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Hypermutation Rate Normalized by Chronological Time

Clifford L. Wang and Matthias Wabl

The incidence of mutation often coincides with cell division. During the DNA synthesis that precedes cell division, imperfect copying and repair can lead to mutations. For this reason, mutation rates are almost always expressed with units of mutations per cell generation, be it bacteria (1, 2), yeast (3), plants (4), animals (5), or Ig somatic hypermutation (6–13). Activated B cells can mutate their DNA at a rate much higher than the normal, spontaneous rate—a phenomenon called Ig somatic hypermutation. Mutations that occur in the Ig V region can lead to more appropriately expressed mutations per day than per cell generation.

In this study, we manipulated the number of cell generations by varying the rate at which cultures of a mouse cell line were replenished with fresh medium. We found that the frequency of mutants does not necessarily increase with the number of cell generations. On the contrary, a greater number of divisions can lead to a lower frequency of mutants, indicating that cell division is not a rate-limiting step in the hypermutation process. Thus, when comparing mutation rates, we suggest that rates are more appropriately expressed as mutations per day than per cell generation.

Materials and Methods

Vector construction

Con structs were based on the Moloney murine leukemia virus retroviral vector contained in p102.21 (kindly provided by J. B. Lorens, Rigel Pharma, South San Francisco, CA). They contain the internal ribosome entry site (IRES) and puromycin resistance gene of pIRESpuro3 (BD Clontech). Mutations were introduced into enhanced GFP (EGFP) (see Fig. 2A) at the fluorescent chromaphore to create the reporter genes GFP-CT (EGFP C198G, T199C) and GFP-CG (EGFP C201T, G203C, C204T). GFP contains the premature stop codon (TAG) at bases 319–321 and was used previously (27).

Cell culture

Retroviral vectors were packaged using PhoenixEco cells (ATCC SD 3444; American Type Culture Collection). 18-81 cells were infected with the vectors and selected in the presence of 2.5 μg/ml puromycin for 7 days. To start experiments with zero fluorescent mutants, fluorescent cells were removed by FACS. After FACS sorting, cells were grown with 1.5 μg/ml puromycin and fluorescent cells were detected by flow cytometry. All 18-81 cultures were grown in RPMI 1640 medium with 10% FCS. Cells were split at the same time daily by different ratios to control the number of cell generations. The splitting ratios and volumes (milliliters) in terms of culture plus fresh media are: 1/2, 10 + 10; 2/5, 8 + 12; 1/3, 7 + 14; 2/7 + 6; 1/4, 5 + 15; 1/5, 4 + 16.

DNA sequencing

DNA from GFP-positive cells was isolated, and the GFP reporter genes were PCR-amplified using Pfu polymerase (Stratagene). The PCR products were incubated with Taq polymerase to add deoxyadenosine overhangs and then cloned into pCR2.1-TOPO (Invitrogen Life Technologies). The plasmids were then amplified in Escherichia coli and sequenced.

Quantitative RT-PCR

Expression of mouse AID and the GFP reporter constructs were measured using quantitative RT-PCR. RNA was extracted from cells and cDNA produced by standard application of reverse transcription and random hexamer primers. PCR was performed using the ABI PRISM 7700 machine. AID and reporter expression was normalized to the amount of expressed mouse β-actin and hypoxanthine phosphoribosyltransferase (HPRT). PCR primers and probes used were AID: 5′-GAAATTCTCTGCGGCTAAGCC-3′, 5′-TCCGAAGTCATGCAAGTCGTCC-3′, 5′-[6-FAM]-TCGGCGCATCCTTTTGCC
ideal for this study because it is almost completely nonadherent

3

mean fluorescence of GFP-negative labeled GFP

based reporters. A

FIGURE 2. Measuring mutation rates with GFP-based reporters. A. Sequences of reporters GFP-CT, GFP-CG, and GFP* compared with GFP (EGFP of BD Clontech). Fluorescence was restored when the boxed nucleotides were mutated. Base locations are given for the starting and ending bases of the sequences. B. Reporters were expressed using retroviral vectors. Vector components: LTR, long terminal repeat; ps, retroviral packaging signal; GFP rep., GFP reporter gene; Puro®, puromycin resistance gene. C. Flow cytometry of GFP expression. The mean fluorescence of GFP-negative cells was set to 3.0. Cells with fluorescence >100 (bar labeled GFP*) were counted as GFP-positive.

and allowed cultures to be split precisely with reproducible growth conditions each day.

Because the cell line proliferates so rapidly, the cell population was able to reach the same stationary cell concentration each day, even when the cultures were split by different amounts daily. For this study, we found that the number of cell generations per day could be varied by the amount that the culture was split and replenished with fresh medium. For example, a culture split by 1/2 (1 part culture plus 1 part fresh medium), on average, undergoes one cell division by the next day. A culture split by 1/4 averages two cell generations per day. Staining with CFSE, a vital dye that enables tracking of cell division, and monitoring with flow cytometry confirmed that varying the ratio of cell splitting changed the average number of cell generations (Fig. 1). However, because the measurement of hypermutation rates in our experiments requires several weeks and because CFSE could only be detected in our cells during the first 4 days after staining, CFSE tracking could not be used concomitantly with our mutation rate measurement experiments.

The mutation reporter genes (Fig. 2A) were variants of GFP that become fluorescent when mutated. GFP-CT becomes fluorescent when base 199 mutates from C to T; GFP-CG becomes fluorescent when base 203 mutates from C to G; GFP* becomes fluorescent when the premature stop codon reverts to a codon assigned to an amino acid. The GFP reporter genes were expressed in 18-81 cells using a retroviral vector (Fig. 2B) and mutations that restored fluorescence were detected by flow cytometry (Fig. 2C). Sequencing the reporter genes from fluorescent cells verified that fluorescence was not restored by unexpected, proximal mutations. In all cases GFP-CT sequences mutated C to T (10/10) and GFP-CG sequences mutated C to G (9/9). Sequences of GFP* from fluorescent cells all contained mutations at base 321; these mutations were primarily G to C (11/12) but also G to T (1/12).

Accumulation of mutations was monitored continually over 32 days (Fig. 3A). Each measured time point consisted of data from 10⁶ cells. Because many cells could be easily analyzed via flow cytometry, we were able to calculate mutation rates (Fig. 3B) more accurately than by mere DNA sequencing.

We measured mutation frequencies from cultures ranging from 1.0 to 2.3 generations per day. From these mutation frequencies we calculated mutation rates. Under these culture conditions, cultures could be stably and continuously maintained. Cells appeared to be

FIGURE 1. Proliferation of cells determined by CFSE staining. 18-81 cells were spun down, stained with CFSE, washed, and resuspended into the original culture medium (supernatant) at the original cell concentration. After 1 day, the mean CFSE fluorescence per cell was measured by flow cytometry. Cell division led to loss of fluorescence; thus, cells having undergone more generations would have less fluorescence. Because cells split with a higher fraction of fresh media had less fluorescence, we conclude that the different cell splitting fractions led to different amounts of cell generations. We note though, that loss of fluorescence, in part, could also have occurred independent of cell generation (e.g., CFSE leakage).


Results

Mutation rates were measured in 18-81 mouse cells. 18-81 is a cell line of B lineage that expresses endogenous AID and hypermutates its Ig V region (11). Like activated B cells, 18-81 divides quickly (as low as a 5 h doubling time) and also undergoes Ig class switching, generally a change from μ H chain to γ2b. 18-81 was also ideal for this study because it is almost completely nonadherent
healthy and viable staining showed <1% dead cells. To our surprise, we found that the number of mutations did not correlate positively with the number of cell generations. This was true of C to T, C to G, and the TAG reversion mutations. In all cases, maximal mutation rates were observed when cells produced 1.3 generations per day and decreased as the number of cell generations per day was increased. We found that at one generation per day, the mutation frequency was low. Though the mutation rate of GFP* was approximately twice that of GFP-CT and GFP-CG, any similarities or differences in rates could be due to differences in sequence or position of the mutation site within the GFP reporter gene. Nonetheless, it was surprising that the mutation rates were approximately the same over the range of cell growth conditions. Because C to T mutations presumably require only AID activity and DNA synthesis, and C to G mutations likely require AID plus additional downstream factors and DNA synthesis, this could indicate that AID is a rate-limiting factor while downstream factors (uracil-DNA-glycosylase, mismatch repair, error-prone polymerases) are not.

In our experiments we varied the media replenishing (i.e., splitting) ratio of the cultures, thereby controlling the number of cell generations per day. Clearly, more cell generations did not necessitate more mutations. When we measured the expression levels of AID (Fig. 4A) and the GFP reporter (Fig. 4B), we found that expression varied over our range of culture splitting conditions. AID expression was optimal when cultures were split by 2/5 and 1/3 (1.6 and 1.8 generations per day, respectively); reporter expression was highest when cultures were split by 1/2 and 1/3 (1.0 and 1.3 generations per day, respectively) and decreased if cells proliferated more.

**Discussion**

In addition to AID, hypermutation is thought to require transcription of the template DNA (6, 17, 28, 29). If levels of AID and reporter transcription both correlate positively with the mutation rate, and are independent of each other, then the product of both factors might also reflect the mutation rate. That is, rate \( \frac{\text{AID}}{\text{GFP}} \). When the AID and reporter expression levels from our experiments are multiplied (Fig. 4C), the resulting curve does reflect the changes in mutation rate that occurred with varying the number of generations per day (Fig. 3B). By the terms of this model, in the cultures where cells divide on average only 1.3 times per day, high reporter transcription plus moderate levels of AID can result in a high mutation rate. Furthermore, high mutation rates can occur when there is high AID expression but less reporter transcription. The correlation of the mutation rate with AID and reporter expression suggests that AID and transcription are primary limiting factors in hypermutation. Although cell division is likely an integral downstream step in the mutation process, the fact that the correlation occurs independently of cell division (i.e., does not require cell generation number as a dependent variable) suggests that even if AID and GFP were held constant, varying the number of generations (under the conditions tested here) would not greatly affect

![Figure 3](https://example.com/figure3.png)

**FIGURE 3.** Rate of hypermutation at different rates of cell generation. _A_, Mutations causing reporters to become GFP-positive were monitored continuously. Reporters tracked accumulation of specific mutations; GFP-CT (C→T), GFP-CG (C→G), and stop codon reversion, GFP*/ (GFP rev.). The rate of cell generations (gen./day) was determined by the daily cell splitting ratio. _B_, Mutation rates as a function of cell generation rate. C→T (□), C→G (●), stop codon reversion (▲). If mutation rate increased proportionally with that of cell generation, the mutation rate would have the slope of the dashed line.
levels.

However, the protocol also causes a role in our experimental system here.

of Ig genes in B cells (31) and such factors may or may not play suggested that other limiting factors exist specifically for mutation export (31, 32) and phosphorylation of AID (33) have recently affect the rate of hypermutation. For example, nuclear import and pledge to cell division in these cells.”

Our results do not imply that there cannot be other factors that affect the rate of hypermutation. For example, nuclear import and export (31, 32) and phosphorylation of AID (33) have recently been suggested to affect the rate of hypermutation. It has been suggested that other limiting factors exist specifically for mutation of Ig genes in B cells (31) and such factors may or may not play a role in our experimental system here.

The only variable that we sought to manipulate was the number of cell generations per day. However, the protocol also causes variation in the freshness of the growth medium (a highly diluted culture has more fresh medium), and time spent at cell growth saturation, which might influence phenotypic expression. The more diluted cultures spend less time at saturation before the GFP is measured. Could a variation in “phenotypic lag”, i.e., a delay in gene expression, have influenced our results? A delay between the time when the mutation occurs and the time when a sufficient amount of GFP can be detected by flow cytometry could skew rate measurements over a period of time that is comparable in length to the phenotypic lag. However, our measurements of mutation frequencies were taken over a month, a period of time that is likely much longer than any lag that might occur between the incidence of mutation and expression of GFP fluorescence. Therefore, irrespective of any short lags in expression, the cultures will have reached a steady state, and the measured rates will not be affected by a phenotypic lag.

We found that fewer generations, and thus more time spent at saturation, gave higher frequencies of mutations. Accumulation of metabolites in the growth medium and time spent at saturation might be also mutagenic. Furthermore, variation of cell concentration or nutrients could affect hypermutation in ways unrelated to replication. But unless there are factors of a higher magnitude of importance than AID or DNA transcription, this would not invalidate our proposition that cell division is not a rate-limiting step for hypermutation.

In the study of mutations, whether Ig hypermutation or not, mutation rates are nearly always expressed on a per cell generation basis. This is because in most cases, mutations are introduced during DNA replication. However, in our study, we find that hypermutation is likely an important exception—cell division is not rate limiting. It follows then that rates should rather be reported on a per time (e.g., mutations/base pair/day) basis.

We have shown that increased cell generations not only do not necessitate increased mutation, but also that high cell proliferation can lead to decreased AID levels and mutation rates. Although our results were obtained using a cell line and may not accurately reflect an in vivo system, in the animal, it might make sense to decrease the mutation rate when cells are dividing quickly; once B cells with good Ab affinity are selected to undergo increased clonal proliferation, reducing AID levels could prevent further mutagenesis at the Ig loci or other locales throughout the genome. Although it has already been recognized that AID and transcription are important factors for hypermutation, we demonstrated here that AID activity and transcription alone are largely sufficient to explain the incidence of mutation. These activities, unlike cell division, are likely the dominant rate-limiting steps.

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Disclosures

The authors have no financial conflict of interest.

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