.*, *Nat. Genet.* **6**, 273 (1994)] and that mutations in hypoxanthine-guanine phosphoribosyltransferase (HPRT) were different in HCT-15 than in other lines (*19*).
15. Primers used for genomic amplification of *GTBP* sequences were 5′-GTATGAAGAAACTACATACAGC-3′ and 5′-AAGTCCAGTCTTTCGAGCC-3′, yielding a 243-bp product corresponding to codons 777 to 858.
16. F. S. Leach *et al.*, *Cell* **75**, 1215 (1993).
17. A genomic P1 clone for hMSH2 was described previously (*16*). A P1 clone containing GTBP was obtained by similar methods, with the use of the primers described in (*15*). Two-color FISH was done as described in P. Lichter *et al.* [*Science* **247**, 64 (1990)] and C. Lengauer *et al.* [*Cancer Res.* **52**, 2590 (1992)].
18. B. Liu *et al.*, *Cancer Res.* **54**, 4590 (1994); R. D. Kolodner *et al.*, *Genomics* **24**, 516 (1994).
19. N. P. Bhattacharyya, A. Skandalis, A. Ganesh, J. Groden, M. Meuth, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 6319 (1994).
20. Y. M. Ionov *et al.*, *Nature* **363**, 558 (1993); S. N. Thibodeau, G. Bren, D. Schaid, *Science* **260**, 816 (1993); L. A. Aaltonen *et al.*, *ibid.*, p. 812; R. Wooster *et al.*, *Nat Genet.* **6**, 152 (1994); A. Merlo *et al.*, *Cancer Res.* **54**, 2098 (1994).
21. N. C. Nicolaides *et al.*, *Nature* **371**, 75 (1994).
22. S. M. Powell *et al.*, *N. Engl. J. Med.* **329**, 1982 (1993).
23. We thank P. Modrich for helpful discussions, P. Modrich and W. G. Thilly for MT1 and TK6 cells, and T. Gwiazda for preparation of the manuscript. Supported by Fonds zur Förderung der wissenschaftlichen Forschung and by National Cancer Institute grants CA62924, CA35494, CA47527, and CA09320. B.V. is an American Cancer Society research professor and an investigator of the Howard Hughes Medical Institute.

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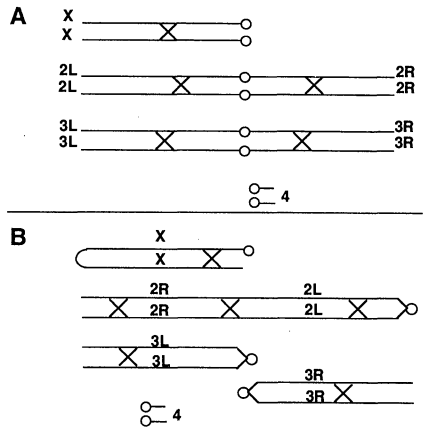

Fig. 1. A schematic diagram of the karyotype of *Drosophila melanogaster* females in wild-type (**A**) and All-Compound females (**B**). With the exception of the obligately achiasmate fourth chromosomes, in the All-Compound females each set of homologs is appended to the same centromere (exchanges are denoted by an "X" between the chromosome arms). The proper designation for such females is *C(1)DX, y f/Y; C(2)EN, c bw/0; C(3)L, h; C(3)R; 4/4*. These females were created by crossing *X/Y; C(2)EN/0; C(3)L; C(3)R; 4/4* males to *C(1)DX, y f/Y; 2/2; 3/3; 4/4* females and recovering the *C(1)DX, y f/X; C(2)EN/2; C(3)L; C(3)R/3; 4/4/4* triploids. Such triploids were then backcrossed to *X/Y; C(2)EN/0; C(3)L; C(3)R; 4/4* males and the All-Compound females were recovered as segregants from this cross that expressed the yellow, forked, curved, brown, and hairy phenotypes. All chromosomes used in this experiment are described in (*14*).

Induction of Metaphase Arrest in *Drosophila* Oocytes by Chiasma-Based Kinetochore Tension

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In normal *Drosophila melanogaster* oocytes, meiosis arrests at metaphase I and resumes after oocyte passage through the oviduct. Thus, metaphase arrest defines a control point in the meiotic cell cycle. Metaphase arrest only occurs in oocytes that have undergone at least one meiotic exchange. Here it is shown that crossovers between homologs attached to the same centromere do not induce metaphase arrest. Hence, exchanges induce metaphase arrest only when they physically conjoin two separate kinetochores. Thus, the signal that mediates metaphase arrest is not the exchange event per se but the resulting tension on homologous kinetochores.

Control of the metaphase-to-anaphase transition is a central component of cell cycle regulation. Meiotic arrest at either metaphase I or II before fertilization is a common component of oogenesis in a wide variety of organisms (*1*). In *Drosophila melanogaster* females, meiotic arrest occurs at metaphase I in stage 13–14 oocytes (*2, 3*). At this point in the meiotic cycle, the exchange bivalents are tightly massed at the metaphase plate. The separation of these bivalents requires the release of sister-chromatid cohesion distal to the site of crossing-over, an event that occurs concomitantly with the initiation of anaphase as the egg passes through the oviduct some 2 hours to a week later (*3, 4*).

Metaphase arrest also halts the precocious poleward movement of nonexchange achiasmate chromosomes, which begins during prometaphase (*5*). During prometaphase, smaller achiasmate chromosomes move away from the main chromosomal mass and by the time of metaphase are positioned between the plate and the poles on long tapered spindles. Larger achiasmate chromosomes frequently remain close to the main chromosomal mass until the onset of anaphase. The release of metaphase arrest is heralded by the completion of the poleward journey of the achiasmate chromosomes. Thus, the triggering of anaphase must allow both the release of sister-chromatid cohesion on exchange bivalents and the inactivation of the antipolar forces acting on nonexchange chromosomes.

Exchange events are required for metaphase arrest, and even a single crossover event is sufficient to induce metaphase arrest (*6*). These conclusions are based on the finding that the vast majority of stage 13–14 oocytes homozygous for any one of four recombination-deficient mutations do not display metaphase arrest. On the basis of these observations, we have proposed that metaphase arrest is the result of tension created on the kinetochores as a consequence of chiasma formation (*6*).

Alternatively, it was possible that recombination events trigger metaphase arrest by means of a chemical signal released by the structures or proteins involved in executing the exchange events (for example, recombination nodules). Distinguishing between these two alternatives required construction of *Drosophila* females in which recombination events occur but tension on the kinetochore does not result. This can be accomplished for an individual pair of chromosome arms by use of a type of chromosome aberration known as a compound chromosome, in which both homologs are attached to the same centromere (*7*). Meiotic recombination events occur between the arms of compound chromosomes at normal frequencies (*7*); however, the resulting crossover events do not conjoin homologous centromeres (Fig. 1).

We constructed females in which all of the arms in the genome that are normally capable of recombination are arranged as compound chromosomes (that is, in which the X, second, and third chromosomes are

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