Probing Immunoglobulin Gene Hypermutation with Microsatellites Suggests a Nonreplicative Short Patch DNA Synthesis Process

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Summary
As the rate of Ig gene hypermutation approximates the level of nucleotide discrimination of DNA polymerases (10^{-3} to 10^{-4}), a local inhibition of proofreading and mismatch repair during semiconservative replication could generate the mutations introduced by the process. To address this question, we have constructed transgenic mice that carry a hypermutation substrate containing a “polymerase slippage trap”: an Ig gene with a mono or dinucleotide tract inserted in its V region. The low amount of slippage events as compared to the number of mutations, the absence of transient misalignment mutations at the border of the repeats, and the dissociation between the amount of frameshifts and mutations when the transgene is put on mismatch repair-deficient genetic backgrounds, suggest that Ig gene hypermutation occurs by an error-prone short patch DNA synthesis taking place outside global DNA replication.

Introduction
Hypermutation of Ig genes was described almost 30 years ago as a mechanism resulting in the introduction of random mutations in rearranged V genes in B cells undergoing an immune response. This process results in the “maturation” of the response. B cells expressing higher affinity receptors specific for the immunizing antigen being preferentially selected (Weigert et al., 1970). These events have been thoroughly studied at the cellular and molecular levels. V gene hypermutation occurs in centroblasts that divide at an exceptionally high rate (6-8 hr) in the dark zone of germinal centers during a T-dependent immune response (reviewed by MacLennan and Gray, 1986). The mutant cells selected by the antibody-antigen complexes present at the surface of follicular dendritic cells thereafter differentiate in Ig memory and/or plasma cells (reviewed by Rajewsky, 1996). At the molecular level, most of the recent advances have been obtained by using an Ig transgene functioning as a hypermutation substrate (O’Brien et al., 1987). These experiments have highlighted the role of the enhancer and the nuclear matrix attachment (MAR) regions to target the process, whereas, surprisingly, the specific promoter and the V region have appeared dispensable (Betz et al., 1994; Yélamos et al., 1995). It has also been shown that the process is targeted to a region of approximately 1 kb within and flanking the V region, that it is initiated at the promoter region, that it favors transitions over transversions, and that it occurs at a rate of about 10^{-3} to 10^{-4} per bp per cell generation (McKean et al., 1984; Lebecque and Gearhart, 1990; reviewed by Neuberger and Milstein, 1995; Storb 1996).

Several molecular models that do not involve semiconservative DNA replication have been proposed to explain this unique process of adaptive mutation targeted to a single locus (reviewed by Reynaud et al., 1996; Wabl and Steinberg, 1996). The original model posited that a single- or double-strand break in the V region was followed by gap formation and error-prone repair (Brenner and Milstein, 1966). It was also suggested that a reverse transcriptase specific to the V region could be responsible for the process because of its low level of fidelity (Steele and Pollard, 1987).

More recently it has been suggested that hypermutation could be linked to transcription. The experimental basis for this proposition came from two different transgenic experiments: the demonstration, on one hand, that hypermutation could be reinitiated on the constant region of an Ig transgene by inserting a promoter upstream of this region (Peters and Storb, 1996) and that the amount of mutation was proportional to the transcriptional activity of the Ig transgene (Goyenechea et al., 1997). Both proposed mechanisms incorporated the assumption that an induced stalling of RNA polymerase in the presence of a putative mutator factor generates gaps that can be repaired in an error-prone fashion.

During DNA replication, misincorporations, deletions, and insertions of bases are at first corrected by the proofreading moiety of DNA polymerases and then by the mismatch repair complex. Estimates for polymerase fidelity, obtained mainly in bacteria and yeast (reviewed by Kunkel, 1992), suggest that a local inhibition of proofreading and mismatch repair targeted to the V gene during replication would give approximately the rate of mutations observed during Ig affinity maturation (10^{-3} to 10^{-4} per bp per cycle). DNA polymerases slip when they copy simple repetitive sequences made of tandem repeats of one or a small number of bases that are scattered as microsatellites in the genome of eukaryotes (Streisinger et al., 1966). The slippage that can result in addition or deletion of repeat units is corrected for small repeat numbers (< 8) by the proofreading activity of DNA polymerases, but most of the alterations occurring on longer runs are repaired by the postreplicative mismatch repair process (Tran et al., 1997), as shown by instability of microsatellites occurring in yeast and mammalian cells in which mismatch repair is inactive (Fishel et al., 1993; Leach et al., 1993; Strand et al., 1993). Such microsatellites can therefore be used as a diagnostic of mismatch repair deficiency because of their high level of instability.

To address the question of whether a local repression
of postreplicative mismatch repair and/or polymerase proofreading during semiconservative replication generates Ig hypermutation, we designed an Ig transgene containing a mono or a dinucleotide run inserted in the variable region and analyzed framenesshifts and nucleotide substitutions in the transgene in normal mice or in mice carrying a null mutation of one of the components of the mismatch repair complex: MSH2 (de Wind et al., 1995) or PMS2 (Baker et al., 1995). The results show that the incidence of slippage events occurring on the V region repeat during hypermutation is strikingly low in normal animals when compared to the frequency of base substitutions. In the mismatch null background, the rate of base substitutions is reduced, while the amount of framenesshifts is similar to the control animals. The low frequency of repeat alterations, the absence of transient misalignment mutations, and the dissociation between the rate of base substitutions and framenesshifts in the mismatch repair-deficient background, support the existence of an Ig mutagenic polymerase that proceeds by short patch error-prone DNA synthesis outside global DNA replication.

Results

Ig Transgenes Containing a Polymerase Slippage Trap

To study the role of postreplicative mismatch repair and polymerase proofreading during Ig hypermutation, we have constructed two transgenes based on the mouse rearranged V\textsubscript{K}Ox1-J\textsubscript{K}5 light chain construct (L\textsubscript{K} transgene, provided by M. Neuberger), previously described as a target for hypermutation (Sharpe et al., 1991).

In the L\textsubscript{K}(TG\textsubscript{1})\textsubscript{11} transgene, a dinucleotide run of eleven TG was inserted in the KpnI restriction site at the border of the CDR1 and FR2 of the V region and in the HpaI site at the beginning of the C region (Figure 1). The L\textsubscript{K}(T\textsubscript{1})\textsubscript{18} transgene was made by inserting a mononucleotide run constituted of 18 T in the same KpnI restriction site of the V region (Figure 1). The insertion of the mono and direpeat tracts causes a shift in the reading frame, creating a stop codon at the beginning of the CDR2 in both constructs, thus rendering both transgenes nonfunctional.

Several transgenic lines analyzed showed a variable incidence of mutation of the transgenes in Peyer's patch PNA\textsuperscript{high} B cells. One line, showing the highest mutation rate, was selected for each construct for further study. They both contained two copies of either the L\textsubscript{K}(TG\textsubscript{1})\textsubscript{11} or L\textsubscript{K}(T\textsubscript{1})\textsubscript{18} transgene.

Mutational Events in the L\textsubscript{K}(TG\textsubscript{1})\textsubscript{11} and L\textsubscript{K}(T\textsubscript{1})\textsubscript{18} Transgenes

Peyer's patch PNA\textsuperscript{high} B cells undergoing chronic antigen stimulation were isolated at 6–7 months (Gonzalez-Fernandez and Milstein, 1993), and sequences of the V region were analyzed after PCR amplification of 577 bp between the leader and the J\textsubscript{K}5 segments of the light chain transgene. L\textsubscript{K}(TG\textsubscript{1})\textsubscript{11} Transgene

PNA\textsuperscript{high} B cells from seven mice were collected for transgene analysis. Sixty-six percent of sequences were mutated, the average mutation frequency being 4.6 mutations per V sequence (307 bp) (Table 1). As previously described for the normal L\textsubscript{K} transgene (Betz et al., 1993), there is a bias for transitions (62%) over transversions (38%) (data not shown). Three deletion events of 11, 17, and 29 bp, respectively, were found in the V region. The distribution of mutations reveals the presence of several hot spots, for example Ser-77, an intrinsic hot spot of the V\textsubscript{K}Ox1 sequence. Two major hot spots are found at the border of the dinucleotide run, which match the consensus RGYW (Rogozin and Kolchanov, 1992) (G being mutated) on the sense DNA strand (third base of Trp-35), or the antisense DNA strand (third base of Tyr-36 after the poly(TG) tract) (Figure 2). These hot spots are in fact contained in the natural KpnI site that was doubled at each side of the (TG\textsubscript{1})\textsubscript{11} tract after its insertion. The Trp-35(III) hot spot is not prominent in normal functional transgenes, in part because its mutation frequently creates a stop codon at this position. There is probably also some structural enhancement of mutation targeting to these sites due to the (TG\textsubscript{1})\textsubscript{11} insertion. Curiously, the intrinsic Ser-31 hot spot was not observed.

L\textsubscript{K}(T\textsubscript{1})\textsubscript{18} Transgene

In the mouse analyzed, 67% of sequences were mutated, with an average of 2.4 mutations per V sequence

Table 1. Somatic Mutations in V Region of L\textsubscript{K}(TG\textsubscript{1})\textsubscript{11} and L\textsubscript{K}(T\textsubscript{1})\textsubscript{18} Transgenes from PNA\textsuperscript{high} Peyer's Patch B Cells

<table>
<thead>
<tr>
<th>Transgene</th>
<th>Number of Sequences</th>
<th>Total Number of Mutations</th>
<th>Mutated Sequence (%)</th>
<th>Mutation per Sequence</th>
<th>Mutation per Mutated Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>L\textsubscript{K}(TG\textsubscript{1})\textsubscript{11} 7 months</td>
<td>115</td>
<td>527</td>
<td>66</td>
<td>4.6</td>
<td>7.2</td>
</tr>
<tr>
<td>L\textsubscript{K}(TG\textsubscript{1})\textsubscript{11} 2 months</td>
<td>53</td>
<td>213</td>
<td>86</td>
<td>4.0</td>
<td>4.6</td>
</tr>
<tr>
<td>L\textsubscript{K}(T\textsubscript{1})\textsubscript{18} Msh2\textsuperscript{2/2} 2 months</td>
<td>45</td>
<td>57</td>
<td>55</td>
<td>1.2</td>
<td>2.3</td>
</tr>
<tr>
<td>L\textsubscript{K}(T\textsubscript{1})\textsubscript{18} Pms2\textsuperscript{2/2} 2 months</td>
<td>45</td>
<td>46</td>
<td>35</td>
<td>1.0</td>
<td>2.9</td>
</tr>
<tr>
<td>L\textsubscript{K}(T\textsubscript{1})\textsubscript{18} 7 months</td>
<td>88</td>
<td>210</td>
<td>67</td>
<td>2.4</td>
<td>3.6</td>
</tr>
</tbody>
</table>
Error-Prone Short Patch DNA Synthesis in Hypermutation

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The influence of sequence modifications on the mutation pattern of VκOx transgenes has also been noted by Goyenechea and Milstein (1996). Mutations occur at a much lower frequency within the (T)\textsubscript{18} than in the (TG)\textsubscript{11} repeat, even with the correction of their respective mutation rates (Figure 3).

A Low Frequency of Frameshifts Is Observed in the V Region of L\textsubscript{k}(TG)\textsubscript{11} and L\textsubscript{k}(T)\textsubscript{18} Transgenes

The occurrence of frameshifts within the (TG)\textsubscript{11} or the (T)\textsubscript{18} run cannot be assessed by direct sequencing: the PCR itself introduces frequent frameshifts, even with the high fidelity Pfu polymerase, due to the inherent instability of such templates at the high elongation temperature used in the PCR technique (Hite et al., 1996). The PCR background level of sequence alteration, estimated by sequencing unmutated transgenes from Pepper's patch PNA\textsuperscript{high} B cells, was around 20% for the L\textsubscript{k}(TG)\textsubscript{11} construct. For L\textsubscript{k}(T)\textsubscript{18}, the background level was as high as 60% (cf. Figure 3), correlating with the lower thermal stability of a run of 18 T.

Frameshifts in mono and dinucleotide tracts were thus estimated by single-cell PCR, followed by direct analysis on sequencing gels, as classically performed to diagnose alterations at natural microsatellite loci. Single Pepper's patch B cells from two 7-month-old L\textsubscript{k}(TG)\textsubscript{11} mice were obtained in 96-well plates by automated single-cell deposition coupled to fluorescence-activated cell sorting. 135 and 205 bp around the (TG)\textsubscript{11} tract in V and C regions, respectively, were amplified from single PNA\textsuperscript{high} and PNA\textsuperscript{low} cells and the product revealed after gel migration by hybridization with a (TG)\textsubscript{29} probe. In V regions, frameshifts were observed in 15 out of 122 PNA\textsuperscript{high} B cells (Figure 4; Table 2), giving a 12% frequency of slippage events. Among these, eight represent a one-repeat contraction, six a one-repeat expansion, and one a three-repeat expansion. No instability above the level of the experimental background was observed for the C region in PNA\textsuperscript{high} B cells (Table 2) or the V region in PNA\textsuperscript{low} B cells (data not shown). Thus, the frameshift/mutation transgenic line selected. Here again, the presence of the (TG)\textsubscript{11} run instead of (TG)\textsubscript{11} seems to modify the targeting of similar to that observed with the dinucleotide transgene, with an average of one frameshift for 73 mutations.

For the L\textsubscript{k}(T)\textsubscript{18} transgenic, four frameshifts were observed in the V region of 61 PNA\textsuperscript{high} B cells from a 7-month-old animal (two +1 and −1 events). No slippage events were observed among 67 single PNA\textsuperscript{high} B cells (data not shown). Thus, the frameshift/mutation ratio for the transgene containing a monorepeat is very similar to that observed with the dinucleotide transgene, with an average of one frameshift for 73 mutations.

Analysis of the L\textsubscript{k}(TG)\textsubscript{11} Transgene on Mismatch Repair-Deficient Backgrounds at 2 Months

V sequences were analyzed at 2 months from three normal L\textsubscript{k}(TG)\textsubscript{11} transgenic mice, one L\textsubscript{k}(TG)\textsubscript{11}/Msh2\textsuperscript{−/−}...
Figure 3. Mutations of the Poly (TG)\textsubscript{11} and (T)\textsubscript{18} Tracts Inserted in V\textsubscript{k}Ox1 Gene

(A) The 55 sequences harboring base substitutions within the (TG)\textsubscript{11} repeat in the Lk(TG)\textsubscript{11} transgene from 6- to 7-month-old mice were compiled from a sample of 115 sequences. 

(B) The 19 sequences harboring base substitutions within the (T)\textsubscript{18} repeat in the Lk(TG)\textsubscript{18} transgene from 7-month-old mice were compiled from a sample of 88 sequences. Deletions are indicated by an asterisk, insertions by arrows. The consensus RGYW hot spots are overlined. See comments in the text about the PCR origin of deletions and insertions in mono and dinucleotide sequences. No transient misalignment mutations are observed on either side of the poly (TG)\textsubscript{11} and (T)\textsubscript{18} tracts.

mouse, and one Lk(TG)\textsubscript{11}/Pms2\textsuperscript{2/2} mouse. Eighty-six percent of sequences were mutated, with an average of 4.0 mutations per sequence in normal transgenic mice (Table 1). Remarkably, this mutation level is only slightly lower than at 6-7 months, suggesting that mutations start accumulating at this particular transgene insertion site at earlier stages than previously described for other transgenic lines (Gonzalez-Fernandez et al., 1994) and also reach a mutation plateau earlier. The difference between the two stages resides, as reported by these authors, in the distribution of mutations. There are no sequences bearing more than 12 mutations at 2 months, whereas 10% of V sequences at 6-7 months harbor between 13-21 mutations.

The frequency of mutations was 3- to 4-fold lower in mismatch repair-deficient mice carrying the Lk(TG)\textsubscript{11} transgene, with 1.2 and 1.0 mutations per V sequence for Msh2\textsuperscript{2/2} and Pms2\textsuperscript{2/2}, respectively (Table 1). The proportion of unmutated sequences was increased (45% and 65%, respectively), but sequences carrying as many as nine mutations were observed in both knockout animals. Five deletions and insertions were found in the Msh2\textsuperscript{2/2} mice (two deletions of 1 bp and one of 6 bp) and Pms2\textsuperscript{2/2} mice (two insertions of 1 and 5 bp) for a total of 103 mutations. In contrast, sequences from normal animals at 2 months showed two deletions (1 and 12 bp) per 213 mutations, thus a 5-fold lower ratio.

The analysis of nucleotide substitutions in mismatch repair knockout animals revealed a marked increase in transitions as compared to normal animals (Table 3). Moreover, in the Msh2\textsuperscript{2/2} mouse, only G to A and C to T transitions were observed. This bias appears to result from the preferential targeting of a few consensus hot spot motifs in the Msh2\textsuperscript{2/2} mouse, each of which display this G to A or C to T transition pattern (Figure 2). A similar targeting of mutations to G and C nucleotides in Msh2\textsuperscript{2/2} mice was reported by Phung et al. (1998) although the quantitative level of mutations was normal after immunization of these mice (cf. Discussion). Although transitions were also increased in the Pms2\textsuperscript{2/2} background, such targeting was not observed. A similar targeting of a few hot spots was described in J H4 flanking sequences obtained from Peyer's patch PNA\textsuperscript{high} B
Figure 4. Instability of the Dinucleotide (TG)11 Repeat in the VvOx1 Region in PNAhigh B Cells

The instability of (TG)11 dinucleotide tract was analyzed in single Peyer’s patch PNAhigh B cells from 6- to 7-month-old Lk(TG)11 transgenic mice by amplification of a 135 bp DNA fragment and hybridization with a (TG)11 probe after electrophoresis and transfer. Arrowheads indicate a detectable length alteration in the repeat run.

Table 2. Percent of Nucleotide Substitutions in VvOx1 Gene from Peyer’s Patch PNAhigh B Cells of 2-Month-Old Lk(TG)11 Normal and Mismatch Repair−Deficient Mice

<table>
<thead>
<tr>
<th></th>
<th>G</th>
<th>A</th>
<th>T</th>
<th>C</th>
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<tbody>
<tr>
<td>From</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>19.7</td>
<td>5.6</td>
<td>3.2</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>15</td>
<td>13.6</td>
<td>4.8</td>
<td></td>
</tr>
<tr>
<td>T</td>
<td>4.2</td>
<td>3.8</td>
<td>5.6</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>4.2</td>
<td>1.9</td>
<td>18.3</td>
<td></td>
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</table>

Transitions

<table>
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<tr>
<th></th>
<th>G/A</th>
<th>T/C</th>
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<tbody>
<tr>
<td>G</td>
<td>87.6-80.5</td>
<td>45.6-41.4</td>
</tr>
<tr>
<td>T</td>
<td>23.9</td>
<td>42.0-39.1</td>
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Transversions

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<th>12.4-19.5</th>
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No Transient Misalignment Mutations in Lk(TG)11 or Lk(T)18 Transgenes

Intronic JH4-JH5 sequences from human peripheral memory B cells have been determined in an independent study (Levy et al., submitted). Such intronic sequences contain frequent short homopolymeric stretches, in particular a run of six G, located 110 bp downstream of JH4. Deletions, insertions, and mutations are observed in this run; however, the most frequent event corresponds to another category of mutations, described as “transient misalignment mutagenesis” (Kunkel and Soni [1988], cf. Discussion). This corresponds to the addition of one or two G residues, always at the 5’ side of the (G)6 repeat, replacing the normal adjacent base(s) and thus not creating an insertion (Figure 5). This sort of mutation occurs six times in a random sample of 27 sequences. The same type of event occurs on other repeats in the human intron sequence on both the 5’ and 3’ sides but also on the primer or template strand. In some rare repeats, there is a total absence of slippage events.

No instance corresponding to a transient misalignment mutation (e.g., a replacement of the sequence immediately flanking the repeat by TG or T nucleotides) was observed among 346 transgene V sequences (Figure 3). On the contrary, these transient misalignment mutations were also found on natural small repeats present in the leader intronic sequence of the transgene.
we have constructed mice carrying an Ig transgene with microsatellites outside of Ig genes remain proofreading and mismatch repair during replication, of postreplicative mismatch repair. A mono or dinucleotide run inserted in its V region and stable in Peyer's patch PNA high B cells undergoing the hypermutation process. This was performed for the D6Mit59 and the D7Mit91 loci by single molecule DNA dilution. An instability of mutations drops (3- to 4-fold) in Peyer's patch B cells. The analysis of Cascalho et al. (1998), such transient misalignment mutations observed in this study suggest that the putative error-prone polymerase enters DNA polymerases slip when they copy microsatellites made of mono or dinucleotide repeats that are present throughout the genome. It has been shown that these polymerase slippage events are in most cases repaired for longer nucleotide runs (>8) by the mismatch repair complex, the 3'-5' exonuclease moiety of DNA polymerases being mainly capable of correcting the immediately produced insertions or deletions adjacent to the extended 3' terminus of DNA. To determine whether Ig hypermutation was accompanied by a local repression of proofreading and mismatch repair during replication, we have constructed mice carrying an Ig transgene with a mono or dinucleotide run inserted in its V region and followed the correlation between the level of mutations and slippages occurring in different genetic backgrounds. No Microsatellite Instability in Peyer’s Patch PNA<sup>high</sup> B Cells

To address the question of whether a general inhibition of mismatch repair could occur during the hypermutation process, instability at two dinucleotide microsatellite loci was measured in Peyer’s patch PNA<sup>high</sup> B cells from two L<sub>k</sub>(TG)<sub>11</sub> transgenic mice at 6–7 months of age. This was performed for the D6Mit59 and the D7Mit91 loci by single molecule DNA dilution. An instability of 1.6% (2/125) for D6Mit59 (Figure 6) and 1.7% (1/57) for D7Mit91 (data not shown) was observed, and similar values were obtained for PNA<sup>low</sup> B cells. Peyer’s patch PNA<sup>high</sup> B cells from nontransgenic mice gave the same results (data not shown).

Discussion

DNA polymerases slip when they copy microsatellites made of mono or dinucleotide repeats that are present throughout the genome. It has been shown that these polymerase slippage events are in most cases repaired for longer nucleotide runs (>8) by the mismatch repair complex, the 3'-5' exonuclease moiety of DNA polymerases being mainly capable of correcting the immediately produced insertions or deletions adjacent to the extended 3' terminus of DNA. To determine whether Ig hypermutation was accompanied by a local repression of proofreading and mismatch repair during replication, we have constructed mice carrying an Ig transgene with a mono or dinucleotide run inserted in its V region and followed the correlation between the level of mutations and slippages occurring in different genetic backgrounds. Normal versus knockout for the mismatch repair part-region. Thus, hypermutation is not accompanied by a general deficit of mismatch repair activity.

We analyzed two lines of transgenic mice carrying an Ig light chain transgene with a repetitive tract of 11 TG inserted in the V and C regions (L<sub>k</sub>(TG)<sub>11</sub> transgene) or 18 T in the V region only (L<sub>k</sub>(T)<sub>18</sub> transgene). The analysis of chronically stimulated Peyer’s patch B cells from these animals at 6–7 months showed that the transgene can target mutations with an average of 4.6 mutations per L<sub>k</sub>(TG)<sub>11</sub> V sequence and 2.4 per L<sub>k</sub>(T)<sub>18</sub> V sequence. Surprisingly, the level of frameshifts was very low for both transgenes with approximately one frameshift every 70 to 80 base substitutions. These frameshifts were mainly insertion or deletion of one repeat occurring in the same proportion with occasionally a larger event involving several repeat units.

DNA polymerases introduce deletions and insertions when they copy repeated sequences, but they also introduce specific base substitutions at the border of such runs, if they lack proofreading activity. These events occur, as proposed by Kunkel and Soni (1988), by displacement of a repeat unit during dissociation of the DNA strands, as in the generation of frameshifts, but this time followed by immediate realignment after copying. This process replaces sequences at the border of the run by an additional repeat, if taking place on the newly synthesized strand. Intrinsic sequences around immunoglobulin genes contain many natural short homopolymers. During the course of a study on mutated intronic Ig sequences from human memory B cells (Levy et al., submitted), such transient misalignment mutations were frequently found on these repeats. Moreover, these mutations were also found on short repeats in the leader intronic sequence of the transgene. These slippage events, which are therefore part of the hypermutation process, were surprisingly never observed on either side of the (TG)<sub>11</sub> or (T)<sub>18</sub> repeat on transgene V sequences.

Mutation hot spots with defined consensus motifs have been described in Ig mutated sequences (Rogozin and Kolchanov, 1992; Betz et al., 1993). Two such hot spots have been created in the V transgene on both sides of the mono and dinucleotide run by its insertion into the KpnI restriction site at the border of the CDR1 and FR2 regions. The relatively low number of frameshifts compared to mutations and the absence of transient misalignment mutations observed in this study suggest that the putative error-prone polymerase enters DNA polymerases slip when they copy microsatellites in or next to these hot spots and only copies very short segments of DNA. While the (TG)<sub>11</sub> run carries some mutations, it may not provide good entry sites for the mutator, implying that the mutagenic DNA polymerase does not cross the border between the repeat and the V sequence. Hence, the size of the patches filled by the mutagenic polymerase may not exceed the length of the repeat (18–22 bp) (Figure 7). One important question that remains is whether this short patch mutagenic event occurs prior or during replication and what the role is of postreplicative mismatch repair.

The natural microsatellites outside of Ig genes remain stable in Peyer’s patch PNA<sup>high</sup> B cells undergoing the hypermutation process. This was also observed to be the case for the dinucleotide repeat inserted in the C<sub>k</sub> region. Thus, hypermutation is not accompanied by a general deficit of mismatch repair activity.

When the L<sub>k</sub>(TG)<sub>11</sub> transgene is put on a null mutation genetic background for PMS2 or MSH2, the frequency of mutations drops (3- to 4-fold) in Peyer’s patch B cells. Despite the fact that these results are similar to the one of Cascalho et al. (1998), interpretations are different. To account for the reduced frequency of mutations observed in memory B cells of Pms2<sup>−/−</sup> mice, these authors postulated an active participation of postreplicative mismatch repair in the hypermutation process. In their proposition, mismatch repair would increase the frequency
of mutations introduced in the V gene during replication by directing their repair to the “wrong” parental strand. Their model, which is based on a study of chronically stimulated B cells by environmental antigens, does not account for the normal mutation rate observed by several groups when these mismatch repair-deficient mice are immunized with a defined hapten (Frey et al., 1998; Phung et al., 1998; Winter et al., 1998). We have proposed elsewhere that mutations cannot accumulate in chronically stimulated B cells of mismatch repair-deficient mice because these fast dividing cells are most probably not able to recycle into germinal centers as a consequence of the extent of DNA alterations they suffer, while in contrast B cells undergoing a short response after immunization are relatively unaffected (Frey et al., 1998).

Strikingly, the drop in base substitutions in the mismatch repair-deficient background, in particular in Msh2−/− mice as also shown by others for endogenous Ig genes (Frey et al., 1998; Jackson et al., 1998; Phung et al., 1998; Winter et al., 1998). Specifically, an increase in G to A and C to T transition mutations by a nicking enzyme (Brenner and Milstein, 1966) or polymerase pausing (Peters and Storb, 1996; Goyenechea et al., 1997) cannot be ascertained at this stage. The role of mismatch repair deficiency beyond postreplicative repair is not well understood (Mellon et al., 1996), and whether it could interfere with single mutations, slippage events, or larger insertions/deletions introduced during the process (Wu and Kaartinen, 1995; Goossens et al., 1998; Wilson et al., 1998) remains a clear possibility (reviewed by Kim and Storb, 1998; Frey et al., 1998).

Which event recruits the mutagenic DNA polymerase, single- or double-strand breaks in the region, (Brenner and Milstein, 1966) or the induced stalling of RNA polymerase II during transcription (Peters and Storb, 1996; Goyenechea et al., 1997) cannot be ascertained at this stage. What the error-prone polymerase could be also remains speculative. However, considering the characteristics of the process: short patch synthesis, low processivity, low fidelity, transient misalignment mutations, and large deletions/insertions, it is tempting to speculate that this enzyme may be evolutionary related to DNA polymerase β (Kunkel, 1985).

**Experimental Procedures**

**Transgenes and Transgenic Mice**

The Lx transgene (given by M. Neuberger) contains the rearranged VxOx1-I-Jκ variable region, the intronic η enhancer, the rat κ enhancer, and 8 kb of the 3′ region with the 3′ enhancer (Sharpe et al., 1991). Two transgenes were constructed: Lx(TG)11, with a dinucleotide repeat inserted in the V and C region, and Lx(TG)18, with a mononucleotide tract inserted only in the V region. The two primers containing the poly TG and T tracts were designed for insertion at the KpnI site at the border of CR1 and FR2 in the V region: (TG)₃TGAC and (TG)₁₁.
(AC)ₖAGTAC for the former, (T₉)₂GTAC and (A₅)GTAC for the latter. After annealing, they were introduced by cohesive end ligation after partial KpnI digestion of the Lx transgene plasmid. For introducing the dierepeat tract in the Cx region, the primers used were (TGG)₄ and (AC)₆. The heteroduplex obtained after annealing was introduced by blunt end ligation into the unique Hpal restriction site in the rat Cx region. The number of repeat units obtained in the final construct was higher than in the primer used, i.e., (TGG)₄ and (AC)₆. This is most probably due to slippage events during primer annealing and/or bacterial transformation. Transgenic mice were obtained by injecting vector-free DNA EcoRI fragment into mouse zygotes (C57BL/6 × DBA/2, F2 eggs). Founder animals were identified by PCR analysis of the VxOx1 gene (VoxOx BACK and LxFOR oligonucleotides; Betz et al., 1993) in tail DNA samples. The transgene copy number was estimated by Southern blot. Founders were bred with DBA/2 animals. The Lx(TG)₉ transgenic mice were bred for two generations with Msh²/² mice (strain 129, obtained from H. te Riele) and Prms²/² or Prms²/² mice (strain B6, obtained from R. M. Liskay) to get homozygous mismatch repair–deficient transgenic animals.

Isolation of Peyer’s Patch B Cells and V Gene Sequence Analysis
After dissection of Peyer’s patches from small intestine of 2- to 12-month-old animals, single-cell suspensions were double-stained with phycoerythrin-labeled CD45R/B220 antibody (Pharmingen) and fluorescence-labeled peanut agglutinin (PNA, Vector). B220⁺ PNA⁻ and B220⁺ PNA⁺ were fractionated by flow cytometry on a FACS Vantage apparatus (Becton-Dickinson). Genomic DNA was prepared according to Reynaud et al. (1992). The oligonucleotide primers for VxOx1-j₅ gene amplification were as follows: LxOx5'-CAAGATCTGGTCTCTGCTGAACT-3' (the EcoRI site is underlined) and j₅-5'-CATCTCAGATGTTCCACGTCAC-3' (the XbaI site is underlined). DNA was amplified from 5 × 10⁶ cell aliquots by the high-fidelity Pfu polymerase (Stratagene) for 35 cycles, 45 sec at 94°C, 1.5 min at 60°C, and 2 min at 72°C. Gel-purified PCR products were cloned by PCR Script Amp SK(+) cloning kit (Stratagene). Sequences were done using IRDiochrome Terminator Cycle Sequencing kit (ABI PRISM) and analyzed by ABI PRISM 310 Genetic Analyzer.

Sorting of Single Cells
Cells were sorted using the FACS Vantage with an automatic cell deposition unit (Becton-Dickinson). Single cells were directly sorted into 96-well PCR plates (Perkin-Elmer) containing 26 μl of PCR buffer 1X (Perkin-Elmer) and 1 μl of proteinase K (10 μg/ml; Boehringer Mannheim). The samples were digested for 30 min at 56°C followed by 10 min at 95°C. Plates were stored at −20°C until use.

Microsatellite Analysis
A 135 bp DNA fragment including the mono and dinucleotide repeat in the transgene V region was amplified from single cells. The oligonucleotide primer pairs were Vw5'-CATCTTGTGGGCCAGGTGTCC-3' and Vw5'-5'CATTCTTGGGAGGTGGTC-3'. PCR was performed in 30 μl volume with 15 pmol of each primer and 1 U of Taq polymerase (Perkin-Elmer). The DNA was amplified for 40 cycles with 1 min at 94°C, 1 min at the annealing temperature, and 1 min at 72°C. For the first cycle, the annealing temperature was 72°C, and it was decreased by 2°C in each of the next five cycles. The remaining cycles were annealed for 1 min at 62°C. Amplification of 205 bp in the Cx region and the poly(TG)₉ tract was used performing 15 pmol of the primers Cx5'-GCTGCTAGATGCTGACCACTACGTTACCTG-3' and Ck15'-CGGAATTCATCATGGAGGTGCTGACCACTACGTTACCTG-3' for 45 sec at 94°C, 1 min at 55°C, and 1 min at 72°C (added XbaI and EcoRI restriction sites are underlined).

The PCR products were fractionated on a 7% or 5% polyacrylamide denaturing sequencing gel for VxOx and Cx, respectively. The DNA was visualized by autoradiography after blotting onto Hybond N+ membrane (Amersham) and hybridization with a 32P-labeled DNA probe constituted by 29 GT repeats or 46 T repeats. The D6Mit59 and D7Mit91 loci were amplified by PCR using MapPars primers from Research Genetics. PCR conditions were as described by Baker et al. (1995) with 40 cycles. Amplification was performed on single molecule dilution from 6- to 7-month-old Lx(TG) mice. Transgenic and normal mice were analyzed similarly.

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