A functional assay for mutations in tumor suppressor genes caused by mismatch repair deficiency

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Received May 25, 2001; Revised and Accepted September 19, 2001

The coding sequences of multiple human tumor suppressor genes include microsatellite sequences that are prone to mutations. Saccharomyces cerevisiae strains deficient in DNA mismatch repair (MMR) can be used to determine de novo mutation rates of these human tumor suppressor genes as well as any other gene sequence. Microsatellites in human TGFB2, PTEN and APC genes were placed in yeast vectors and analyzed in isogenic yeast strains that were wild-type or deletion mutants for MSH2 or MLH1. In MMR-deficient strains, the vector containing the (A)_{10} microsatellite sequence of TGFB2 had a mutation rate (mutations/cell division) of $1.4 \times 10^{-4}$, compared to a mutation rate of $1.7 \times 10^{-6}$ in the wild-type strain. In MMR-deficient strains, mutation rates in PTEN and APC were also elevated above background levels. PTEN mutation rates were higher in both msh2 (4.4 $\times 10^{-5}$) and mlh1 strains (2.3 $\times 10^{-5}$). APC mutation rates in the msh2 strain (2.4 $\times 10^{-6}$) and the mlh1 strain (1.7 $\times 10^{-6}$) were also significantly, but less dramatically, elevated over background. Mutations selected for in the yeast screen were identical to those previously observed in human tumor samples with microsatellite instability (MSI). This functional assay has applicability in providing quantitative data about microsatellite mutation rates caused by MMR deficiency in any human tumor suppressor gene sequence. It can also be applied as a genetic screen to identify new genes that are vulnerable to such microsatellite mutations and thus may be involved in the neoplastic development of tumors with MSI.

INTRODUCTION

Hereditary non-polyposis colorectal cancer (HNPCC) is a familial cancer syndrome caused by germline mutations in DNA mismatch repair (MMR) genes such as hMSH2 and hMLH1 (1). The loss of these MMR genes causes microsatellite instability (MSI), a phenomenon where mutations occur at significantly elevated rates in microsatellite tracts of genomic DNA (2). The study of MMR genes in Saccharomyces cerevisiae was vital in defining the role of MMR in human cancer (3,4). Similarly, the study of microsatellite tracts in MMR-deficient strains of S.cerevisiae has increased our understanding of the role of MSI in human tumors (5–7).

In cancers from individuals with HNPCC, the elevation in mutation rates causes inactivation of specific tumor suppressor gene, producing a selective growth advantage and thus contributing to stepwise neoplastic progression (8). Multiple genes have been identified with microsatellite mutations including TGFB2, IGFR2, MSH3, MSH6, PTEN, BAX and MBD4 (8–15). The majority of these microsatellites consist of mononucleotide repeats which accumulate 1 bp deletions or insertions.

Observational studies have identified these microsatellite mutation hotspots; candidate genes are sequenced for microsatellite mutations in a series of tumors and cell lines exhibiting MSI. Recently, criteria have been developed to standardize diagnostic testing of MSI in primary human tumors, permitting comparison among genes that display high MSI (MSI-H) versus those with mutations attributable to background levels (MSS or MSI-L) (16). Whereas these tumor studies accurately identify mutations and their frequency among the tumor sample sets, they lack the ability to quantify rates of mutation caused by MMR deficiency (17). In addition, there are inherent difficulties in analyzing de novo mutations in coding region microsatellites. Fluctuation analysis with mammalian cell lines has been developed for artificial dinucleotide tracts and is technically challenging (17–20). As an added complication of tumor studies, comparing the effects of individual MMR genes on MSI is difficult because tumor samples and cell lines are frequently not isogenic and oftentimes have not been fully characterized for loss of MSH2 or MLH1.

We have adapted a plasmid frameshift assay to evaluate MSI within the coding regions of human tumor suppressor genes in yeast strains lacking either msh2 or mlh1 (5). Relying upon the highly conserved function of MMR genes between humans and S.cerevisiae, this genetic screen permits the quantitative analysis and identification of de novo mutations caused by MMR deficiency in any human microsatellite sequence. Several plasmids (pHJ series) were constructed in which a portion of the tumor suppressor cDNA sequence was placed upstream and in-frame of URA3. These vectors were transformed into three isogenic haploid strains: wild-type, msh2 deletion mutant and mlh1 deletion mutant. De novo mutations in the tumor suppressor sequence lead to frameshifts that prevent the appropriate translation of the URA3 marker. These events can be seen as growth on 5-fluororotic acid (FOA) and
subsequently be scored (21). For fluctuation analysis, the method of the median was used to determine mutation rates, the number of FOA\textsuperscript{8} events per cell division (22).

For this initial study, we measured the mutation rates of microsatellite sequences in three tumor suppressor genes, TGFBR2, PTEN and APC. Transforming growth factor β-receptor II inhibits cell proliferation and contains an (A)\textsubscript{10} tract that is frequently mutated in MSI-positive tumors such as colon adenocarcinomas (8). The phosphatase tumor suppressor PTEN contains two (A)\textsubscript{5} tracts, one in exon 7 and the other in exon 8, the latter being the primary mutation hotspot in endometrial carcinomas with MSI (11,13). The adenomatous polyposis gene APC plays a key role in sporadic and familial colon cancers and contains a large number of microsatellites. We chose a region of APC with two (A)\textsubscript{5} and one (T)\textsubscript{5} microsatellites previously shown to have mutations in MSI-positive colon carcinomas (9).

RESULTS

Mutation rate analysis of positive control and background levels in MMR-deficient strains

As a positive control to test mutation rates in the MMR-deficient strains, we designed a centromeric plasmid (pHJ5) with a dinucleotide tract, (GT)\textsubscript{160} upstream and in-frame of URA3 (Fig. 1A). This plasmid also contained a LEU2 marker for plasmid retention. As has been demonstrated in previous work, this microsatellite tract mutates at significantly elevated rates in MMR-deficient yeast strains (5,6,23). In our hands, mutation rates (FOA\textsuperscript{8}/cell division) in msh2 were 4.6 × 10\textsuperscript{4}, a 71.4-fold elevation over wild-type background and in msh1 were 3.4 × 10\textsuperscript{4}, a 53-fold elevation over wild-type.

As an additional control, vector alone (pCI-HA) was transformed into all strains to measure background mutation rates which can occur in URA3 alone, leading to FOA\textsuperscript{8}. Vector alone had the lowest mutation rates among all the plasmids tested. In wild-type strains, the mutation rate for vector alone was 2.0 × 10\textsuperscript{7}. Mutation rates were 1.0 × 10\textsuperscript{6} in msh2, a 5-fold increase over wild-type and were 0.8 × 10\textsuperscript{6} in msh1, a 4-fold increase over wild-type. These mutation rates are a direct reflection of mutations occurring in URA3.

Analysis of mutation rates in tumor suppressor microsatellites in MMR-deficient strains

A series of constructs (pHJ series) were designed using a low-copy number centromeric vector which is stably maintained with one to two copies per cell. Use of centromeric vectors minimizes the possibility that plasmid copy number could influence mutation rates among the different plasmids. Each construct incorporated an ~400 bp region, containing tumor suppressor coding region microsatellites and in-frame of a URA3 marker (Fig. 1B). These vectors also contain a LEU2 selectable marker for plasmid retention. The plasmids pHJ3 (PTEN), pHJ4 (APC) and pHJ9 (TGFBR2) were transformed into isogenic msh2, msh1 and wild-type strains. All plasmids demonstrated a stable Ura+ phenotype, indicating that the encoded Ura3p was not affected by the N-terminal fusion of the selected tumor suppressor.

Using the method of the median, fluctuation analysis was repeated independently three to four times for each plasmid to determine mutation rates in each strain and the results were averaged (22). For all tumor suppressor genes, mutation rates were significantly higher in MMR-deficient strains in comparison with the wild-type strain (Table 1). The plasmid pHJ9, containing the TGFBR2 (A)\textsubscript{10} tract, showed dramatically elevated mutation rates in comparison with the other vectors. The TGFBR2 plasmid mutation rates were similar between either msh2 or msh1 backgrounds. Mutation rates in pHJ3, containing the PTEN microsatellites, were slightly higher in msh2 strains than msh1 strains (1.84 times). For pHJ4, containing APC microsatellites, mutation rates were surprisingly low with only 11- and 8-fold increases over wild-type mutation rates for msh2 and msh1, respectively.

Analysis of tumor suppressor microsatellite mutations

Mutations caused by MMR deficiency were identified in the tumor suppressor regions of the vectors (Table 2). Plasmids were recovered from FOA\textsuperscript{8} colonies and the tumor suppressor regions sequenced. Plasmids were isolated from independent experiments, separate samples and represent independent mutations. FOA\textsuperscript{8} mutations were detected more frequently in TGFBR2 than in PTEN and APC, the difference attributable to significantly higher mutation rates of the TGFBR2 region. Overall, the types of mutations from the msh2 and msh1 strains had a similar distribution to those found in MSI-positive tumors, with 90% being 1 bp deletions.

Plasmids lacking mutations in the TGFBR2, PTEN and APC tumor suppressor microsatellites had mutations in URA3 as was determined in a subset that was sequenced. URA3 mutations occurred mostly in PTEN and APC plasmids, given the lower mutation rates of their microsatellites. These mutations occurred in three microsatellites tracts in URA3: (A)\textsubscript{5} codons 58–60, (T)\textsubscript{5} codons 67–69 and (T)\textsubscript{6} codons 85–87. To account for the difference caused by mutations occurring in the tumor suppressor sequence versus URA3, we calculated an estimated tumor suppressor mutation rate using the ratio of plasmids with tumor suppressor mutations over the total number sequenced (Fig. 2). Given that no mutations were identified in the PTEN and APC plasmids rescued from wild-type background, we used 1.0 × 10\textsuperscript{8} which is an estimate of the general background mutation rate. In concordance with the plasmid mutation rate, the estimated tumor suppressor mutation rates of the TGFBR2 sequence were significantly higher than mutation rates of the PTEN or APC sequences.

Mutations revealed by the yeast analysis reflected sites previously identified in MSI-positive human tumors (Table 2). The (A)\textsubscript{10} microsatellite of TGFBR2 was found to be highly prone to mutations. Mutations in the TGFBR2 microsatellite consisted of 1 bp deletions and insertions, matching the location, type and frequency of TGFBR2 mutations found in human MSI-positive colon adenocarcinomas (8). The mutations in PTEN were distributed over several microsatellites and consisted mostly of 1 bp deletions (Fig. 3). The majority of mutations were found in the exon 8 (A)\textsubscript{5} tract, located in codons 321–323, the site frequently altered in MSI-H endometrial carcinomas (11,13). The other PTEN (A)\textsubscript{5} tract in codons 265–267 had a lower frequency of mutations. In APC, the majority of mutations were found in the (A)\textsubscript{5} tract located...
in codons 756–758 where microsatellite mutations have previously been found in MSI-positive colon adenocarcinomas (9). Also, a single base pair substitution (G→T) leading to A762S was also identified in several independent, separate experiments for determining mutation rate.

**DISCUSSION**

Taking advantage of the conservation of MMR between yeast and humans, we have adapted *S.cerevisiae* as a model organism system for examining MSI in human tumor suppressor genes. Whereas MSI has been thoroughly studied in yeast using plasmid frameshift assays, this work is the first to demonstrate that human tumor suppressor microsatellites behave in MMR-deficient yeast strains similarly to MSI-positive tumors.

A quantitative analysis for individual tumor suppressor microsatellites reveals significant differences in MMR-deficient strains. The *TGFBR2* plasmid had the highest mutation rates among the three tumor suppressor sequences tested, correlating with the high frequency of mutations seen in human MSI-positive tumors. The *PTEN* and *APC* plasmids also showed an increase in mutation rates although not as dramatic as *TGFBR2*. In general, the mutation rates in different microsatellite sequences correlate with the lengths of the mononucleotide tracts as seen in the shorter length tracts in *PTEN* (A)₈ or *APC* (A)₅ compared to the (A)₁₀ microsatellite of *TGFBR2*. The significantly lower mutation rates of the tested *PTEN* and *APC* microsatellites indicate that they have a reduced probability of accumulating mutations than the highly mutation prone *TGFBR2* (A)₁₀ microsatellite. One can hypothesize that mutations in the *TGFBR2* microsatellite may be a relatively early event compared to other microsatellites (i.e. *APC*) in MMR-deficient cells. A small difference in mutation rates between *msh2* and *mlh1* strains for the *PTEN* plasmid was found, but this may not be statistically significant. Plasmid copy number was unlikely to have a significant effect on mutation rate given our use of centromeric vectors, but it could be a minor factor leading to some of the smaller differences noted in the *PTEN* vector.

Sequencing of the *de novo* tumor suppressor mutations showed that the spectrum and location of microsatellite mutations in MMR-deficient yeast were the same as those found in human tumors, namely 1 bp insertions and deletions. The (A)₁₀ microsatellite of the *TGFBR2* was found to accumulate 1 bp deletions and insertions which match the type and frequency of *TGFBR2* mutations found in human MSI-positive colon adenocarcinomas (8,24). In the first studies, over 90% of the MSI-positive colon carcinoma samples and cell lines were found to have deletion and insertion mutations in the (A)₁₀ tract in *TGFBR2* and decreased cell surface expression of the receptor. The MSI-negative tumors showed normal *TGFBR2* expression.

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**Figure 1.** pHJ microsatellite vectors. (A) The positive control vector pHJ9 contains the DNA sequence (GT)₁₆G in-frame of *URA3*. (B) The coding region microsatellite vectors contain a portion of cDNA sequence of a tumor suppressor gene upstream and in-frame of *URA3*. pHJ3 incorporates a region of the *PTEN* between codons 226 and 360 with four microsatellites. pHJ4 incorporates a region of *APC* between codons 733 and 867 with three (five repeat unit) microsatellites. pHJ9 incorporates a region of the *TGFBR2* between codons 68 and 201 with one microsatellite. Microsatellites with five or greater repeat units are designated as bars within the gene insert. Codons refer to the location within the tumor suppressor gene ORF.
This finding has since been corroborated in multiple studies (25).

In comparison with TGFBR2’s microsatellite, mutations were found less frequently in the PTEN and APC microsatellites, a direct reflection of their significantly lower mutation rates and in concordance with tumor studies. Among the various PTEN microsatellites, the (A)_8 tract located in exon 8 which is located in codons 321–323 of PTEN and is known to frequently contain mutations in MSI-positive endometrial carcinomas.

Table 1. Mutation rates at human tumor suppressor genes in MMR-deficient strains versus wild-type strains

<table>
<thead>
<tr>
<th>Tumor suppressor gene (plasmid)</th>
<th>Strain genotype</th>
<th>Plasmid mutation rate (FOA&lt;sup&gt;+&lt;/sup&gt;/cell division)</th>
<th>Fold induction over wild-type rate</th>
<th>Estimated tumor suppressor mutation rate</th>
<th>Fold induction of estimated rate over wild-type rate</th>
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<tr>
<td>TGFBR2 (pHJ9)</td>
<td>msh2</td>
<td>1.4 × 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>82</td>
<td>1.2 × 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>1.5 × 10&lt;sup&gt;3&lt;/sup&gt;</td>
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<tr>
<td>TGFBR2 (pHJ9)</td>
<td>mlh1</td>
<td>1.4 × 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>82</td>
<td>1.3 × 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>1.6 × 10&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>TGFBR2 (pHJ9)</td>
<td>WT</td>
<td>1.7 × 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>–</td>
<td>7.9 × 10&lt;sup&gt;-4&lt;/sup&gt;</td>
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<tr>
<td>PTEN (pHJ3)</td>
<td>msh2</td>
<td>2.3 × 10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>40</td>
<td>2.9 × 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>290</td>
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<td>PTEN (pHJ3)</td>
<td>mlh1</td>
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<td>1.3 × 10&lt;sup&gt;-5&lt;/sup&gt;</td>
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<td>PTEN (pHJ3)</td>
<td>WT</td>
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<td>–</td>
<td>1.0 × 10&lt;sup&gt;-8&lt;/sup&gt;</td>
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<td>APC (pHJ4)</td>
<td>msh2</td>
<td>1.7 × 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>8</td>
<td>3.1 × 10&lt;sup&gt;-7&lt;/sup&gt;</td>
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<td>APC (pHJ4)</td>
<td>mlh1</td>
<td>2.4 × 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>11</td>
<td>1.6 × 10&lt;sup&gt;-7&lt;/sup&gt;</td>
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<tr>
<td>APC (pHJ4)</td>
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<td>2.1 × 10&lt;sup&gt;-7&lt;/sup&gt;</td>
<td>–</td>
<td>1.0 × 10&lt;sup&gt;-8&lt;/sup&gt;</td>
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</table>

Figure 2. Distribution of mutations in the microsatellites of PTEN. Mutations from both msh2 and mlh1 strains were collated and shown as a distribution against the region of PTEN in pHJ3. The majority of mutations occurred in the exon 8 (A)_8 tract which is located in codons 321–323 of PTEN and is known to frequently contain mutations in MSI-positive endometrial carcinomas.

Figure 3. Tumor suppressor mutation rates for individual tumor suppressor sequences. Mutation rates represent FOA<sup>+</sup>/cell division. Tumor suppressor mutation rates were estimated by multiplying the overall plasmid mutation rate against the proportion of vectors containing mutations in tumor suppressor sequence over the total number sequenced. Rates were plotted on a log scale to facilitate comparison.

APC compared with only 20% of the MSI-negative tumors having APC microsatellites mutations.

In FOA<sup>R</sup> colonies where mutations were not found in the tumor suppressor region, mutations occurred in URA3. Based upon our determination of mutation rates in the vector alone control, URA3 mutations account for a relatively low fraction of mutations and a minimal background mutation rate. We also calculated an estimate of the mutation rate dependent on tumor suppressor sequence alone, independent of de novo mutations in the URA3. These estimated tumor suppressor mutation rates showed the same general trends as the plasmid mutation rates with the TGFBR2 microsatellite sequence having dramatically higher mutation rates than the PTEN or APC microsatellites.

Sequence context influences MSI as demonstrated by the difference in the number of mutations found in the two (A)<sub>6</sub>
tracts in PTEN. Mutation frequency was highest in the exon 8 (A)$_8$ tract, as has been seen with MSI-positive endometrial cancers. This suggests some influence of the surrounding sequence that contributes to the exon 8 microsatellites vulnerability to MSI. In APC, the (A)$_5$ tract located in codons 756–758 had the highest frequency of mutations. Compared to the other APC microsatellites, the tract in codons 756–758 may have an increased susceptibility to DNA mismatches given the presence of an (A)$_3$ tract located 1 bp upstream of the (A)$_5$ region. A substitution mutation (A762S) was also identified in APC but its functional significance is unknown pending further investigation. One limitation of this assay in terms of sequence context is its inability to account for chromosomal context. Application of yeast artificial chromosomes containing large regions of human genome sequence as substrates may prove to be useful in studying chromosomal context. This genetic assay is limited in its ability to detect mutations that lead to in-frame deletions or insertions. However, in human tumor samples with MSI mutations, in-frame mutations are almost never found with the vast majority being either 1 bp deletions or insertions.

The use of S. cerevisiae in studying the behavior of tumor suppressor gene MSI has a number of advantages. The application of this frameshift assay complements human tumor studies by providing mutation rates for critical genes with a coding region microsatellite. Mutation rate data will aid in discriminating true MSI-H targets versus events that are the result of background levels of mutation and unlikely to be of significance to MSI-related tumorogenesis. Mutation rate data can lead to inferences about mutation events occurring in HNPCC-related neoplastic development. Using isogenic strains from collections such as those available from the Saccharomyces Genome Deletion Project, the individual contribution of different MMR genes to MSI can be determined and compared (27). This system can evaluate the influence of sequence context on MSI more readily than models using human tumor cell lines. Finally, this assay can be adapted as a genetic screen to identify new genes containing hypermutable microsatellite targets involved in the pathogenesis of MSI-positive tumors.

**MATERIALS AND METHODS**

**Yeast strains**

Saccharomyces cerevisiae haploid strains were obtained from the Research Genetics and originate from the Saccharomyces Genome Deletion Project (27). The following strains were used in this work: BY4741 (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0), YD0750 (MATa leu2Δ0 met15Δ0 ura3Δ0).

**Table 2. Microsatellite mutations in tumor suppressor genes in pHJ3, pHJ4 and pHJ9**

<table>
<thead>
<tr>
<th>Tumor suppressor gene</th>
<th>Strain genotype</th>
<th>Total number of mutations</th>
<th>FOA$^2$ clones sequenced</th>
<th>Microsatellite</th>
<th>Codon location</th>
<th>Microsatellite sequence arranged by codons</th>
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<td>APC mlh1</td>
<td>8</td>
<td>44</td>
<td>(A)$_5$</td>
<td>756–758</td>
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<td></td>
<td></td>
<td>(A)$_5$</td>
<td>846–848</td>
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<td>del A (1)</td>
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<td></td>
<td></td>
<td></td>
<td>(GA)$_4$</td>
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<td>del A (2)</td>
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<td>PTEN mlh1</td>
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<td>del A (1)</td>
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FOA$^2$ refers to growth on 5-fluororotic acid.
**MH1**: KanMX4 his3Δ1) and YD6240 (MATa leu2Δ0 met15Δ0 ura3Δ0 MSH2::KanMX4 his3Δ1).

**Plasmids**

As previously described, the plasmid, pCI-HA, is a low copy number centromeric vector containing two markers (LEU2 and URA3) and has a unique BamHI site located upstream and of URA3 (28). Gap repair was used to create a series of constructs with tumor suppressor microsatellites. PCR primers were designed to incorporate an ~400 bp region encompassing the selected microsatellite, upstream and in-frame of URA3. Location of the primer is based on the cDNA sequence ORF. To mediate the homologous recombination for gap repair, the 5′ flanks of each primer are derived from sequence encompassing the BamHI site of pCI-HA. For pH3 containing the PTEN microsatellites, the primers used were 5′-CCCTATGTCC-CCGACTATGCGATCTCCTCCTCAATTCAGGACCCA-CAGA-3′ and 5′-ATGAGTACGACAGCAGTTTCAATTAT-AGGTATGGCCCTGACTCCTGGATTTGACG-3′ (nt 678–1080). For pH4 containing the APC microsatellites, the primers used were 5′-GCCGGCTATCCCTATGACGTCCCGGACTATGCAGGATCCGGCCGCCTAGGCCTGCGAAGTACAAGG-3′ and 5′-ATGAGTACGACAGCAGTTTCAATTAT-AGGTATGGCCCTGACTCCTGGATTTGACG-3′ (nt 2196–2601).

For pH7 containing TGFB2R microsatellite, the primers used were 5′-GGCCGGCTATCCCTATGACGTCCCGGACTATGCAGGATCCGGCCGCCTAGGCCTGCGAAGTACAAGG-3′ and 5′-ATGAGTACGACAGCAGTTTCAATTAT-AGGTATGGCCCTGACTCCTGGATTTGACG-3′ (nt 202–603). PCR templates included the vector pSAR-MT containing the APC ORF and pH2-3F containing the TGFB2R ORF. PTEN was amplified from a cDNA library derived from human lymphocytes (Clontech). PCR reactions were subjected to 35 cycles of 94°C for 30 s, 50°C for 30 s and 72°C for 30 s. The vector pCI-HA was cut with BamHI and the linearized vector was co-transformed with the PCR product into BY4741 (wild-type). Plasmids were rescued from yeast strains showing a Leu+, Ura+ phenotype and transformed using into Escherichia coli using electroporation. Plasmids showing the correct insert were selected. The positive control plasmid, pH5, was derived from pRS415, a centromeric vector with a LEU2 marker and pSH44, a vector containing a dinucleotide tract, (GT)nt, upstream and in-frame of URA3 (23). The plasmid pHSH44 was digested with HindIII and the 4 kb fragment containing both the dinucleotide tract and URA3 marker was gel purified. This fragment was ligated into HindIII-cut pRS415. All plasmids were sequenced to confirm the presence of the insert, its orientation and sequence.

**Mutation rate analysis**

The MSI rate for individual yeast strains was determined by fluctuation analysis using the method of the median from samples of 11 or 15 independent cultures (22,29). A new mutation in the tumor suppressor region of the plasmid results in a frameshift. Introduction of these mutations produces a selectable phenotype which is Ura– and cells are able to grow on FOA8 (21). pH3, pH4, pH5, pH9 and pCI-HA were transformed into isogenic haploid wild-type (BY4741) and deletion mutant strains of MSH2 (YD6240) and MLH1 (YD0750). Leu+, Ura+ colonies were selected initially. Transformed yeast strains were subsequently grown on Leu− media for 3 days. Individual colonies were isolated, suspended in water and dilutions were plated on Leu−, FOA plates (to measure FOA8) and Leu− plates (to monitor viable cells). For each strain, fluctuation analysis was repeated independently three to four times and mutation rates were determined by averaging the results. Mutation rates represent FOA8 events/cell division. Fold induction over wild-type was calculated by dividing the MMR-deficient strain rate over the wild-type strain rate. Tumor suppressor mutation rates were estimated by multiplying the overall plasmid mutation rate against the proportion of vectors containing mutations in the tumor suppressor sequence over the total number sequenced.

**Plasmid rescue, PCR and DNA sequencing**

FOA8 colonies were patched on Leu−, FOA plates. Plasmids were recovered using methods described previously (30). Primers specific for the pCI-HA and encompassing the tumor suppressor insert region were designed: 5′-GTTCCTGAC-TATGGGCGGCTA-3′ and 5′-AAGTCTGACCCCTATTGG-3′. The tumor suppressor insert was amplified by PCR using the following protocol: 35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 30 s. The PCR product was purified. DNA sequencing of pH3, pH4 and pH9 PCR products was performed using the first primer listed and Big Dye terminator sequencing chemistry as per the manufacturer’s protocol (Perkin-Elmer). Sequencing reactions were precipitated and run on an Applied Biosystems 377 DNA sequencer.

**ACKNOWLEDGEMENTS**

We thank I.Chikashi for the pCI-HA plasmid, T.D.Petes for the pSH44 plasmid, B.Vogelstein for the pSAR plasmid and B.Schiemann for the pH2-3F plasmid. We thank I.Ji for her help in construction of the pHJ5 vector. We thank M.Vidal, E.Swisher and J.Boeke for insightful comments about this work. This work was supported by a Howard Hughes Medical Institute Physician-Scientist Fellowship (H.P.J.) and NIH R01 CA27632 (M.C.K.).

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