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Increased Transcription Levels Induce Higher Mutation Rates in a Hypermutating Cell Line

Jürgen Bachl,† Chris Carlson,* Vanessa Gray-Schopfer,* Mark Dessing,* and Carina Olsson*

Somatic hypermutation, in addition to V(D)J recombination, is the other major mechanism that generates the vast diversity of the Ab repertoire. Point mutations are introduced in the variable region of the Ig genes at a million-fold higher rate than in the rest of the genome. We have used a green fluorescent protein (GFP)-based reversion assay to determine the role of transcription in the mutation mechanism of the hypermutating cell line 18-81. A GFP transgene containing a premature stop codon is transcribed from the inducible tet-on operon. Using the inducible promoter enables us to study the mutability of the GFP transgene at different transcription levels. By analyzing stable transfectants of a hypermutating cell line with flow cytometry, the mutation rate at the premature stop codon can be measured by the appearance of GFP-positive revertant cells. Here we show that the mutation rate of the GFP transgene correlates with its transcription level. Increased transcription levels of the GFP transgene caused an increased point mutation rate at the premature stop codon. Treating a hypermutating transfection clone with trichostatin A, a specific inhibitor of histone deacetylase, caused an additional 2-fold increase in the mutation rate. Finally, using Northern blot analysis we show that the activation-induced cytidine deaminase, an essential trans-factor for the in vivo hypermutation mechanism, is transcribed in the hypermutating cell line 18-81. The Journal of Immunology, 2001, 166: 5051–5057.

Materials and Methods

Plasmid construction

For the plasmid pI, the AseI-SalI fragment of plasmid pEGFP-N1 (Clontech, Palo Alto, CA) was replaced by the XhoI-SalI fragment from plasmid pHHC 13-3 (22) containing the tet operon. To generate the plasmid pI-Enh, the XbaI fragment from plasmid phyp#2 (23) containing the Ig heavy chain large intron enhancer (ELi) was blunt-ended and cloned into the I fragment from plasmid phyp#2 (23) containing the Ig heavy chain large intron enhancer (ELi) was blunt-ended and cloned into the AflI site of plasmid pI. The premature stop codon was introduced at aa position 107 of the EGFP gene by oligonucleotide-directed in vitro mutagenesis using the oligo 5′-GGA CGA CCGCAA CTA TAC CCG CGC CGA GG-3′. To verify expression levels, all constructs were generated with and without the premature stop codon. Constructs pI and pI-Enh were linearized before transfection with Alw44I and then cotransfected with

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Article references and specific details are not included in this text but are part of the full document.
plasmid pRT-TA (20) by electroporation in a Bio-Rad electroporator (Hercules, CA). The copy number of the stably integrated plasmids ranged from one to four copies.

Cell cultures
The hypermutagenic active pre-B cell line 18-81 was cultured in RPMI medium containing 10% FCS, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 IU/ml penicillin-streptomycin, and 50 mM 2-ME.

Flow cytometry
Before expansion the cell cultures were purged of pre-existing revertant cells by sorting on a MoFlo high speed sorter (Cytomation, Fort Collins, CO) using the sort mode: single one drop. PKH-26 (Sigma, St. Louis, MO) staining was performed according to the recommendation of the manufacturer. On day 0, 3–5 million nonfluorescent cells were seeded into 100 ml of fresh medium. The volume of the culture was slowly expanded up to 500 ml over the course of 4 days. Each day (every 24 h) a sample was removed, and the numbers of fluorescent and nonfluorescent cells among a million viable cells were analyzed by flow cytometry. The mutation rate was determined by applying the curve fit function from program CA-Cricket Graph III 5.2 (Computer Associates International, Islandia, NY). For phenotypic analysis, cells were washed once in PBS and resuspended with PBS and 1% BSA. Flow cytometric analysis was performed on a FACS-Calibur (BD Sciences, Mountain View, CA).

Preparation of mRNA samples and Northern blot analysis
Northern blot analysis was performed essentially as previously described (24). mRNA was extracted from expanding cultures with Oligotex Direct mRNA Midi Kit (Qiagen, Chatsworth, CA). For each Northern blot, 1 μg of mRNA was loaded per lane. The probes used were fragments from the GFP gene, the GAPDH gene and the activation-induced cytidine deaminase (AID) gene. The probes were radiolabeled using a random primer labeling kit (Roche). The PCR primers for the GFP gene were: sense, 5'-GTG CCC ATC CTG GTC GAG CTG GAC-3'; antisense, 5'-GGCGAGGGCGATGAC-3'. PCR amplification was performed with Taq polymerase using the buffer supplied by the manufacturer (Roche). The PCR primers for the GFP gene were: sense, 5'-GTG CCC ATC CTG GTC GAG CTG GAC-3'; and antisense, 5'-GGCGAGGGCGATGAC-3'. The purified PCR product of 617-nt length was directly sequenced with the GFP nested primer (5'-GGCGAGGGCGATGAC-3') using the dyeodeoxy chain termination method with T7 polymerase (Amersham Pharmacia Biotech, Arlington Heights, IL.).

RT-PCR and nucleotide sequence analysis
Total RNA was extracted from sorted revertant cells with TRizol (Life Technologies, Gaithersburg, MD), and cDNA was prepared by RT with Superscript II reverse transcriptase (Life Technologies) using a oligo(dT)11 primer. PCR amplification was performed with Taq polymerase using the buffer supplied by the manufacturer (Roche). The PCR primers for the GFP gene were: sense, 5'-GTG CCC ATC CTG GTC GAG CTG GAC-3'; and antisense, 5'-GGCGAGGGCGATGAC-3'. The purified PCR product of 617-nt length was directly sequenced with the GFP nested primer (5'-GGCGAGGGCGATGAC-3') using the dyeodeoxy chain termination method with T7 polymerase (Amersham Pharmacia Biotech).

Results
GFP-expressing revertant cells are analyzed by flow cytometry
To determine the influence of transcription levels on the mutability of a reporter gene, we generated construct pl (Fig. 1A) and stably transfected it into cell line 18-81. In construct pl the GFP gene is driven by the inducible tet-on promoter, Ptet. Transcription at Ptet is induced by supplementing Dox to the growth medium and can be modulated by altering the Dox concentration. The GFP gene contains a premature TAG stop codon and is therefore nonfunctional. In stably transfected clones revertant cells express the functional GFP. Flow cytometric analysis enables the enumeration of revertant cells and the measurement of GFP expression levels within individual revertant cells, shown as the relative fluorescence intensity (RFI). The RFI value of revertant cells can be used as an approximate and indirect measurement for the transcription level of the GFP transgene (17).

To determine the reversion frequency of the GFP transgene, a stable transfection clone is initially purged of pre-existing revertant cells by FACS. GFP-negative sorted cells (3-5 × 10^6) were seeded into growth medium at different concentrations of Dox and expanded for 4 days. The appearance of revertant cells was monitored by daily removal of 1–2 × 10^6 viable cells from the expanding cultures and subsequent flow cytometric analysis. To measure the actual reversion rate, the number of cell divisions during the experimental period has to be assessed. Therefore, before the purging of pre-existing revertant cells, the transfection clones were stained with the red fluorescent cell linker, PKH-26 (Sigma). With every cell division the cells lose half their PKH-26 intensity. Thus, the mean PKH-26 intensity can be used as a direct measure of the number of cell divisions in the expanding cultures. The assessment of revertant cells and PKH-26 intensity can be performed simultaneously by flow cytometry at different detection channels.

Mutation rate is coupled to transcription level
In a screen of roughly 100 pl transfection clones, three independent transfection clones were identified that were Dox inducible, had a very low basal transcription level in the absence of Dox, and showed a significant number of revertant cells. Fig. 2 shows the 5-day flow analysis for one of the transfection clones, pl#1, at different Dox concentrations. Transfection clone p#1 harbors three copies of the transgene, as determined by Southern blot analysis (data not shown). In Fig. 3 the flow cytometry data of Fig. 2 are plotted graphically as a function of reversion frequency vs cell divisions. Very few revertant cells were detected in the absence of Dox (culture A). This is not surprising, because transcription of the transgene is required to express GFP in revertant cells. With increasing Dox concentrations, higher numbers of revertant cells were generated. The mutation rate in culture B is 0.8 × 10^-5 mutations/base pair/generation, in culture C it is 2.1 × 10^-5 mutations/base pair/generation, and in culture D it is 5.0 × 10^-5 mutations/base pair/generation. The mean GFP expression level of the revertant cells (shown as RFI values) increased with higher Dox concentrations. The RFI values were 650 for culture B, 1023 for culture C, and 1681 for culture D. The increase in transcription levels reached a threshold at a Dox concentration of 0.8 μg/ml, beyond which no further effect was seen. To verify the correlation between RFI values and the transcription level of the GFP transgene, Northern blot analysis was performed on the steady state mRNA levels of the GFP transgene in cultures A, B, C, and D (Fig. 4). The RFI values of the revertant cells corresponded to the mRNA level of the GFP transgene and can be used as an indirect measurement of the transcription activity of the GFP transgene.

A potential mutagenic effect of Dox is not responsible for the increased mutation rate
The higher mutation rates at increased Dox concentrations could be due to a potential mutagenic effect of Dox. To address this possibility, a constitutively transcribed GFP transgene, driven by the thymidine kinase promoter and the large intron enhancer (18), was stably transfected into cell line 18-81. The reversion frequency

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**FIGURE 1.** GFP reporter constructs to monitor the mutator activity. A, Construct pl contains the GFP gene with a premature TAG stop codon. The GFP gene is driven by the inducible tet-on promoter (Ptet). B, In construct pl-Enh the heavy chain large intron enhancer is present 3′ of the GFP gene.
of three independent transfection clones was then analyzed in the presence or the absence of Dox. The transfection clones displayed no significant differences in their mutation frequencies when expanded at 1.0 μg/ml Dox or in the absence of Dox. The RFI values of the revertant cells were also unaffected by the addition of Dox (data not shown).

**Mutation rate responds directly to changes in transcription level**

It is also possible that the decreased mutation rate at lower transcription levels (cultures B and C) is due to undetected, low GFP-expressing revertants, which are hidden in the GFP-negative cell population. These putative, GFP<sup>low</sup> revertants should become detectable by raising the Dox concentration and thus increasing the GFP expression level. To address this possibility, culture B was split on day 2. Half of the culture continued to expand at 0.1 μg/ml Dox (culture B), while the Dox concentration for the other half of the culture was raised to 1.0 μg/ml (culture E). Similarly, the Dox concentration for half of culture C was raised to 1.0 μg/ml Dox (culture F). If the same number of revertants had accumulated over the first 2 days in cultures B, C, and D, then a similar frequency of revertants would be expected on day 3 in cultures D, E, and F upon full transcription induction. Alternatively, if cultures B and C generated fewer revertants than culture D during the first 2 days, a lower frequency of revertants would be expected upon full transcription induction.

Raising the Dox concentration to 1.0 μg/ml caused a rapid increase in the mutation rate in cultures E and F to the level of culture D. The mutation rates (4.0 × 10<sup>-5</sup> mutations/base pair/generation for culture E and 4.7 × 10<sup>-5</sup> mutations/base pair/generation for culture F) were comparable to that for culture D (5.0 × 10<sup>-5</sup> mutations/base pair/generation). The RFI values for revertant cells also reached comparable levels in culture D (1681 RFI), E (1619 RFI), and F (1769 RFI), indicating the enhanced transcriptional activity in cultures E and F after raising the Dox concentration. Thus, alterations of the transgenic transcription level caused rapid changes in the mutation rate at the premature stop codon. However, the absolute number of revertants remained lower in both cultures E and F than in culture D. This confirms that at lower transcription levels of the GFP transgene, fewer revertant cells were generated.

**FIGURE 2.** Flow cytometric analysis of GFP-positive cells and PKH-26 intensity for pI transfection clone 1 at various Dox concentrations. GFP-negative cells were expanded at Dox concentrations of 0, 0.1, 0.2, and 1.0 μg/ml for 4 days. To determine the reversion frequency, 10<sup>6</sup> viable cells were daily removed from the expanding cultures and analyzed. The mean number of cell divisions in the culture was assessed by PKH-26 staining. On day 0 the GFP-negative cells were stained with PKH-26. The PKH-26 intensity in the daughter cells is only 50% of the PKH-26 intensity of the parental cell (after every cell division). Representatively, the PKH-26 data for the 1.0 μg/ml Dox culture are shown. GFP-positive cells and PKH-26 staining were assessed simultaneously at different detection channels. x-axis, Fluorescence intensity on log scale; y-axis, cell count.

**FIGURE 3.** Increase in the frequency of GFP-positive cells with higher Dox concentrations. The flow cytometry data of Fig. 2 are plotted as a function of GFP-expressing revertant cells per 10<sup>6</sup> cells vs number of cell divisions. Cultures A, B, C, and D were expanded at Dox concentrations of 0, 0.1, 0.2, and 1.0 μg/ml, respectively. On day 2 culture B was split into cultures B and E, and culture C was split into cultures C and F. Cultures B and C were further expanded at the original Dox concentration. For cultures E and F the Dox concentration was raised to 1.0 μg/ml. RFI, mean RFI of the GFP-expressing cells. The mutation rate in cultures E and F refers exclusively to a 2-day period at 1.0 μg/ml Dox. The mutation rate and the RFI value in culture A were below the detection limit (n.d.).
TSA treatment boosts the mutation rate

TSA treatment of cells can increase the transcription level of endogenous genes and transgenes (25–28). TSA blocks histone deacetylases and causes nonspecific opening of the chromatin structure by hyperacetylation of histones (29, 30). To determine the effect of TSA on the mutability of the GFP transgene, cells from culture D were further expanded at different TSA concentrations (17 and 34 nM). Culture D was consequently split on day 2 into three equal cultures (D, G, and H), all of which were maintained at 1.0 μg/ml Dox. Culture G was supplemented with 17 nM TSA, culture H was supplemented with 34 nM TSA, and culture D contained no TSA. Addition of TSA caused several dose-dependent effects on the cells (Fig. 5). First, the generation time was prolonged for cultures G and H. Second, the RFI values for revertant cells increased to 1941 for culture G and 2917 for culture H, indicating higher transcription levels of the GFP transgene compared with 1681 in culture D. Northern blot analysis confirmed increased steady state mRNA levels of the GFP transgene in the TSA-treated cultures (data not shown). Third, the frequency of revertant cells was higher in the TSA-treated cultures than in culture D. The prolongation of the generation time and the higher revertant frequency both translate into increased mutation rates for culture G (9.4 × 10⁻⁵ mutations/base pair/generation) and culture H (1.4 × 10⁻⁵ mutations/base pair/generation). Therefore, TSA treatment caused a 2-fold increase in the mutation rate.

Hypermutation in the presence of the ELi

The presence of the Ig enhancers is required for an efficient targeting of the Ig mutator system (12, 23). In particular, the ELi appears to play an essential role in the hypermutation mechanism, although by itself the ELi is not sufficient to confer maximum levels of hypermutation (31). Construct pi-Enh (Fig. 1B) was generated and stably transfected into 18-81 to address whether targeting of the inducible GFP transgene can be improved. Construct pi-Enh is identical with construct pi, except that it includes the ELi downstream of the GFP gene. In contrast to construct pi, 80% of the pi-Enh transfection clones had a significant number of revertant cells (data not shown). Thus, the presence of the large intron enhancer greatly increases targeting of the GFP transgene by the 18-81 mutator system. The pi-Enh transfection clones showed an elevated level of background transcription for the GFP transgene even in the absence of Dox. The ELi presumably activates the Ptet promoter independently of Dox. Yet, addition of 1.0 μg/ml Dox increased the transcription level 2- to 10-fold for most of the pi-Enh transfection clones. Fig. 6 shows the reversion analysis for four independent pi-Enh-transfected clones grown in either the presence or the absence of Dox. The mutation rates for all four pi-Enh transfection clones ranged from 2.5 to 4.6 × 10⁻⁶ mutations/base pair/generation without Dox and from 1.6 to 2.2 × 10⁻⁵ mutations/base pair/generation at 1.0 μg/ml Dox. Thus, the mutability of the GFP transgene increased when 1.0 μg/ml Dox was added to the medium. The RFI levels of the four pi-Enh clones ranged from 251 to 311 without Dox and from 1237 to 2033 at 1.0 μg/ml Dox, and increased mutation rates again coincided with higher RFI values and thus consequently with higher transcription levels. Yet, the transfectant clone with the highest transcription level (pi-Enh#2) did not display the highest mutation rate. The differences in the mutation rate among the four transfection clones

FIGURE 5. Increased mutability caused by treatment with TSA. On day 2 culture D (1.0 μg/ml Dox) was split into three cultures: D, G, and H. All three cultures were further expanded at 1.0 μg/ml Dox. TSA was added to a final concentration of 17 nM in culture G and to 34 nM in culture H.
were also not related to the copy number of the transgene. Transfection clones 1 and 3 contained one copy of the transgene each, transfection clone 2 contained three copies, and transfection clone 4 contained four copies (Southern blot analysis not shown). This indicates that other parameters besides the transcription levels and the copy number of the transgene may influence the mutability of the GFP transgene (32).

**AID is transcribed in the hypermutating cell line 18-81**

A recent study by Muramatsu et al. (33) implicated a crucial role of AID in both somatic hypermutation and Ig class switching. Somatic hypermutation was drastically reduced in AID gene knockout mice, indicating that the expression of the AID gene is essential for the hypermutation mechanism (33, 34). To investigate the expression of the AID gene in the hypermutating cell line 18-81, Northern blot analysis of total RNA was performed (Fig. 7). Two AID transcripts, 1.9 and 1.3 kb in length, were detectable in the hypermutating cell line 18-81. In contrast, various control cell lines (28-C-9, 702Z/3, and Raw 8.1) that failed to hypermutate the GFP transgene were devoid of detectable AID transcripts. Both AID transcripts in the cell line 18-81 were shorter than the predicted length of the murine AID transcript at 2.3 kb and will require further characterization. Yet, the presence of AID transcripts in the hypermutating cell line 18-81 compared with nonhypermutating cell lines supports the assumption that its mutator mechanism is indeed closely related to the in vivo somatic hypermutation process.

**Point mutations in the TAG stop codon caused the reversion**

The genetic event that caused the reversion of the GFP transgene was determined for eight pl-Enh revertants (obtained from eight independent pl-Enh transfection clones) and three pl revertants (obtained from three independent pl transfection clones). All 11 revertants were caused by point mutation in the TAG stop codon. Nine revertants had a G to C transition, and two revertants had a G to T transversion. The strong GC bias is characteristic of mutation hot spots in the Ig genes (35–38). Due to the limited pool of our sequencing data (~4 kb), obtained by sequencing 11 revertant cells, only one additional point mutation, a G to T transversion, was detected. This additional point mutation also occurred in an Ig hot spot motif, yet on the opposite strand (data not shown). This indicates that the mutator system of cell line 18-81 may recognize Ig hot spots on both DNA strands.

**Discussion**

The mutator system of the cell line 18-81 shares key characteristics with the somatic hypermutation process of Ig genes in mouse and human. 1) Predominantly, point mutations are introduced (19, 39–41). 2) GC base pairs, which are embedded in the RGYW motif (generally the underlined base is initiated), can become mutational hot spots (37, 42). 3) The promoter is exchangeable for targeting by the mutator system, yet the distance from the promoter influences the mutability of a transgene (43). 4) The targetability of a transgene is affected by the presence of Ig enhancers (43). In addition, we show here that the AID, an essential trans-acting factor for the somatic hypermutation process (33, 34), is transcribed in the cell line 18-81. Transcription of the AID gene has also been shown in the constitutively hypermutating human Burkitt lymphoma cell line Ramos (34), indicating the requirement of AID expression for somatic hypermutation-active cell lines. Despite these similarities the mutator mechanism of cell line 18-81 exhibits two differences. First, the mutator system of cell line 18-81 almost exclusively mutates GC base pairs, whereas in mammalian Ig loci, AT and GC base pairs are mutated equally (38, 44). Second, the mutation rate in 18-81 appears to be 2 orders of magnitude lower than the expected mutation rate in vivo (2, 23). Both exceptions are shared by all Ig-hypermutating cell lines described to date (such as Ramos, BL-2, and CL-01) and are reminiscent of the Ig hypermutation mechanism in Xenopus and nurse shark (45, 46). Therefore, the Ig hypermutating cell lines, including cell line 18-81 might display the basic Ig-mutator system of shark and Xenopus, which is missing certain functions of the further evolved murine and human mutator systems.

We have adapted our previously published GFP-based reversion assay (17) to investigate the influence of transcription levels on the mutator mechanism of the cell line 18-81. The inducible tet-on promoter enables us to modulate the transcription levels in individual transfection clones and directly measure the mutation rate of the GFP reporter gene.

The importance of transcription for the Ig hypermutation mechanism has been demonstrated in vivo by several groups (8, 9, 15, 47, 48). In particular, Fukita et al. (15) demonstrated a direct correlation between the pre-mRNA transcription levels and the mutation frequency at the Ig heavy chain locus. Our studies, using the inducible GFP reversion assay, provide direct evidence that this correlation also exists in the hypermutating cell line 18-81. Increasing the transcription level of the GFP transgene by modulating the Dox concentration resulted in higher mutation rates at a premature stop codon. The mutator of cell line 18-81 is not an all-or-nothing system, because intermediate transcription levels at 0.1 and 0.2 μg/ml Dox resulted in a corresponding gradual increase in mutation rates. The mutator system responds rapidly to changes in the transgenic transcription level. The model proposed by Peters and Storb (9) accounts for such a correlation between transcription level and mutator activity. This model predicts that a specific mutator factor binds to the initiating RNA polymerase complex. Binding of this putative mutator factor is thought to cause premature pausing of the elongating RNA polymerase. Stalling of the RNA polymerase acts as a signal to attract the nucleotide excision repair (NER) system (49–51). The NER system introduces a single-strand gap in the DNA adjacent to the stalled RNA polymerase. A putative, error-prone DNA polymerase, which is part of the B cell-specific NER system, would then introduce point mutations in the repaired region during the fill-in reaction (52, 53).

However, the hypermutation rate in NER knockout mice is not decreased (54, 55), suggesting that the mechanism of somatic hypermutation may be more complex than predicted by that model. Alternatively, the transcription machinery may not directly interact with the mutator factor(s). Instead, transcription could be required to alter the chromatin structure and thus provide increased accessibility to mutator factor binding sites. For V(D)J recombination in T lymphocytes, a linkage of histone H3 acetylation status at a transgenic V(D)J recombination reporter gene and its accessibility for V(D)J recombination machinery were recently demonstrated (56).

TSA, an inhibitor of histone deacetylases, has been shown to change the chromatin structure by hyperacetylation of histones (57, 58). Adding TSA to the fully induced pl transfection clone caused a further dose-dependent increase in the mutation rate. TSA treatment also increased the transcription level beyond that of the fully Dox-induced (i.e., 1.0 μg/ml) GFP transgene. This elevation of the GFP transcription level would be the most straightforward explanation for the hypermutation-boosting effect of TSA. Alternatively, treating the cells with TSA could up-regulate the expression of the mutator factor(s) in the cell line 18-81, because the effect of TSA is nonspecific and therefore not restricted to the GFP transgenic locus (59).

Does TSA treatment influence the mutability of the GFP transgene in the absence of Dox-induced transcription? Our attempts to
answer this question have been unsuccessful to date, because we found that TSA treatment before Dox induction abolishes the inducibility of the GFP transgene by Dox. We are presently determining whether we can overcome this problem.

The ELI plays probably a dual role for the somatic hypermutation mechanism: it contains a putative cis-element(s) to target the mutator system, and it provides a transcriptional activation site(s) for the heavy chain gene. The presence of the ELI in plasmid p-Enh increased the targeting of the GFP transgene by the mutator system of the cell line 18-81. The search for the cis-element(s) has been hindered particularly by the lack of a suitable model system. Deletions within the ELI (including the matrix attachment region) regularly resulted in a marked reduction of both the mutability and the transcription levels (13). Yet, to elucidate the putative cis-element(s) in the ELI it will be necessary to ensure that manipulations of the enhancer do not also affect the transcription level of the reporter gene. The inducible GFP system described here provides transcription independently of the enhancer and may therefore be a useful tool to search for the mutator targeting element(s).

Depending on its integration site, a transgene lacking the ELI can nevertheless become hypermutable, as seen for the rare hypermutable pl transcription clones. The GFP gene may have integrated into one of the Ig loci or, alternatively, the mutator mechanism is not exclusively restricted to the Ig loci. In vivo data indicated that the Bcl-6 gene in humans, but not that in mice, can be hypermutated (60–63). It remains to be shown whether the Bcl-6 locus shares mutator targeting elements with the Ig loci or whether the mutations in the Bcl-6 gene are somewhat the “cortical damage” of the mutator system.

In summary, the inducible GFP reversion assay is a powerful tool to study the influence of transcription levels on mutability. This system is easily applicable and may prove useful to test other cell lines. Our results using TSA show that the complex interaction of several parameters, such as generation time, expression level, and mutability, can be assessed simultaneously. In addition, the GFP-based system can be exploited for a systematic screening of drugs, to find inhibitors and stimulators of the mutator system.

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