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Variation in the extent of microsatellite instability in human cell lines with defects in different mismatch repair genes

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Mismatch repair deficiency results in the elevation of mutation rates in tumors, which is especially pronounced in simple repeat sequences (microsatellites). We have investigated the relationship between microsatellite mutagenesis and certain combinations of mutations in mismatch repair genes, using a frameshift reversion assay to determine the spontaneous mutation rates of a dinucleotide microsatellite in two cancer cell lines, HCT116, which has defects in *hMLH1* and *hMSH3*, and HEC-1-A, which has defects in *hPMS2* and *hMSH6*. We found a 10-fold difference in mutation rates between these two cell lines. In addition, a mutant *hPMS2* allele, *PMS134*, which has been reported to have a dominant negative effect, was expressed in mismatch repair-proficient telomerase-immortalized hTERT-1604 fibroblasts and mutation rates were determined. Expression of *PMS134* did not elevate mutation rates in hTERT-1604. Combined, these results suggest that mutations in different mismatch repair genes can lead to varying degrees of microsatellite instability. It is also likely that there is heterogeneity in the mutations that are acquired in the absence of mismatch repair, such that some mismatch repair-defective cancer cells also contain mutations in other genes coding for proteins involved in the maintenance of genetic stability.

Introduction

Microsatellite instability is found in most tumors from patients with hereditary non-polyposis colorectal cancer (HNPCC) (Ionov *et al*., 1993; Thibodeau *et al*., 1993). HNPCC is the most common form of inherited susceptibility to colorectal cancer, comprising an estimated 5% of all colorectal cancers (Lynch, 1999). Mismatch repair deficiency was originally demonstrated to result in microsatellite instability in the yeast *Saccharomyces cerevisiae* (Strand *et al*., 1993). The mismatch repair system is the post-replication DNA repair pathway that is responsible for the correction of errors generated by DNA polymerases during DNA synthesis (Fishel *et al*., 1993; Leach *et al*., 1993; Parsons *et al*., 1993; Strand *et al*., 1993; Prolla *et al*., 1994; Umar *et al*., 1994). Mutations have been identified in five known mismatch repair genes, *hMLH1*, *hPMS2*, *hMSH2*, *hMSH3* and *hMSH6*, in HNPCC patients and/or in sporadic tumors (Fishel *et al*., 1993; Leach *et al*., 1993; Nicolaides *et al*., 1994; Papadopoulos *et al*., 1994; Yin *et al*., 1997). A high fraction of sporadic mismatch repair-defective colorectal cancers have been shown to have lost activity of the *hMLH1* gene as the result of promoter inactivation (Koike *et al*., 1997; Cunningham *et al*., 1998).

Considerable progress has been made in the understanding of the human mismatch repair pathway. hMSH2 forms a heterodimer with hMSH6 or hMSH3 and acts as the MutS-α or MutS-β complex, respectively; these complexes are involved in the recognition and binding of replication errors (Drummond *et al*., 1995; Acharya *et al*., 1996). The hMSH2–hMSH6 complex has a higher affinity for mispaired bases and single repeat insertion/deletion loops, while the hMSH2–hMSH3 complex functions predominantly in the recognition of larger insertion/deletion loops (Acharya *et al*., 1996; Umar *et al*., 1998). The MutL complex, composed of hMLH1 and hPMS2, is necessary for the excision of the sequence containing the replication error (Li and Modrich, 1995; Nicolaides *et al*., 1998). hMLH1 has also been shown to form a complex with hPMS1 (Leung *et al*., 2000). Recently, a new partner for hMLH1, hMLH3, was identified (Lipkin *et al*., 2000). Although the yeast homolog of this protein has a minor role in mismatch repair (Flores-Rozas and Kolodner, 1998), the contribution of hMLH3 to the human mismatch repair pathway has not yet been determined; it appears that it does not substitute for hPMS2 in the *hPMS2*$^{-}$ endometrial cancer cell line HEC-1-A (Lipkin *et al*., 2000). Other proteins that are involved in DNA metabolism and are thought to function in mismatch repair include PCNA and EXO1 (Tishkoff *et al*., 1998; Kokoska *et al*., 1998; Flores-Rozas *et al*., 2000).

The inactivation of mismatch repair is clearly one major factor that leads to microsatellite instability. Since mismatch repair genes are mutated in HNPCC families and disruption of mismatch repair genes results in microsatellite instability in cultured cells, the current view of mismatch repair inactivation is that loss of function of one mismatch repair protein, in the absence of functionally redundant homologs, simply leads to complete loss of mismatch repair activity; however, recent studies have shown that mismatch repair inactivation may be much more complex than previously thought. Baranovskaya *et al.* (2001) showed that mutation rates were higher in *hMLH1*/*hMSH6* double mutants than in *hMLH1* single mutants in mismatch repair-defective colorectal cancer cell lines that were otherwise isogenic. As they suggest, this observation could be explained by the presence of an alternative protein to hMLH1, yet to be identified, which would be functionally dependent on hMSH6. Alternatively, there may be residual mismatch repair activity when hMLH1 is absent, even if there is no alternative protein. Mismatch repair proteins seem to act as part of a large protein complex (Prolla *et al*., 1994; Flores-Rozas *et al*., 2000); it is possible that, in the absence of hMLH1, this complex might form but would be unstable and have significantly reduced activity. The additional mutation in *hMSH6* may prevent the formation of such a complex completely, resulting in the total absence of mismatch repair activity.

We have compared mutation rates in human cancer cell

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lines with mutations in different combinations of mismatch repair genes, in order to determine whether mutations in different mismatch repair genes can lead to different microsatellite mutation rates, possibly as a consequence of varying degrees of destabilization of mismatch repair complexes. We have also investigated the effect of a truncating mutation of *hPMS2* (*PMS134*) (Parsons *et al*., 1995) on mismatch repair activity and microsatellite mutation rates in mismatch repair-proficient diploid human fibroblasts. *PMS134* has a dominant negative phenotype in patients; the protein has been shown to bind hMLH1, but to lack the hPMS2 catalytic site (Nicolaides *et al*., 1998). We found that the expression of this mutant gene in the presence of normal hPMS2 in telomerase-expressing human fibroblasts was insufficient to result in microsatellite instability. These studies provide further insight into mismatch repair and microsatellite instability as complex processes.

Materials and methods

Spontaneous mutation rate analyses

A subclone (H6) of HCT116 cells (Papadopoulos *et al*., 1994; Li and Modrich, 1995; Bennett *et al*., 1997) was obtained from Dr B.Vogelstein (Johns Hopkins University); HEC-1-A cells (Risinger *et al*., 1995; Glaab *et al*., 1998) were obtained from Dr J.I.Risinger (National Institutes of Health). These cancer cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% defined calf serum (Hyclone) at 37°C in an atmosphere of 5% CO_2 in air. hTERT-expressing 1604 human lung fibroblast cells (hTERT-1604) (Ouellette *et al*., 2000) were obtained from Dr R.A.Schultz (University of Texas Southwestern Medical Center); hTERT-1604-derived cell lines were maintained under the same conditions in DMEM with 10% fetal bovine serum (Hyclone).

All $(CA)_{17}$ repeat-containing cell lines in this study were produced by electroporation with *Sca*I-linearized pBsd-$(CA)_{17}$ plasmid DNA (1 µg per 1×10^7 cells) (Figure 1A) (N.A.Yamada, J.M.Parker and R.A.Farber, submitted for publication). pBsd-$(CA)_{17}$ contains a *tk–neo* fusion gene, in which the *neo* coding region was placed out of frame in the (–1) direction by insertion of an oligonucleotide containing the microsatellite. Cells were exposed to 10 µg/ml blasticidin (Invitrogen) for 10 days. Stably transfected independent clones were isolated and expanded for determination of spontaneous mutation rates by fluctuation analysis.

Preliminary experiments to determine mutation frequencies were conducted on approximately 15 independent transfectants by plating in 100 mm dishes in G418 (500 µg/ml) at densities ranging from 5×10^3 to 5×10^5 cells per dish; at this time, fluctuation tests were also begun for each transfected clone by initiating at least 10 subcultures with a very small number of cells (5 cells/well for the cancer cell lines and 100 cells/well for the hTERT-1604-derived cell lines) in 24-well plates in medium without G418. The cells in 100 mm dishes were grown in G418 for at least 10 days; plates were stained with Giemsa (Gibco BRL) when discrete revertant colonies appeared. Colonies were counted and mutation frequencies were determined. At least three independent transfectants with at least one $G418^R$ colony but with a reversion frequency of $<1\%$ were chosen for fluctuation analysis. We avoided the use of transfectants that produced no revertants, since it is likely that these clones contain inactivating mutations in the *neo* coding region of pBsd-$(CA)_{17}$; clones with a $>1\%$ reversion frequency were not used because they were likely to have had frameshift mutations in the microsatellite that arose during the propagation of the plasmid in bacteria or very early in the expansion of the transfected clone, such that all or nearly all of the cells in the population were G418-resistant. Of the clones that met the above conditions, the first three or four clones to grow to a sufficient number for fluctuation analysis were chosen for reversion rate determinations.

Cells from each of the 10 subcultures were plated in two 100 mm dishes in G418. For hTERT-derived cell lines, 5×10^5 cells were plated per dish, and for HCT116 and HEC-1-A, 5×10^3 cells were plated per dish. At this time, 300 cells from each subculture were plated into each of two dishes in the absence of G418 for determination of colony-forming efficiency (CFE). Cultures with G418 were fed every 5 days and cultures without G418 were fed every 7 days until colonies were visible. One revertant from each subculture was isolated and expanded for PCR analysis. Plates were stained with Giemsa and colonies were counted.

PCR analysis was conducted by amplification of the microsatellite region with fluorescently labeled primers and direct size comparison of parental cell and revertant PCR products by capillary electrophoresis on an ABI 310 Genetic Analyzer (Applied Biosystems). The reverse HSVtk primer (5′-GATTGGTCGTAATCCAGGAT-3′) was 5′-end-labeled with a HEX fluorescent label; the forward HSVtk primer (5′-CAACGGCGACCTGTATAACG-3′) was unlabeled. Temperature cycling conditions for PCR were as follows: 94°C for 10 min; 30 cycles of 30 s at 94°C, 30 s at 59.5°C and 30 s at 72°C; with a final 10 min extension at 72°C. Samples were loaded onto the capillary electrophoresis instrument in deionized formamide with 0.3 µl of TAMRA-labeled size standards. POP4 polymer was used with the 310 Collection software (v.2.0) for sample processing. GeneScan Analysis software (v.3.1.2) was used for sample analysis. PCR analysis was also used to determine the copy number of the plasmid as described previously, where revertants from clones with more than one copy of the plasmid retain a parental PCR profile, in addition to the mutant PCR profile (Hanford *et al*., 1998). Examples of PCR results are shown in Figure 2.

The number of revertants in each subculture was corrected for CFE and the fraction of clones with frameshift mutations from each transfectant in the microsatellite was determined by PCR. Mutation rates were calculated using

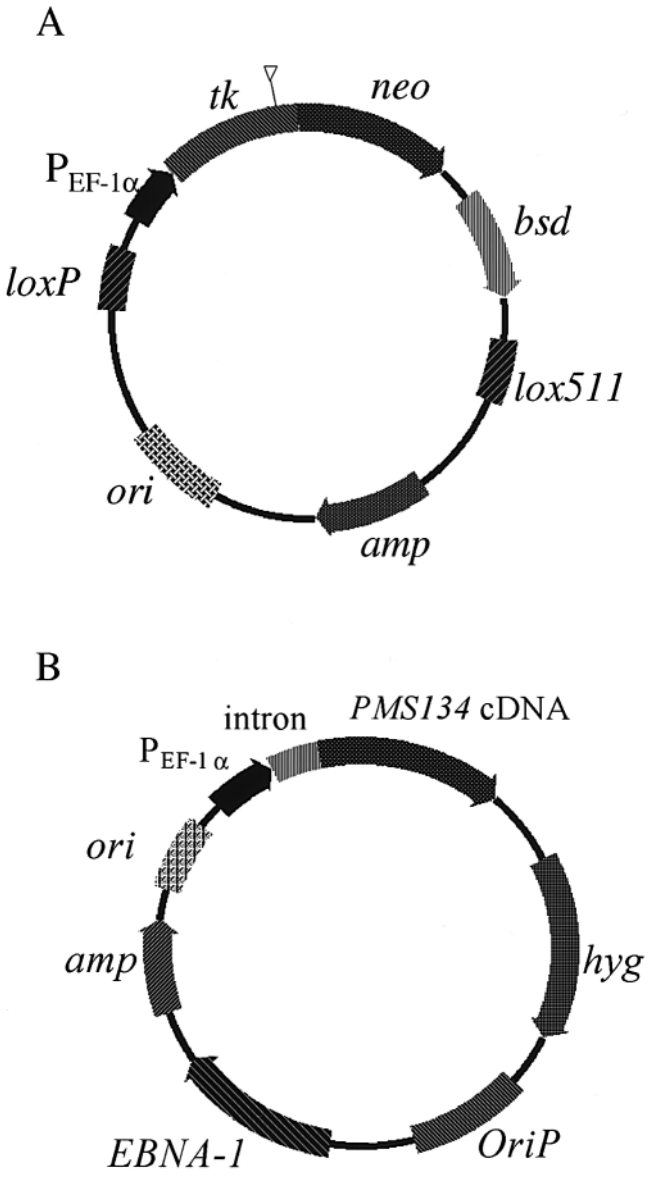

Fig. 1. Plasmids. (**A**) pBsd-$(CA)_{17}$. Microsatellites were inserted at the restriction site (*Aat*II) indicated by the triangle. The EF-1α promoter drives the *tk–neo* gene. *bsd*, blasticidin resistance gene; *ori*, bacterial origin of replication; *amp*, ampicillin resistance gene; *loxP/lox511*, Cre recombinase recognition sequences (not used in this study). (**B**) pEEP4-PMS134. The EF-1α promoter drives *PMS134* expression. A rabbit β-globin intron is included between the promoter and the *PMS134* cDNA. *hyg*, hygromycin resistance gene; *oriP*, Epstein–Barr virus origin of replication; *ori*, bacterial origin of replication; *amp*, ampicillin resistance gene.

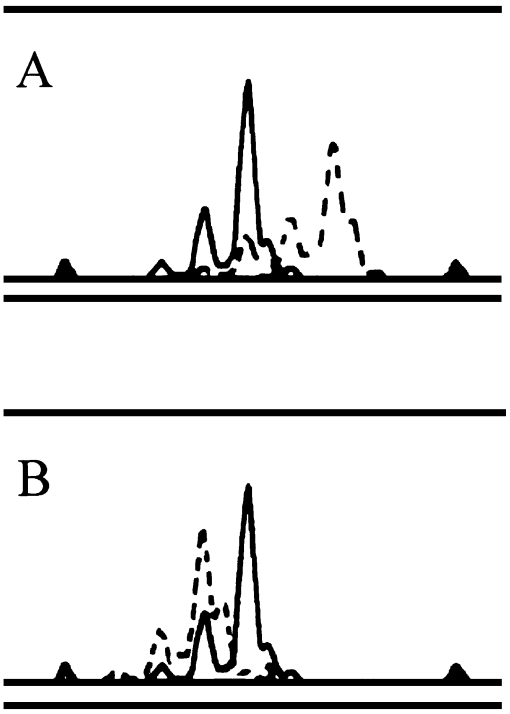

Fig. 2. Examples of PCR results from spontaneous G418^R revertants. The dotted lines indicate PCR products from revertant DNA and the solid lines are from parental DNA. (**A**) 4 bp insertion; (**B**) 2 bp deletion.

the Luria and Delbrück (1943) method of the mean. Statistical analyses were conducted as described by Wierdl *et al.* (1997). Briefly, mutation rates were determined for each of the 10 subcultures for every independent transfectant using the Lea and Coulson (1949) equation. The median of these mutation rates was determined and Fisher's exact test was conducted on the numbers of subcultures that were above versus below the median for the two cell lines being compared.

Construction of PMS134-expressing cell lines

The expression vector containing the *PMS134* gene (pSG5-PMS134) (Nicolaides *et al*., 1998) was obtained from Dr N.C.Nicolaides (Genaera Corp.). *PMS134* is a mutant form of the *hPMS2* gene which has a nonsense mutation at codon 134. The *PMS134* cDNA was cleaved from pSG5-PMS134 with *Bam*HI and ligated into pEEP4. pEEP4 is an episomal expression vector containing the human EF-1α promoter (Figure 1B). The orientation and sequence of the *PMS134* fragment was confirmed by direct sequencing of plasmid DNA. Sequencing was carried out in an ABI 310 Genetic Analyzer (Applied Biosystems), using the BigDye Terminator Cycle Sequencing Ready Reaction Kit.

Cell lines expressing the PMS134 protein were developed by transfection of pEEP4-PMS134 (30 μg) into two hTERT-1604 derived cell lines, Bsd-$(CA)_{17}$-1 and Bsd-$(CA)_{17}$-4, which have $(CA)_{17}$ mutation rates near the median of all four hTERT-1604 transfectants that were tested. Cells containing this plasmid were selected in hygromycin (100 μg/ml) and independent transfectants were isolated. Mutation rates of these clones containing the *PMS134* gene were determined as described above.

Western blot analysis of PMS134

Western blotting was conducted by a modification of the procedure described by Nicolaides *et al.* (1998), to confirm that the *PMS134* fragment was expressed. Nuclear and cytoplasmic extracts were made using the Nuclear Extract Popper kit (Pierce). Protein levels were quantitated with Coomassie Dry Protein Assay Plates (Pierce), following the manufacturer's instructions. Western blotting was conducted using polyclonal antibodies generated against residues 2–20 of hPMS2 (E-19; Santa Cruz Biotechnology). After electroblotting, gels were stained with GelCode Blue Stain Reagent (Pierce) to confirm that equal amounts of protein were loaded. Examples of western blots are shown in Figure 3. hPMS2 (110 kDa) and PMS134 (14 kDa) proteins were distinguished by size.

In vitro mismatch repair assays

In vitro mismatch repair assays were carried out as described by Thomas *et al.* (1991). NR9268 electrocompetent *Escherichia coli* cells, CSH50 *E.coli*

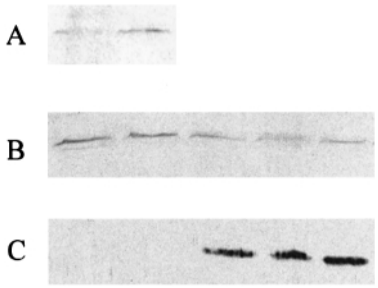

Fig. 3. Examples of western blotting results for PMS134 on cytoplasmic extracts from hTERT-1604-derived cells. (**A**) Wild-type hPMS2 (110 kDa). Lane 1, hTERT-1604 non-transfected; lane 2, hTERT-1604 Bsd-$(CA)_{17}$-4 vector only. (**B**) Wild-type hPMS2. Lane 1, hTERT-1604 Bsd-$(CA)_{17}$-4 vector only; lane 2, hTERT-1604 Bsd-$(CA)_{17}$-1 vector only; lane 3, 4-PMS134-10; lane 4, 4-PMS134-12; lane 5, 1-PMS134-8. (**C**) PMS134 (14 kDa). Lane 1, hTERT-1604 Bsd-$(CA)_{17}$-4 vector only; lane 2, hTERT-1604 Bsd-$(CA)_{17}$-1 vector only; lane 3, 4-PMS134-10; lane 4, 4-PMS134-12; lane 5, 1-PMS134-8.

Table I. Spontaneous microsatellite mutation rates of mismatch repair-defective and -proficient cells

Cell line	Mismatch repair defect	Mutation rate (mutations/cell/generation)
HCT116	*hMLH1*$^-$, *hMSH3*$^-$	1.9×10^{-3}[a]
		2.9×10^{-3}
		3.0×10^{-3}
HEC-1-A	*hPMS2*$^-$, *hMSH6*$^-$	3.0×10^{-4}
		3.9×10^{-4}
		5.8×10^{-4}
hTERT-1604	Mismatch repair proficient	2.8×10^{-6}
		5.8×10^{-6}[b]
		6.0×10^{-6}[b]
		6.2×10^{-6}

Median values are in bold.
[a]This clone has two copies of the plasmid integrated in the genome, so that the mutation rate per microsatellite may be as low as half of the rate reported here.
[b]These rates have been reported elsewhere (N.A.Yamada, J.M.Parker and R.A.Farber, submitted for publication).

cells and 3′ and 5′ nicked heterodupulex substrates were obtained from Dr T.A.Kunkel (National Institute of Environmental Health Sciences). Fifty micrograms of crude cell extract were used in each experiment. Heteroduplex substrates were incubated in cell extracts at 37°C for 15 min. Mock-treated controls were conducted with water in place of cell extracts. Proteins were digested with proteinase K and precipitated with SDS. Chloroform:isoamyl alcohol (24:1) was used to extract the substrates, which were subsequently precipitated with isopropanol. Salts were removed by washing the DNA pellet with 70% ethanol and the mismatch repair reaction product was air dried. Repair products were resuspended in water and electroporated into NR9268 cells. Electroporated cells were plated in soft agar in the presence of CSH50 α-complementation cells, IPTG (Sigma) and X-Gal (Gibco BRL) and incubated at 37°C. Blue, white and mixed-burst plaques were scored the following day. Percent repair was determined by comparing the percentage of mixed-burst plaques from extract-treated and mock-treated substrates using the equation: % repair = 1 – (% mixed-burst plaques from extract-treated substrates/% mixed-burst plaques from mock-treated substrates).

Results

We have used a reversion assay to determine the rates of frameshift mutations in microsatellites in two cancer cell lines with different mismatch repair defects. Microsatellite mutation rates in HCT116, which is a colorectal cancer cell line with *hMLH1* and *hMSH3* mutations (Papadopoulos *et al*., 1994; Li and Modrich, 1995; Bennett *et al*., 1997), were 10-fold higher ($P < 0.001$) than those in HEC-1-A, which is an endometrial

Table II. Spontaneous microsatellite mutations found in mismatch repair-proficient and -deficient cell lines

Cell line	4 bp insertion	Deletions			Other[a]	Total mutations
		2 bp	8 bp	14 bp		
HCT116	0 (0%)	23 (96%)	0 (0%)	0 (0%)	1 (4%)	24
HEC-1-A	1 (5%)	19 (95%)	0 (0%)	0 (0%)	0 (0%)	20
hTERT-1604	14 (39%)	20 (56%)	0 (0%)	2 (5%)	0 (0%)	36
hTERT-1604-PMS134	11 (38%)	13 (45%)	4 (14%)	0 (0%)	1 (3%)	29

[a]'Other' mutations were a 2 bp insertion in HCT116 and a 16 bp deletion in hTERT-1604-PMS134.

Table III. Spontaneous mutation rates of *PMS134*-expressing cell lines

Cell line	Mutation rate (mutations/cell/generation)
hTERT-1604 Bsd-$(CA)_{17}$-4	6.0×10^{-6}[a]
4-PMS134-10	1.9×10^{-6}
4-PMS134-12	2.3×10^{-6}
hTERT-1604 Bsd-$(CA)_{17}$-1	5.8×10^{-6}[a]
1-PMS134-8	4.6×10^{-6}

[a]These rates have been reported elsewhere (N.A.Yamada, J.M.Parker and R.A.Farber, submitted for publication).

Table IV. Mismatch repair activity of *PMS134*-expressing clones

Cell line	(CA) loop, 3′ nick	G:G mismatch, 5′ nick
hTERT-1604 Bsd-$(CA)_{17}$-4	59.5%	42.3%
		38.4%
hTERT-1604 Bsd-$(CA)_{17}$-4-PMS-12	55.1%	44.2%
		64.1%
HEC-1-A	<0%	Not determined

Percent repair activity represents the repair of heteroduplex substrate relative to mock-treated substrates.

cancer cell line with *hPMS2* and *hMSH6* mutations (Risinger *et al*., 1995; Glaab *et al*., 1998), as shown in Table I. This difference was consistently observed in each of the three independent transfectants analyzed from each cell line. As expected, both cancer cell lines had significantly higher mutation rates than the mismatch repair-proficient hTERT-1604 cells; the HCT116 rates were three orders of magnitude higher and the HEC-1-A rates two orders of magnitude higher than those of the normal fibroblasts.

PCR results showed that in both HCT116 and HEC-1-A, 2 bp deletions were the predominant type of mutation, while in hTERT-1604 cells, the frequencies of 2 bp deletions and 4 bp insertions were similar (Table II). No large deletions were seen in either mismatch repair-defective cell line. One revertant of an HCT116 clone carrying two copies of the plasmid sequence contained two mutations, a 2 bp deletion and a 2 bp insertion. Because the *neo* coding region is in the (–1) reading frame in the parental cells, the 2 bp insertion would not be expected to restore the reading frame and is, therefore, likely to be coincidental. There was no microsatellite of the parental type in this revertant, which indicates that there were no more than two copies of the plasmid in the original transfected clone.

In order to investigate the effect of a single mutation in a mismatch repair gene on microsatellite mutation rates in diploid human fibroblasts, we introduced an episomal vector expressing the mutant *hPMS2* variant that was reported to have a dominant negative phenotype (Nicolaides *et al*., 1998) into the two hTERT-1604 transfectants with median microsatellite mutation rates. Two independent clones were analyzed in order to avoid the possibility that the site of integration of the microsatellite reporter might be a factor in assessing the effect of *PMS134* expression on microsatellites. *PMS134* expression did not have a significant effect on microsatellite mutation rates in either transfectant (Table III). We investigated whether PMS134 protein was expressed in the nucleus by western blotting of both nuclear and cytoplasmic extracts and found that it was expressed only in the cytoplasmic fraction (Figure 3). The types of microsatellite mutations were also similar in the presence or absence of PMS134; 4 bp insertions and 2 bp deletions were both common. Small numbers of large deletions were observed in both clones: 8 bp deletions in *PMS134*-expressing cells and 14 bp deletions in parental cells. One insertion of 16 bp was found in the *PMS134*-expressing cell line (Table II).

We investigated the *in vitro* mismatch repair activity of the *PMS134*-expressing cell line to determine whether the absence of an elevation in microsatellite mutation rates resulted from retention of mismatch repair activity in these cells, even in the presence of the mutant protein. We found that the parental hTERT-1604 cells and the *PMS134*-expressing cells were equally proficient in 5′ and 3′ nick-directed *in vitro* mismatch repair of both mismatches and loops (Table IV). Combined, these data suggest that the expression of *PMS134* alone does not decrease mismatch repair activity or induce microsatellite instability in diploid human fibroblasts.

Discussion

We have found that cancer cell lines with mutations in different mismatch repair genes vary in their microsatellite mutation rates. A cell line (HCT116) with defects in *hMLH1* and *hMSH3* had a 10 times higher mutation rate than a line (HEC-1-A) with defects in *hPMS2* and *hMSH6*. Mutations in multiple mismatch repair genes appear to result in more severe phenotypes than those resulting from single gene mutations (Amin *et al*., 2001; Baranovskaya *et al*., 2001). It is also likely that mutations in different mismatch repair genes may lead to different degrees of microsatellite instability. If the mutator phenotype in mismatch repair-defective cells occurred only as a result of the loss of any one of the mismatch repair genes with non-overlapping functions, we would expect to see the same microsatellite mutation rates in all cells with mismatch repair gene defects, regardless of which gene was mutated. In further support of the idea that mutations in multiple mismatch repair genes can elevate microsatellite mutation rates beyond those that occur in cells with a single mismatch repair defect, we previously observed a higher mutation rate in another

colorectal cancer cell line, LoVo, than in HCT116 (Hanford *et al*., 1998); LoVo is deficient in *hMSH2*, but is also thought to have at least one additional, unidentified repair defect (Umar *et al*., 1994). These results suggest that there may be a selective advantage for tumor cells with mutations in multiple mismatch repair genes; cells with multiple mutations and, consequently, higher mutation rates may be more likely to acquire mutations in cancer-related genes.

The lower mutation rate observed in HEC-1-A may be the result of residual mismatch repair activity that could not be detected by the *in vitro* mismatch repair assay, whereas in HCT116, the loss of hMLH1 and hMSH3 may have further reduced mismatch repair activity. Because mismatch repair proteins function in higher order complexes (Prolla *et al*., 1994; Flores-Rozas *et al*., 2000; Amin *et al*., 2001), it is possible that the complex in HEC-1-A can function even in the absence of hPMS2 and hMSH6, albeit with lower stability and efficiency, especially given that both hPMS2 and hMSH6 have candidate alternative proteins (hMLH3 and hMSH3, respectively) (Umar *et al*., 1998; Lipkin *et al*., 2000). The importance of the interplay and formation of functional mismatch repair complexes is evident from observations of decreased mismatch repair activity and increased mutation rates in the *HPRT* gene in cells that overexpress hMSH3 (Drummond *et al*., 1997; Marra *et al*., 1998). It has been shown recently that the combined loss of hMLH1 and hMSH6 results in a stronger mutator phenotype than loss of hMLH1 alone in colorectal cancer cells (Baranovskaya *et al*.., 2001), which also suggests the importance of a higher order complex formation in mismatch repair. In addition, it has been demonstrated in yeast that strong mutator phenotypes occur when two weak mutator mutations are present in the same strain, which supports the hypothesis that additive mutations can result in further destabilization of the mismatch repair complex (Amin *et al*., 2001).

The data presented here on the *PMS134* mutation may support the concept that additive mutations can lead to disruption of the mismatch repair complex. Although *PMS134* was reported to have a dominant negative effect on mismatch repair in Syrian hamster Tk-ts13 fibroblasts (SH cells) (Nicolaides *et al*., 1998), we found that expression of the truncated hPMS2 fragment alone was not sufficient to drive microsatellite instability or the inactivation of mismatch repair in diploid human fibroblasts (Tables II and III). hTERT-1604 cells are mismatch repair proficient (Table III). We have previously shown that the expression of telomerase has no effect on microsatellite mutation rates and that these cells are an appropriate model of normal human fibroblasts for the study of microsatellite mutations (Roques *et al*., 2001). Under conditions of normal human DNA metabolism, as in hTERT-1604 cells, PMS134 was expressed only in the cytoplasm and did not have a dominant negative phenotype. The pEEP4 vector has been used to successfully express other proteins that are transported into the nucleus (Yamada and Farber, 2002); therefore, we did not specifically include nuclear localization signals in the vector. Because this mutation was identified in an HNPCC patient (Parsons *et al*., 1995), it is possible that the microsatellite instability observed in the patient was the result of a combined effect of *PMS134* expression and some other unidentified genetic defect. PMS134 may have been expressed only in the cytoplasm in the patient as well, since the cDNA does not appear to contain a nuclear localization signal; however, it is possible that there were other factors that resulted in PMS134 localization in the nucleus in the patient. It is not known whether PMS134 was expressed in the nucleus or in the cytoplasm in the SH cells; however, in those cells, the expression of even wild-type *hPMS2* resulted in a small decrease in mismatch repair activity (Nicolaides *et al*., 1998). Perhaps the expression of human mismatch repair proteins disrupts the hamster mismatch repair complex. If so, a sufficient destabilization of the mismatch repair complex may have occurred by PMS134 expression alone to induce the dominant negative phenotype in SH cells.

The mutation rates presented in this study are ~10-fold higher than those previously reported for HCT116 and hTERT-1604 (Hanford *et al*., 1998; Roques *et al*., 2001). These differences are likely to be related to the promoters that drive the *tk–neo* fusion gene; the previous experiments were conducted with the reporter construct under control of a viral *tk* promoter, while the reporter construct used in the experiments described here was under control of the stable human EF-1α promoter (Gopalkrishnan *et al*., 1999). We speculate that the *tk* promoter may have been inactivated in a fraction of cells, leading to lower apparent mutation rates because of the absence of *neo* gene expression in some cells with microsatellite mutations. It is also likely that the EF-1α promoter leads to higher levels of protein expression than the *tk* promoter, which could have provided greater protection of *neo* revertants from killing by G418.

In addition to possible differences in the rates at which replication errors are repaired, the analysis of mechanisms that lead to mutator phenotypes is complicated by the possibility that cell lines may differ in the rates at which these errors occur. For example, we recently showed that the rate of microsatellite mutations can be elevated in the absence of mismatch repair defects by the overexpression of an error-prone DNA polymerase, pol β (Yamada and Farber, 2002). HCT116 and HEC-1-A originate from cancers in different organs and they may have cell type-specific differences in DNA metabolism. It is also possible that these cells may have acquired mutations in genes that reduce replication fidelity, while they were propagated in the absence of mismatch repair. These mechanisms of generating microsatellite mutations are not necessarily mutually exclusive. We are currently attempting to develop isogenic human fibroblast lines with or without a defect in a specific mismatch repair gene; the interpretation of mutation rate comparisons will obviously be simplified if development of these lines can be accomplished. Cancer cell lines were used in this study, given that no such normal human cell lines are currently available. We conclude that microsatellite instability is a complex phenotype that is likely to involve both increases in the rates at which errors occur and decreases in the rates at which they are repaired.

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