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Mutation Rate of a Microsatellite Sequence in Normal Human Fibroblasts[1]

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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 $<3.1 \times 10^{-8}$ to 44.8×10^{-8} 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 MMR[3]-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.

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[3] The abbreviations used are: MMR, mismatch repair; NHF, normal human fibroblast; G418^R, G418-resistant; hyg, hygromycin; hyg^R, hyg-resistant; CFE, colony-forming efficiency; PD, population doubling; neo, neomycin; tk, thymidine kinase; FBS, fetal bovine serum, HPRT, hypoxanthine phosphoribosyltransferase.

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 *Aat*II 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 *hyg*^{R} 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 G418^R.

pRTM2 plasmid DNA (10 μg) linearized with *Hin*dIII was electroporated into 10^7 NHF1 cells, and transformed clones were selected with hyg B (70 μg/ml). The hyg^R clones were screened for CFE, and clones with the highest CFE were used in fluctuation analyses. Subcultures of each hyg^R clone were seeded at 1000 cells/well in a 24-well culture dish. These were expanded to approximately 2×10^6 cells each. Revertants that express the *neo* gene were selected from each subculture by plating cells at a density of 5×10^5 cells/100-mm dish in medium containing 500 μg/ml G418 (Geneticin; 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 hyg^R and G418^R cells for PCR analysis. With one exception, DNA was isolated from either live or frozen G418^R 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 $MgCl_2$, 50 mM KCl, 0.01% (w/v) gelatin, 0.45% (v/v) NP40, and 1 mg/ml proteinase K. DNA was isolated from frozen cell pellets ($\geq 1 \times 10^6$ 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 proteinase 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 G418^R 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 G418^R 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.

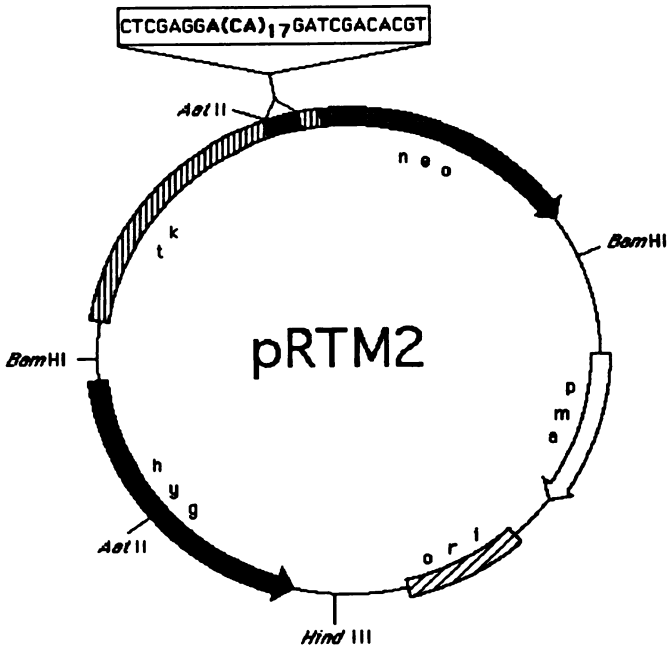

Fig. 1. Diagram of plasmid pRTM2. Bold characters in the box represent the microsatellite sequence. *ori*, origin of replication; *amp*, ampicillin.

DNA Sequencing. DNA sequencing was performed on two G418^R clones using the Thermo Sequenase radiolabeled terminator cycle sequencing kit (Amersham Life Science, Cleveland, OH) according to the manufacturer's instructions for sequencing PCR products. For the G418^R clone that contained multiple copies of the target sequence, we first purified the mutant PCR product from the normal products by PAGE, followed by excision of the band of interest from the dried gel and extraction of the DNA from the gel into 100 μl of water.

RESULTS

We have determined the rate of mutation of a dinucleotide repeat in diploid human fibroblasts (NHF1 cells). The plasmid pRTM2 (Fig. 1), which contains a 17-repeat poly(dC-dA)·poly(dG-dT) sequence [abbreviated $(CA)_{17}$], was introduced into the cells by electroporation, where it became stably integrated into the genome. The CA repeat is located such that it disrupts the reading frame of the downstream portion of the *tk-neo* fusion gene; therefore, no *neo* gene product is produced. Cells transfected with pRTM2 were first selected with hyg. Transfected cells were then used in fluctuation tests to measure frameshift mutation rates in the target CA repeat sequence in G418^R revertants. Because the microsatellite insert puts the *neo* coding region in the -1 reading frame, detectable dinucleotide frameshift mutations include deletions of 20, 14, 8, or 2 bp and insertions of 4 bp. Mutations of these types have been found in other cell types using this system (26, 31).

Determination of the rates of mutation in normal human cells is difficult because of their limited life span in culture. In this assay, we estimate that the cells go through approximately 40 PDs from electroporation through hyg selection and selection of G418^R colonies. Because transfections were carried out in cells at PD = 21, colonies of G418^R NHF1 cells in these experiments are estimated to have gone through a total of 60 PDs.

In early attempts to measure mutation rates in fibroblasts grown in DMEM supplemented with 10% FBS, we did not detect any G418^R mutants. To increase our probability of success in selecting G418^R clones, we supplemented the normal fibroblast growth medium with a 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 clones 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 transfectants 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 $1.8–3.2 \times 10^7$ 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 $<3.1 \times 10^{-8}$ 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 CA repeat units. Clone 13 has an addition of one CA repeat unit and 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

Table 1 *Reconstruction of a G418^R mutant selection using NHF1 cells transfected with the neo in-frame plasmid pRTM1-IF*

No. of NHF1 (pRTM1-IF)[a] cells/dish[b]	Average no. of G418^R colonies/dish	% G418^R colonies recovered
250	8.0	3.2
500	15.5	3.1
1000	21.2	2.1
2000	34.0	1.7

[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. 2*B*). 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.

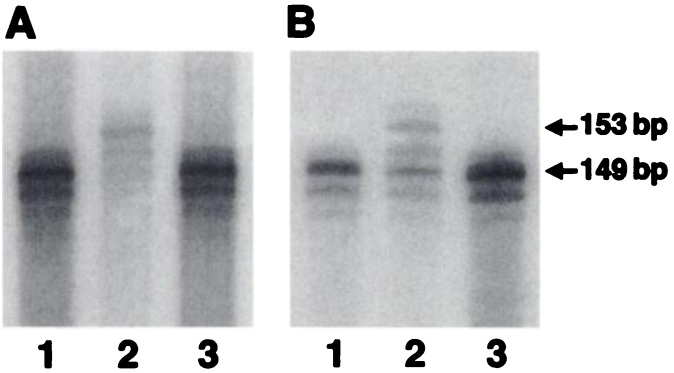

Fig. 2. Size analysis of PCR products of the CA repeats in two G418^R NHF1 clones. The control PCR product is 149 bp in length. *A*, *Lanes 1* and *3*, control PCR products; *Lane 2*, product from a G418^R clone derived from hyg^R clone 13. This product is 153 bp in length. *B*, *Lanes 1* and *3*, control PCR products; *Lane 2*, product from a G418^R clone derived from hyg^R clone 1. This clone contains a PCR product band that is 153 bp in length as well as one that is 149 bp. Intermediate-sized bands result from strand slippage during amplification of the repeat by Taq DNA polymerase (43).

DISCUSSION

The mutation rate in diploid human fibroblasts for a microsatellite sequence composed of a $(CA)_{17}$ repeat was determined to be $<3.1 \times 10^{-8}$ 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 hyg^R 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 G418^R 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 ($<1 \times 10^{-4}$ 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 very low estimated maximal mutation rate ($<4.7 \times 10^{-8}$) at a nonrepetitive target (*HPRT*; Ref. 12).

We and others have estimated the mutation rates for CA microsatellite sequences in SV40-transformed human fibroblasts ($<2.5 \times 10^{-7}$ to 35.8×10^{-7} mutation/cell/generation[4] and $<2.2 \times 10^{-5}$ 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 75× 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 G418^R NHF1 clones that were analyzed by PCR both contained a 4-base addition within the amplified region. Subsequent sequence analysis revealed that the G418^R clone derived from hyg^R clone 1 had a 4-base addition in the $(CA)_{17}$ repeat, and the clone derived from hyg^R 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 intact 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

Table 2 *Mutation rate analysis of reversion to G418 resistance in NHFs stably transfected with a $(CA)_{17}$ repeat*

Hyg^R NHF1 clone no.	10^7 × no. of cells plated	No. of subcultures	No. of G418^R colonies	Average CFE (%)[a]	10^{-8} × mutation rate[b]
5	3.2	16	0	24	<3.1
9	1.9	16	0	8	<5.1
15	3.2	10	1	23	12.7
1	2.8	16	3	13	30.6[c]
13	1.8	10	5	32	44.8

[a] NHF1 PD = 56.

[b] The number of mutations/cell/generation was calculated using the Luria-Delbrück method (28) and the Capizzi and Jameson tables (29) and corrected for CFE.

[c] The hyg^R clone from which these G418^R mutants were derived contains three copies of the target DNA. The rate presented in the table is the actual observed rate/cell. The rate/copy of the target sequence could be 3× lower than that indicated here.

[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 $(CA)_{17}$ 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 $= 7 \times 10^{-9}$ 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).

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