Mutation Rate of a Microsatellite Sequence in Normal Human Fibroblasts

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ABSTRACT

Dinucleotide repeats, because of their repetitive nature, are prone to frameshift mutations, most likely via a DNA-polymerase slippage mechanism. Mutation rates in microsatellite DNA sequences are high in mismatch repair-defective cells. In normal cells, only estimates of maximal rates of mutation in microsatellites have been possible previously, because of the low sensitivity of screening assays for mutations in endogenous sequences. We have measured the spontaneous mutation rate of a dinucleotide repeat in diploid human foreskin fibroblasts. In our system, the mutation target is a (CA)17 repeat contained within a stably integrated plasmid. The repeat disrupts the reading frame of a neomycin (neo) resistance gene within the plasmid. Cells containing frameshift mutations in the CA repeat that correct the reading frame of the neo gene are selected using the neo analogue G418. This system of measuring microsatellite mutation rates is highly sensitive, because there is a specific target within which mutations can be selected. Fluctuation analysis of cells containing the target DNA yielded mutation rates of <3.1 × 10⁻⁶ to 44.8 × 10⁻⁶ mutations/cell/generation. This is the first report of a direct measurement of a spontaneous mutation rate of a microsatellite sequence in normal human cells.

INTRODUCTION

The multistep nature of carcinogenesis implies that multiple mutations are necessary to produce a malignant cell. Because the estimated spontaneous mutation rate in normal human cells (1 × 10⁻¹⁰ mutations/nucleotide/cell generation; Ref. 1) is thought to be inadequate to yield enough mutations to produce cancer, a mutator hypothesis (2, 3) has been invoked as a possible explanation for how cancers develop. Initial mutation rate comparisons between normal and malignant human cells yielded conflicting results: some studies demonstrated a higher rate in malignant cells (4, 5) whereas other studies did not (6, 7). More recently, a mutator phenotype has been observed in MMR3-defective cancer cell lines (8–12), tumors (13–15), and knockout mice (16–18). These cells display enhanced mutation frequencies at repetitive (19–21) and nonrepetitive DNA sequences (8, 9, 12, 22, 23).

It is particularly difficult to measure rates of mutation in microsatellites in normal cells. Previous attempts have involved the screening of endogenous microsatellite loci in individual clones. Because of the low sensitivity of this approach, such mutations have not been detected in clones of diploid human cells. Thus, it has been possible to establish only upper limits on microsatellite mutation rates in these cells (24). We have developed a highly sensitive system for the selection of mutations in a microsatellite sequence introduced into cells by transfection. Using this system, we have calculated spontaneous mutation rates for a dinucleotide repeat in diploid human fibroblasts.

MATERIALS AND METHODS

Cell Culture Conditions. NHF1 cells (25) were cultured in MEM-α supplemented with 20% FBS (Life Technologies, Inc., Gaithersburg, MD) and 20% AmnioMax-C100 (Life Technologies, Inc.). The CFE in this medium at low cell PDs (PD = 21) averaged 30–40%, and at high PDs (PD = 45), the CFE averaged 25–40%. This medium produced faster-growing colonies that were larger (4–5 mm in diameter) and denser than those grown in DMEM containing 10% FBS (≈2 mm in diameter) after 10 days in culture.

DNA Transfection and Fluctuation Analysis. The pRTM2 plasmid (26) (Fig. 1) contains a (CA)17 dinucleotide repeat sequence that was inserted into a fusion gene between the herpes tk gene (tk) and a bacterial gene coding for neo resistance (neo). The repeat was inserted at an AarHI site near the 3' end of the tk portion of the fusion gene, just upstream of the neo portion of the gene. The presence of the microsatellite insert results in the disruption of the reading frame of the downstream region of the tk-neo fusion gene, which is then translated in the −1 reading frame. The site in the tk portion of the fusion gene was used to avoid possible effects of the presence of unusual amino acids in the neo portion of the gene. Cells containing mutations in the CA repeat that restore the reading frame are resistant to the neo analogue G418. The sequence of the entire frameshift target has been reported previously (27). The bacterial hygR gene in pRTM2 was used for the selection of stable transfectants.

The pRTM1-IF plasmid is identical to pRTM2, except that the microsatellite insert contains 16 CA repeats, allowing the tk-neo gene to be read in-frame; therefore, cells stably transfected with this plasmid are G418R. pRTM2 plasmid DNA (10 μg) linearized with HindIII was electroporated into 10⁵ NHF1 cells, and transformed clones were selected with hyg B (70 μg/ml). The hygR clones were screened for CFE, and clones with the highest CFE were used in fluctuation analyses. Subcultures of each hygR clone were seeded at 1000 cells/well in a 24-well culture dish. These were expanded to approximately 2 × 10⁵ cells each. Revertants that express the neo gene were selected from each subculture by plating cells at a density of 5 × 10⁵ cells/100-mm dish in medium containing 300 μg/ml G418 (Genetin; Life Technologies, Inc.). Fluctuation analysis was performed, and mutation rates were calculated using a Chipmunk BASIC computer program written by Eric Bronner (Oregon Health Sciences University, Portland, OR) that is based on the Luria and Delbrück method of the mean (28), using the Capizzi and Jameson tables (29).

PCR. DNA was isolated from hygR and G418R cells for PCR analysis. With one exception, DNA was isolated from either live or frozen G418R colonies. A crude lysate was prepared from either colonies (approximately 5 mm in diameter) or a well of cells from a 24-well dish (approximately 350,000 cells) by incubating at 55°C for 1 h in a lysis buffer containing 10 mm Tris-HCl (pH 8.3), 1.25 mM MgCl₂, 50 mm KCl, 0.01% (w/v) gelatin, 0.45% (v/v) NP40, and 1 mg/ml protease K. DNA was isolated from frozen cell pellets (≥1 × 10⁶ cells) by the method of Miller et al. (30) using cell lysis buffer containing 50 mm Tris-HCl (pH 7.6), 100 mm NaCl, 1.0 mm EDTA, 290 μg/ml protease K, and 2 μg/μl RNase A and incubating at 37°C overnight. The DNA was subsequently precipitated using NaCl and ethanol. In one instance, DNA was purified from a methanol-fixed G418R colony using the guanidine salts reagent DNAzol (Life Technologies, Inc.) and precipitated with ethanol using glycogen as a carrier. Either the crude lysate or purified DNA was then used directly for PCR.

PCR analysis of the target CA repeat was performed as described previously (26) to determine the change in size of the CA repeat. Cycling conditions were as follows: (a) an initial 7-min denaturation at 94°C; (b) 27 cycles of 1 min at 95°C, 2 min at 55°C, and 1 min at 72°C; and (c) a final extension of 6 min at 72°C. In one G418R mutant that contained multiple copies of the pRTM2 plasmid, the number of copies of the CA target sequence was estimated using PhosphorImager analysis (Molecular Dynamics, Sunnyvale, CA) of the PCR products, which were separated on a 6% denaturing polyacrylamide gel.
commercial product that contains human-derived growth factors (AmnioMax-C100) and chose hyg^R clones with the best growth characteristics (i.e., CFE, density of colonies, and growth rate) for fluctuation analysis. The use of AmnioMax-C100 does not increase the life span of the cells, but the more favorable growth properties provide a larger number of healthy nonsenescent hyg^R clones. Thus, we were able to choose clones that were most likely to have the growth potential to allow completion of the experiment.

We performed a reconstruction experiment to determine the potential for survival of G418^R cells at high PDs during selection in G418 in the presence of G418-sensitive cells. Cells transfected with pRTM1-IF, a plasmid similar to pRTM2 but with the neo gene in-frame, were selected directly in G418 (instead of hyg). The cells were then grown without selection to approximately the same number of PDs as their pRTM2-transfected counterparts at the time G418 selection was initiated (PD = 45–53). These G418^R cells were plated at low densities (250–2000 cells/100-mm dish; 4 dishes/dilution) in the presence of 4 × 10^5 G418-sensitive NHF1 cells (PD = 27) and G418 to simulate selection of G418^R mutants from colonies containing the out-of-frame sequence. The results (Table 1) indicate that of the G418^R cells plated, approximately 2–3% were able to form colonies in the presence of G418 and 4 × 10^5 G418-sensitive cells. The CFEs of cells containing the in-frame target in the presence and absence of G418 (plated in the absence of sensitive cells) were 5.0 and 4.7%, respectively. The CFE of these cells was lower than that of their age-matched pRTM2-containing counterparts (25–40%), presumably because the pRTM1-IF transfecteds were not prescreened for growth potential. We made two conclusions from this control experiment: (a) it was possible to select G418^R mutants of normal human cells at high PDs; and (b) the presence of sensitive cells at the density used here may have a small (about 2-fold) effect on the recovery of the G418^R clones.

After transfection with pRTM2, DNA from hyg^R clones was analyzed by PCR to confirm the presence of the CA repeat and the tk-neo fusion gene in the clones. Fluctuation analysis was performed, and mutation rates were calculated (28, 29). Table 2 lists the results for five independent hyg^R clones. A total of 10–16 subcultures were used for a total of 1.8–3.2 × 10^7 cells/hyg^R clone. Before calculation of the mutation rates, the numbers of G418^R colonies were corrected for the CFE of each parental hyg^R clone at the time the plates were established (see Table 2). Rates of reversion to G418 resistance were <3.1 × 10^{-8} to 44.8 × 10^{-8} cell/generation.

The DNA from two of the nine independent G418^R clones was isolated, and the lengths of the dinucleotide repeats were analyzed by PCR. Both of the G418^R clones had 4-base insertions in the PCR products (Fig. 2, A and B). DNA sequencing of these clones showed that the 4-base insertion in clone 1 resulted from the addition of two 1-base insertions of a guanosine, located 6 bases from either end of the repeat. Because the portion of the tk-neo fusion gene downstream of the microsatellite is in the −1 reading frame, the smallest dinucleotide frameshift mutations that would restore the reading frame would be 2-base deletions and 4-base insertions. Frameshifts

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**Table 1 Reconstruction of a G418^R mutant selection using NHFI cells transfected with the neo in-frame plasmid pRTM1-IF**

<table>
<thead>
<tr>
<th>No. of NHF1 (pRTM1-IF)^a cells/dish</th>
<th>Average no. of G418^R colonies/dish</th>
<th>% G418^R colonies recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>250</td>
<td>8.0</td>
<td>3.2</td>
</tr>
<tr>
<td>500</td>
<td>15.5</td>
<td>3.1</td>
</tr>
<tr>
<td>1000</td>
<td>21.2</td>
<td>2.1</td>
</tr>
<tr>
<td>2000</td>
<td>34.0</td>
<td>1.7</td>
</tr>
</tbody>
</table>

*a* NHF1 cells stably transfected with pRTM1-IF; PD = 53.  
*b* Plated in the presence of 4 × 10^5 NHF1 cells in a 100-mm dish.
resulting from the addition of two CA repeat units have been detected in other cell types using this system (26, 31).

One clone (clone 1) contained multiple copies of the dinucleotide target sequence (Fig. 2B). In this clone, there are two different PCR products, one of the same size as that in the parental cells, and one with the 4-base insertion. Phosphorimager analysis revealed that this clone contained three copies of the dinucleotide repeat, one containing the 4-base addition, and two of normal length (data not shown). Because reversion to G418 resistance is dominant, a mutation in only one copy of the microsatellite is necessary to produce a resistant clone. Thus, the mutation rate/copy of the (CA)₁₇ repeat in this clone is probably three times lower than the rate calculated for the clone as a whole.

DISCUSSION

The mutation rate in diploid human fibroblasts for a microsatellite sequence composed of a (CA)₁₇ repeat was determined to be <3.1 × 10⁻⁷ to 44.8 × 10⁻⁸ mutations/cell/generation. This measurement is unique in that we were actually able to detect colonies with mutations in the microsatellite in three of five hyg₆ clones analyzed. Others (24) have reported only the upper limits of mutation rates in endogenous microsatellite sequences for normal fibroblasts, because no mutants were detected. The advantage of the mutation detection system used here is the sensitivity that results from the ability to select mutant clones. Other factors that made it possible to perform this measurement in normal cells include the use of medium supplements that enhanced cell growth and CFE and the preselection of the heartiest clones for use in fluctuation analyses. Preselection of clones with the best growth potential was based on observations made by others that there is a high degree of heterogeneity in doubling potential among individual clones of diploid human fibroblasts in a mass culture (32–34).

The actual mutation rate of the CA repeat sequence in the NHF1 cells could be as much as three to six times higher than we calculated because: (a) only one-third of possible dinucleotide frameshift reversion mutations are detectable (i.e., revert the neo gene to in-frame), as is the case for any frameshift reversion assay; and (b) the recovery of G418₆ cells from a background of drug-sensitive cells was approximately 50% (see “Results”). Despite this possibility, this rate is still much lower than a maximum rate estimate made in diploid fibroblasts for endogenous microsatellite sequences (<1 × 10⁻⁴ mutations/cell/generation; Ref. 24). If the highest rate we obtained (44.8 × 10⁻⁸ mutations/cell/generation) is multiplied by 6, the rate is still only 2.7 × 10⁻⁶ mutations/cell/generation. NHF1 fibroblasts also have a mutation rate (<2.5 × 10⁻⁷ to 35.8 × 10⁻⁷ mutation/cell/generation⁴ and <2.2 × 10⁻⁵ mutations/cell/generation; Ref. 35). These cells have infinite life spans and altered cell cycle characteristics that may contribute to genetic instability. Thus, their mutation rates may not be directly comparable to those of normal cells.

Our mutation system has been used to calculate the rates of mutation in MMR-proficient (MMR⁺) and -deficient (MMR⁻) cancer cell lines (31). It is interesting to compare the mutation rates of NHF1 cells to the median rates of the MMR⁺ fibrosarcoma line HT1080 (9.8 × 10⁻⁶ mutations/cell/generation) and to MMR⁻ cancer lines H6 (hMLH⁺: 1.6 × 10⁻⁴ mutations/cell/generation) and LoVo (hMSH2⁺: 3.3 × 10⁻³ mutations/cell/generation; Ref. 31). The median rate of NHF1 cells (12.7 × 10⁻⁸ mutations/cell/generation; Table 2) is about 75 times lower than that of the MMR⁺ fibrosarcoma line and approximately 1,200–25,000 times lower than that of the MMR⁻ lines (31). From this comparison, the importance of MMR in the maintenance of repetitive sequences is apparent. However, other factors must also contribute to the stability of repetitive DNA, because the MMR⁺ fibrosarcoma line still exhibits a 75X higher mutation rate than the normal fibroblasts. One possible factor that could contribute to the mutation rate of MMR⁻ cancer cells might be aberrant cell cycle checkpoint control, which may allow DNA replication in the presence of DNA damage, resulting in mutations.

The two G418₆ NHF1 clones that were analyzed by PCR both contained a 4-base addition within the amplified region. Subsequent sequence analysis revealed that the G418₆ clone derived from hyg₆ clone 1 had a 4-base addition in the (CA)₁₇ repeat, and the clone derived from hyg₆ clone 13 had a 2-base addition in the (CA)₁₇ repeat and two 1-base insertions outside the repeat. Frameshift mutations in microsatellite sequences commonly result from additions and deletions of repeat units. Because the target sequence in pRTM2 is in the −1 reading frame, a +4 frameshift mutation would be the smallest insertion of CA repeat units that would revert the target to the normal reading frame. Analyses of frameshift mutations in microsatellites have indicated that mutations involving the smallest numbers of repeat units are the most common (26, 36). Our limited data are consistent with this observation.

In addition to the above-referenced reports that mutations in repetitive DNA tend to involve the smallest numbers of repeat units, the literature indicates that in some prokaryotic and in vitro systems, there is a bias toward deletion frameshifts over insertion (37–39), whereas

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Table 2: Mutation rate analysis of reversion to G418 resistance in NHFs stably transfected with a (CA)₁₇ repeat

<table>
<thead>
<tr>
<th>Hyg₆ NHF1 clone no.</th>
<th>10³ × no. of cells plated</th>
<th>No. of subcultures</th>
<th>No. of G418 clones</th>
<th>Average CFE (%)</th>
<th>10⁻⁸ × mutation rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>3.2</td>
<td>16</td>
<td>0</td>
<td>24</td>
<td>&lt;3.1</td>
</tr>
<tr>
<td>9</td>
<td>1.9</td>
<td>16</td>
<td>0</td>
<td>8</td>
<td>&lt;5.1</td>
</tr>
<tr>
<td>15</td>
<td>3.2</td>
<td>10</td>
<td>1</td>
<td>23</td>
<td>12.7</td>
</tr>
<tr>
<td>1</td>
<td>2.8</td>
<td>16</td>
<td>3</td>
<td>13</td>
<td>30.6</td>
</tr>
<tr>
<td>13</td>
<td>1.8</td>
<td>10</td>
<td>5</td>
<td>32</td>
<td>44.8</td>
</tr>
</tbody>
</table>

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4 R. A. Farber, M. G. Hanford, and R. J. Monnat, Jr., unpublished data.
a yeast-based system shows the opposite bias (40). The smallest frameshift mutations that our system can detect are 2-bp deletions and 4-bp insertions. If there was a bias in favor of insertions in NHFs, then the actual mutation rate might be higher than that we observed. We believe that this outcome is unlikely, because we have recently compared the mutation rates in near-diploid immortalized mouse fibroblasts with constructs that contain the (CA)7 repeat in the −1 or +1 reading frame and have found these rates to be similar to each other.5 It will be interesting to make this comparison in the NHFs.

In conclusion, we have measured the mutation rate of a dinucleotide repeat in NHFs using a selectable system for detection of frameshift mutations. The median mutation rate, when expressed per dinucleotide (12.7 × 10−8 mutations/target/generation divided by 17 dinucleotides/target = 7 × 10−9 mutations/dinucleotide/generation), approaches the estimation made by Loeb and Christians (1) of the overall mutation rate in diploid human cells (1 × 10−10 mutations/nucleotide/generation). The difference between the rates may reflect the stability difference between repetitive and nonrepetitive sequences (the estimation by Loeb and Christians was based on data generated using the HPRT system; Ref. 1). Although an alternate hypothesis of mutation-driven clonal expansion (41) is not ruled out on the basis of our data, the low mutation rate in normal cells supports the mutator hypothesis for the development of cancer (3, 42).

ACKNOWLEDGMENTS

We thank Drs. Stanley M. Gartler (University of Washington, Seattle, WA), William K. Kaufmann (University of North Carolina, Chapel Hill, NC), and Tom Petes (University of North Carolina, Chapel Hill, NC) for helpful comments. We are also grateful to Eric Bronner (Oregon Health Sciences University, Portland, OR) for providing the BASIC computer programs for calculation of mutation rates.

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