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Genetics

Mutator phenotypes in human colorectal carcinoma cell lines

(genetic instability/microsatellite/mutation rate/hypoxanthine guanine phosphoribosyltransferase)

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ABSTRACT **Recent studies have revealed that tumors in patients with hereditary nonpolyposis colon cancer are associated with high-frequency alterations of microsatellite sequences. To investigate the mechanisms and consequences of this form of genetic instability, we identified three colorectal carcinoma cell lines that express dinucleotide-repeat instability like that found in hereditary nonpolyposis colon cancer tumors and show increased rates of spontaneous mutation at selectable loci. However, the pattern of hypermutation in these cell lines differed significantly. In one line (HCT116), microsatellite mutations occurred at a remarkably high rate ($\approx 10^{-2}$ mutations per cell per generation), whereas this rate was considerably lower in the two other lines (DLD-1 and HCT15). The rate of mutation at the locus encoding hypoxanthine guanine phosphoribosyltransferase was substantially elevated (200- to 600-fold) in all three tumor cell lines, yet the types of mutations arising differed. A specific frame-shift hotspot accounted for 24% of hypoxanthine guanine phosphoribosyltransferase mutations in HCT116. The frequency of mutations at this site was reduced significantly in DLD-1 and HCT15 lines. These data suggest that the mutator phenotypes in the colorectal carcinoma cell lines could be the consequence of mutator genes affecting different repair or error-avoidance pathways.**

Colorectal tumor development is a multistep process that depends upon the accumulation of mutations in protooncogenes and tumor suppressor genes (1). Considerable progress has been made in the identification of some of these genes, although relatively little is known of the mutational mechanisms that alter them (2). Recent studies have revealed that a number of tumor types [in particular, hereditary nonpolyposis colon cancer (HNPCC)] are associated with a high-frequency variation of microsatellite repeats (3–7). In some HNPCC families this instability is associated with mutations in a gene (*MSH2*) mapping to chromosome 2p16 that encodes a human homolog of the *Escherichia coli* mismatch-repair protein mutS (8, 9). In these patients a mutation of one copy of this gene is inherited. When cells sustain a somatic mutation in the second allele, they presumably become deficient in the correction of replication errors in coding sequences as well as microsatellites (10). The enhanced rate of spontaneous mutation or "mutator phenotype" in such mismatch-repair-deficient cells would be likely to accelerate the mutation-dependent process of tumor development.

Although current evidence clearly links *MSH2* mutations with microsatellite instability in some HNPCC families, alterations of other proteins required for the maintenance of DNA-sequence integrity may produce the same phenotype. HNPCC is a genetically heterogeneous disease, as at least two loci have been mapped in HNPCC families (3, 6). Genes encoding other proteins required for mismatch repair would be obvious candidates. However, aberrations of other processes required for maintenance of DNA-sequence stability, such as exonucleolytic proofreading (11), the repair of spontaneous DNA damage (12), or the regulation of DNA-precursor synthesis (13), would have the same effect of increasing the rate of spontaneous mutation. To identify and characterize the pathways that lead to DNA-sequence instability in human tumors, we screened colorectal carcinoma cell lines for increased mutation rates at both selectable and microsatellite loci. Here, we report the characterization of three lines exhibiting such DNA-sequence instability. These mutator lines exhibited two distinct phenotypes that may result from aberrations of different repair or error-avoidance pathways.

MATERIALS AND METHODS

Cell Lines. The cell lines used in these experiments were obtained from the American Type Culture Collection. The simian virus 40-transformed human diploid fibroblast MRC-5 was obtained from the central cell facility of the Imperial Cancer Research Fund (London). All lines were grown routinely in Dulbecco's modified Eagle's medium (GIBCO/BRL) supplemented with fetal bovine serum (HyClone). The lines were monitored periodically for mycoplasma contamination and found to be free.

Mutant Selections. To isolate mutant strains resistant to 6-thioguanine or ouabain, cultures were trypsinized and plated in 6-thioguanine at 5 μg/ml at a density not greater than 5×10^5 cells per 10-cm dish, or 1 μM ouabain at a maximum density of 10^6 cells per 10-cm dish. Colonies were grown for 2 weeks before picking them for further analysis or staining to calculate mutant frequencies. Mutation rates were determined by the Luria–Dulbruck fluctuation test. Replica cultures with no preexisting mutants were grown to final density ranging from 2×10^5 to 10^7 cells. All the cells in each replica were plated into 6-thioguanine-selective medium at a density not greater than 5×10^5 cells per 10-cm dish. Colonies were grown for 2 weeks before picking mutant colonies for analysis or staining. Mutation rates were calculated by a P_0 calculation or by the method of the mean (14).

Microsatellite Analysis. Six microsatellite loci were assayed for instability. The oligonucleotide primers used to amplify these loci have been published (15–18). Purified genomic DNA was used with these primers and *Taq* polymerase to amplify the microsatellite loci. The products from these reactions were fractionated on 6% polyacrylamide gels and blotted onto Hybond-N$^+$. Blots were probed with one of the

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Abbreviations: HNPCC, hereditary nonpolyposis colon cancer; HPRT, hypoxanthine guanine phosphoribosyltransferase.

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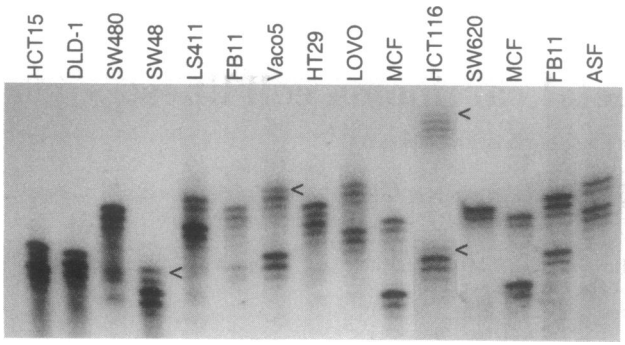

FIG. 1. Analysis of microsatellite-repeat stability in colorectal carcinoma cell lines. DNA purified from the indicated cell lines was analyzed for heterogeneity at the microsatellite locus *D11S527*. Multiple alleles (indicated by arrows) were particularly evident in HCT116 but could also have appeared in SW48 and Vaco5 cells.

PCR primers that had been end-labeled with terminal transferase (GIBCO/BRL).

DNA Sequence Analysis of *HPRT* Mutations. Exon 3-coding sequences of *HPRT* were amplified from DNA purified from mutant strains using the oligonucleotides 5′-GACTGAACGTCTTGCTCGAGATG-3′ and 5′-AATCTACAGTCATAGGAATGGA-3′. This yielded a 160-bp fragment that was fractionated on 6% denaturing polyacryamide gels. The gel was blotted onto nitrocellulose and probed with one of the primers labeled using terminal transferase. This allowed rapid visualization of mutant strains with +1 or −1 frameshifts. To determine whether the alterations of the fragments were the result of frame-shifts within the run of guanine residues in exon 3, the fragments were sequenced with *Taq* polymerase, as described by the manufacturer (GIBCO/BRL).

RESULTS

Microsatellite Instability in Colorectal Carcinoma Cell Lines. Fourteen colorectal carcinoma cell lines were screened for heterogeneity of microsatellite structure. Multiple alleles of the microsatellite locus *D11S527* (15) were most evident in three lines, HCT116, SW48, and Vaco5 (Fig. 1). To examine this instability in greater detail, subclones were isolated from one line clearly exhibiting this heterogeneity (HCT116, ref. 19) and three lines that appeared stable (SW620, DLD-1, and HCT15, refs. 20 and 21). Expansions and contractions of microsatellite length were found in a high proportion of subclones of HCT116 at all six loci tested (Table 1, Fig. 2). These loci mapped to six different chromosomes and consisted of di-, tri-, or tetranucleotide repeats. Variations also were detected in DLD-1 and HCT15 at two of the six loci, but these occurred in a considerably lower

Table 1. Microsatellite variation in colorectal carcinoma cell lines

Locus	Repeat unit	Cell line*			
		SW620	HCT116	DLD1	HCT15
HBAP1	2 bp	0/29	3/29	1/19	1/19
D11S527	2 bp	0/38	24/30	7/37	2/19
D19S178	2 bp	0/19	23/26	0/18	0/18
D14S43	2 bp	0/19	12/14	0/18	0/19
IID	3 bp	0/19	11/53	0/19	0/19
vWF	4 bp	0/19	9/28	0/19	0/19

vWF, von Willebrand factor.
*Fractions represent number of subclones with different alleles over number of subclones screened for these mutations. Microsatellite analysis was done as described.

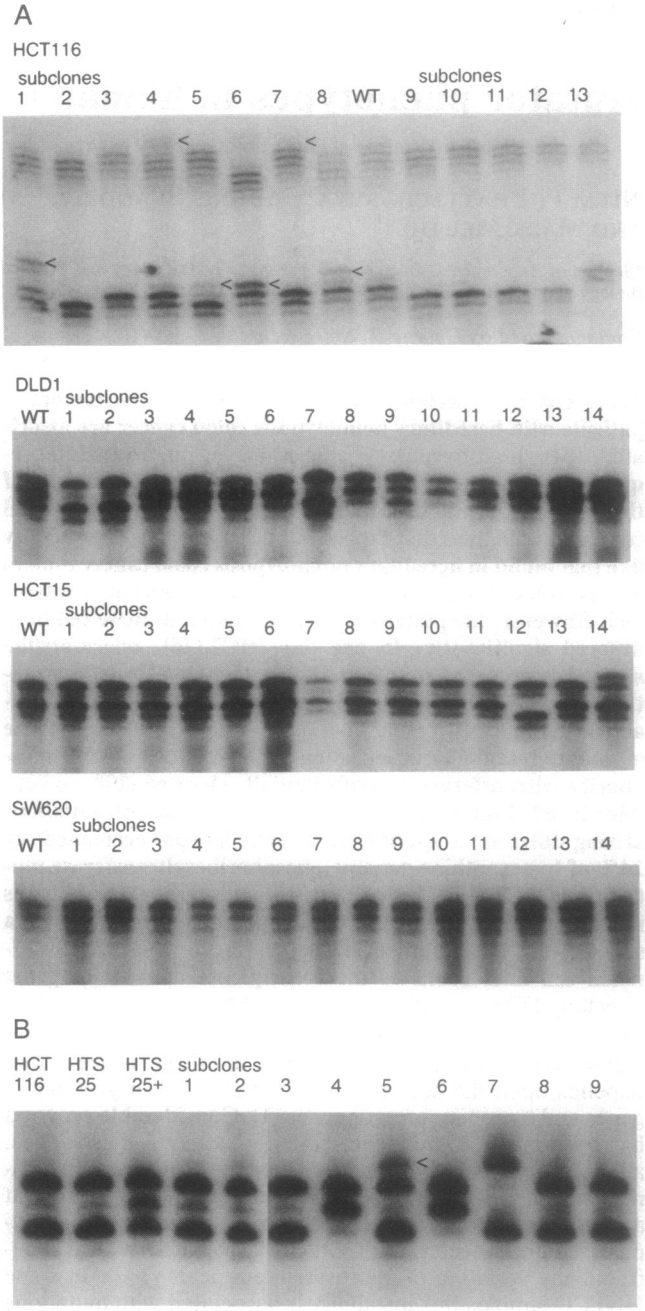

FIG. 2. Microsatellite-length variability in subclones of colorectal carcinoma cell lines. Instability was assayed at *D11S527* (*A*) and *IID* (*B*) loci by amplification of the locus followed by fractionation of the products on a denaturing polyacrylamide gel. The gel was blotted onto Hybond-N$^+$ (Amersham) and probed with one of the primers that was labeled using terminal transferase. In *A*, subclones derived from each of the indicated cell lines were analyzed. In *B*, one of the subclones of HCT116 (designated HTS25) was resubcloned to reduce the frequency of preexisting mutants. The lane marked HTS25+ contains alleles amplified from DNA isolated from the HTS25 subclone after a longer period (≈20 more generations) in culture. The mutant allele accumulates with prolonged culture, consistent with the high rate of mutation at this locus. Each lane represents an independent subclone [WT, parental (wild type) cell line]. Distinctive bands are evident in subclones of all cell lines except SW620. Marked bands (<) indicate specific alleles present in subclones that also contain both wild-type alleles. Presumably, this pattern emerges when a mutation occurs at the microsatellite locus within the first few rounds of cell division after subcloning. These events were used to calculate a mutation rate for microsatellite mutations in HCT116 cells (Table 2).

Table 2. Mutation rates at microsatellite and *HPRT* loci in colorectal carcinoma cell lines

	Cell line					
	HCT116			DLD1	HCT15	MRC-5
	D11S527	*IID*	*HPRT*	*HPRT*	*HPRT*	*HPRT*
Replica cultures, no.	15	16	13	16	23	15
Initial cells, no.	1	1	100	100	100	1000
Final cells, no.	8*	8	$1.2 \pm 0.3 \times 10^6$	$0.2 \pm 0.1 \times 10^6$	$0.6 \pm 0.2 \times 10^6$	9.8×10^6
Cultures with mutants, no.	6	1	13	13	23	5
Mutant colonies, x no.			97 ± 208	17.9 ± 17.2	96 ± 109	1.5 ± 1.6
Mutation rate†						
P_0 calculation	3.8×10^{-2}	0.6×10^{-2}		0.5×10^{-5}		2.8×10^{-8}
Method of the mean			1.5×10^{-5}	1.9×10^{-5}	2.6×10^{-5}	4.1×10^{-8}

*Mutation was scored on the basis of the presence of a new allele detected on 6% polyacrylamide gels after amplification of the microsatellite locus. Because reconstruction experiments show that this protocol can only detect those alleles that represent >10% of the product, we can only detect mutations that produce these alleles in the first three rounds of cell division after subcloning. Thus the final cell number has been set at eight, even though the subclones were grown to a much larger number (>1000) before analysis.

†Mutation rates for the microsatellite loci were calculated by the P_0 method, whereas rates at *HPRT* were calculated by the method of the mean for all four cell lines. P_0 calculations for *HPRT* were also done for DLD-1 and MRC-5.

proportion of subclones. The alterations in the cell lines also differed in structure. Those in HCT116 were highly variable in size, affecting up to 8 bp, whereas alterations in DLD-1 or HCT15 cells only involved single 2-bp repeat units of the microsatellite (Fig. 2*A*). No changes were found in SW620 cells. Thus, microsatellite instability does not appear to be associated with chromosomal instability. SW620 cell line has a hyperdiploid karyotype and the lowest frequency of microsatellite instability, whereas HCT116, DLD-1, and HCT15 lines have near-diploid chromosome complements. Microsatellite instability also may be independent of mutations of p53 and the adenomatous polyposis colon cancer (*APC*) gene, as HCT116 has no detectable mutations of these tumor suppressors, whereas SW620, DLD-1, and HCT15 have mutations of these genes (ref. 22; J.G., unpublished observations).

Rate of Mutation at Microsatellite Loci in HCT116 Cell Line. The variation in microsatellites found in HCT116 could be the result of preexisting heterogeneity in the tumor cell population or it could reflect a continuing high rate of mutation at these loci. The observation that some subclones of HCT116 contained both wild-type and other alleles (Fig. 2*A*, subclones 1, 4–8) indicated that HCT116 cells continued to express this instability. It seemed likely that the mixed allele pattern resulted from a mutation in the first few rounds of replication after subcloning. Subclones in which a mutant allele replaced one of the wild-type alleles presumably were derived from mutant cells preexisting in the population. Therefore, we eliminated preexisting mutants by resubcloning an HCT116 subclone and analyzing isolates for instability at the trinucleotide repeat locus *IID* (17). Again, subclones with multiple alleles were evident (Fig. 2*B*). Thus microsatellite mutations occur at a high rate in HCT116 cells and are detectable in subclones isolated from freshly cloned populations of HCT116 carried for a limited period of time (<30 generations). Furthermore, the detection of new mutant alleles after subcloning enabled us to calculate mutation rates for the microsatellite loci ranging from 0.6×10^{-2} mutations per cell per generation for the trinucleotide repeat microsatellite *IID* to 3.8×10^{-2} mutations per cell per generation for the dinucleotide-repeat microsatellite *D11S527* (Table 2).

A further indication of the high rate of spontaneous mutation in HCT116 is the accumulation of mutant alleles in subclones carried in culture (Fig. 2*B*). In subclone HTS25 there is only a faint band for the mutant allele upon assay soon after subcloning. When this subclone was carried for a longer period of time (≈20 further generations), the mutant allele became more evident.

Frequency and Rate of Mutation at Selectable Loci. To determine whether the destabilization extended to structural genes, the mutant frequencies at two selectable loci were measured in the colorectal carcinoma cell lines. These frequencies were compared with those in MRC-5, a simian virus 40-transformed human diploid fibroblast line. Table 3 shows that the frequencies of 6-thioguanine-resistant colonies were dramatically elevated (>1000-fold) in all three lines exhibiting microsatellite instability. Similarly, the frequencies of ouabain-resistant colonies increased significantly (>80-fold). SW620, which was does not exhibit microsatellite instability, did not have an elevated mutant frequency at either locus. However, the frequency of hypoxanthine guanine phosphoribosyltransferase (HPRT) mutants in SW620 cannot be directly compared because of the presence of two active X chromosomes in this line (J. Harwood and M.M., unpublished observations).

To more precisely compare the occurrence of mutations in these cell lines, we measured the rate of mutation giving rise to 6-thioguanine resistance. Replica cultures of the colorectal carcinoma cell lines were grown to a final density of 0.2–1.2 $\times 10^6$ cells before plating in medium containing 6-thioguanine. After 2 weeks in selective medium, resistant colonies were scored, and the rate of spontaneous mutation was calculated (Table 2). The mutation rates at *HPRT* in HCT116, HCT15, and DLD-1 lines were 200- to 600-fold greater than the rate measured for MRC-5 cells. These *HPRT* mutation rates were also 10- to 1000-fold higher than those previously reported for "normal" diploid fibroblasts [which range from 1.8×10^{-6} (23) to 9.2×10^{-9} mutations per cell per generation (24)]. Again, we were unable to measure the rate of mutation in SW620 cells at *HPRT*. However, the mutation rate at the hemizygous selectable locus encoding adenine phosphoribosyltransferase in SW620 cells was nearly as low [5×10^{-8} (25)] as the rate measured for *HPRT* in MRC-5 cells. Thus the mutation rate at a selectable structural gene was elevated substantially in three colorectal carcinoma lines exhibiting microsatellite variation. It is notable, however, that the *HPRT* mutation rate does not necessarily parallel the degree

Table 3. Mutant frequencies at selectable loci

Cell line	6-Thioguanine^r	Ouabain^r
MRC-5	5×10^{-7}	$<5.0 \times 10^{-8}$
SW620	$<2 \times 10^{-7}$	$<4.0 \times 10^{-7}$
HCT116	6×10^{-4}	6.9×10^{-4}
HCT15	6×10^{-4}	4.0×10^{-6}
DLD-1	4×10^{-3}	1.1×10^{-5}

Superscript r represents resistant.

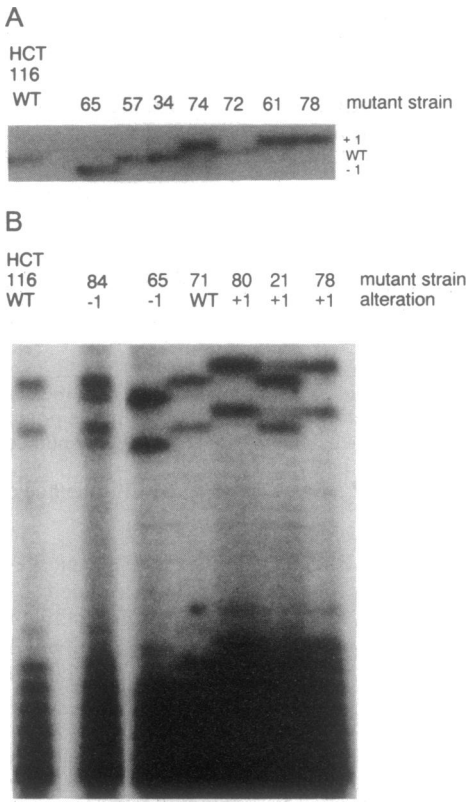

FIG. 3. Frame-shift hotspot in exon 3 of the *HPRT* locus in mutants isolated from HCT116. (*A*) Exon 3-coding sequences were amplified from DNA purified from the indicated mutant strains and fractionated on 6% denaturing polyacrylamide gels. The gel was blotted onto nitrocellulose and probed with one of the primers that was labeled using terminal transferase; this allowed rapid visualization of mutant strains with +1 or −1 frame-shifts. To determine whether the alterations of one of the fragments resulted from frame-shifts within the run of guanine residues in exon 3, the fragments were sequenced. (*B*) Products of reactions containing ddGTP fractionated on a 6% denaturing polyacrylamide gel. Loss (−1) or gain (+1) of guanine residues in the guanine run can be found in several of these mutant strains. In two of these mutants (strains 21 and 84), the alterations are complex, as a wild-type (WT) band and a mutant band with either a +1 or −1 frame-shift can be seen; these may result from a duplication of exon 3 occurring concurrently with the frame-shift. Frame-shifts at this site were found in 24% of HPRT mutant strains obtained from HCT116.

of microsatellite instability, as HCT15 had the highest mutation rate at *HPRT* but displayed only an intermediate level of microsatellite instability.

Molecular Analysis of Mutations at *HPRT*. The mutations occurring at *HPRT* in these colorectal carcinoma cell lines were characterized by Southern blot and DNA-sequence analysis. Southern blots prepared from restriction endonuclease digests of genomic DNAs from the mutant strains did not reveal any changes of the pattern of *HPRT* fragments (data not shown). This is in marked contrast to spontaneous mutations in "normal" human cell lines in which as many as 40% of mutants show partial or complete deletion of *HPRT*-coding sequences (26). Sequence analysis of *HPRT*-coding regions focused on exon 3 as a mismatch-repair-deficient human lymphoblastoid strain developed a hotspot in a short run of 6 guanine residues present in this exon [nt 207–212 (27)]. Spontaneous mutations in other, nonmutator, human cells also appear to cluster in this exon (28), but they are infrequent in this guanine-run [only 2 of 149 mutant alleles reported (26, 28–32)]. Similar to the mismatch-repair-deficient line, 24% of HPRT mutants from the colorectal carcinoma cell line HCT116 (11 of the 46 independent mutants examined) contained guanine insertions within this run (Fig. 3). The frequencies of frame-shifts at this site in HCT15 (2 of 33 mutants or 6%) and DLD1 (1 of 14 mutants examined or 7%) cells were significantly lower. No other hotspots in exon 3 were detected in these mutant strains.

DISCUSSION

There is increasing evidence that DNA-sequence instability is an important component of the development of some sporadic tumors, as well as those occurring in HNPCC patients. The substantially elevated rates of spontaneous mutation at selectable genes and microsatellite loci in the three colorectal carcinoma cell lines investigated here provide further support for this hypothesis. The patterns of microsatellite instability observed in these lines mimic those found in colorectal tumors. Some tumors show a high degree of variability (with respect to both the proportion of loci affected and the size of the alterations, ref. 4), much like the HCT116 line. A second group shows less variation, reminiscent of DLD-1 or HCT15 cell lines. Tumors from HNPCC patients also have a lower frequency of chromosome loss (4); HCT116, DLD-1, and HCT15 cells have near-diploid chromosome constitutions.

The sequence instability in these colorectal carcinoma cell lines extends to coding regions, as the rate of mutation at the *HPRT* locus is elevated 200- to 600-fold compared with a human fibroblast line. Yet differences in the rate and pattern of mutations were evident. We found a dramatic destabilization of microsatellite loci associated with an elevated rate of mutation at *HPRT* and the development of a novel frame-shift hotspot in the HCT116 cell line. We suggest that this mutation hotspot develops specifically as a result of mismatch-repair deficiency. This result is supported by the recent report of a deficiency of mismatch repair in HCT116 (10), together with the occurrence of the same hotspot in a mismatch-repair-deficient lymphoblastoid line (MT-1, ref. 27). The repair deficiencies in these lines apparently result from alterations of different components of the pathway.

In DLD-1 and HCT15 cells, microsatellite variation was less frequent and the frame-shift hotspot was not evident, although mutation rates at *HPRT* were as high as those found in HCT116 cells. The difference in the pattern of mutations in these strains may result from mutations of different components of the mismatch-repair pathway. In *E. coli* eight proteins are required for the repair of mismatches in cell-free extracts (33). However, frame-shifts dominate mutations occurring in mutator strains of *E. coli* [*mutH*, *-L*, or *-S* strains (34)] and yeast [*msh2* or *mlh1* strains (35)] deficient in mismatch repair. Frame-shifts are not as prevalent in mutator strains of *E. coli* and yeast lacking the 3′ → 5′ proofreading exonuclease of the replicative polymerases, although the overall rates of spontaneous mutation are substantially elevated (35, 36). Thus, the differences in the two mutator phenotypes described here may result from alterations in different repair or error-avoidance pathways.

The DNA-sequence instability induced by mutator genes is only one pathway that results in tumor-associated genome instability. For example, SW620 cell line shows very low rates of base substitution but exhibits a substantially altered karyotype and high rates of multilocus deletion (25, 37). This cell line also displays an unusual mechanism of mutation in the form of multiple base substitutions or frame-shifts that apparently occur as a result of a single initiating event (25). Biochemical and genetic analysis of all these different forms of hypermutation should contribute significantly to our understanding of the mechanisms by which cells maintain genome integrity.

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