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Indirect and direct evidence for DNA double-strand breaks in hypermutating immunoglobulin genes

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The generation of a diverse antigen receptor repertoire is fundamental for the functionality of the adaptive immune system. While the V(D)J recombination process that generates the primary antigen receptor repertoire is understood in great detail, it is still unclear by which mechanism immunoglobulin (Ig) genes are further diversified by somatic hypermutation. Using mouse strains that carry a non-functional, predefined $V_HD_HJ_H$ gene segment in their IgH locus we demonstrate DNA double-strand breaks (DSBs) in and around $V_HD_HJ_H$ in B cells undergoing somatic hypermutation. The generation of these DSBs depends on transcriptional activity, and their distribution along the $V_HD_HJ_H$ segment parallels that of point mutations in the hypermutation domain. Furthermore, similar to hot spots of somatic hypermutation, 50–60% of all DSBs occur preferentially at RGYW motifs. DSBs may transiently dissociate the Ig promoter from the intronic enhancer to block further transcription and to initiate an error-prone non-homologous DSB repair pathway. In accord with this model large deletions are frequently produced, along with point mutations, in a $V_HD_HJ_H$ segment inserted together with its promoter into the IgH locus in inverted orientation. Our data suggest that DSBs are reaction intermediates of the mechanism underlying somatic hypermutation.

Keywords: deletion ends; germinal centre; knock-in mice; ligation-mediated polymerase chain reaction; non-homologous DNA double-strand break repair; RGYW motif

1. INTRODUCTION

Somatic hypermutation allows rearranged immunoglobulin (Ig) genes to be further diversified. Mutations in hypermutated Ig genes are confined to the hypermutation domain, a region that spans about 1.5 kb downstream of the Ig promoter. The frequency of mutations increases from 5′ to 3′ along the leader exon, peaks over the rearranged V(D)J exon and decreases into the J-C intron of Ig genes. The mutation rate has been estimated to be 10^{-3} to 10^{-4} base pairs per generation, i.e. six orders of magnitude higher than that of spontaneous mutations (Allen *et al.* 1987; Berek *et al.* 1991; McKean *et al.* 1984). The majority of mutations introduced are point mutations but deletions and duplications also occur (Goossens *et al.* 1998). Somatic hypermutation is not a random process but exhibits a characteristic nucleotide substitution preference. The RGYW motif (A/G, G, C/T, A/T), and preferentially AGC and AGT based RGYWs (both serine codons) have been identified as hot spots of somatic hypermutation (Betz *et al.* 1993; Rogozin & Kolchanov 1992). In contrast to the mechanism generating the primary Ig repertoire, which is understood in quite some detail, the molecular mechanism underlying somatic hypermutation of Ig genes remains to be solved.

In search for *cis*-elements controlling the mutability of rearranged Igκ transgenes, the promoter, the enhancers, but not the rearranged $V_\kappa J_\kappa$ segment itself, have been identified as critical (Betz *et al.* 1994; Yelamos *et al.* 1995). The V gene promoter can be exchanged by other RNA polymerase II-dependent promoters without affecting hypermutation (Betz *et al.* 1994; Tumas-Brundage & Manser 1997). Placing an additional Igκ promoter immediately 5′ of the constant portion of an Igκ transgene leads to the occurrence of somatic hypermutation within 2 kb downstream of each promoter but not between these two areas (Peters & Storb 1996).

As in conventional transgenic mice usually several copies of a transgene integrate randomly into the genome, it is difficult to correlate transcription and somatic hypermutation in such animals (Lacy *et al.* 1983). We therefore generated three mouse strains, each of which carried a single copy of a mutant $V_HD_HJ_H$ gene segment, derived from the V186.2 V_H gene and designated V_HB1-8, in its physiological position in the IgH locus. The transcriptional activities of the three targeted genes are determined by different promoters: either the original promoter of the V186.2 gene (pII); a truncated DQ52 promoter (pΔ) (figure 1*a*,*b*); or a ribosomal RNA polymerase I dependent promoter (pI) (Fukita *et al.* 1998;

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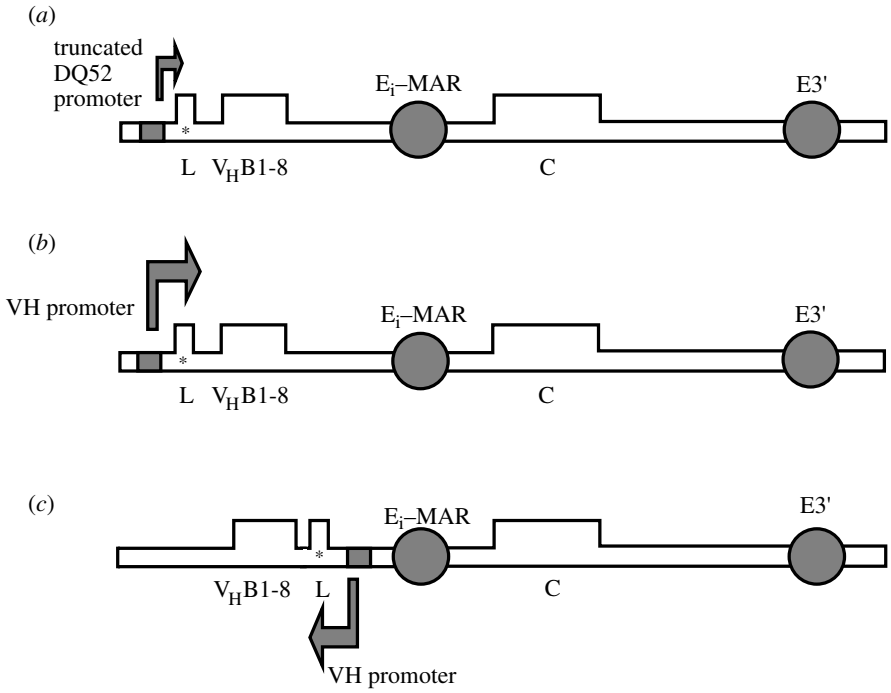

Figure 1. Schematic representation of the promoter–V_HB1-8 transcription unit and the IgH enhancers in pΔ, pII and pII^{inv} 'knock-in' mice. (*a*) B cells of pΔ mice express the targeted V_HB1-8 element under the control of a truncated DQ52 promoter (Jacobs *et al*. 1999). (*b*,*c*) B cells of pII and pII^{inv} 'knock-in' mice express the targeted V_HB1-8 element under the control of its regular IgH promoter. While in pII the IgH promoter–V_HB1-8 transcription unit is in its normal configuration (Fukita *et al*. 1998), in pII^{inv} this unit is inverted, bringing the promoter adjacent to the intronic IgH enhancers (Bross *et al*. 2000). The asterisk in the leader intron represents an introduced stop codon, which renders all 'knock-ins' non-functional mutation reporters.

Jacobs *et al*. 1999). A premature termination codon in the leader intron of the targeted V_HBl-8 renders all inserted transgenes non-productive (figure 1*a–c*). The transcribed regions of the targeted V_HBl-8 are identical in pI, pII and pΔ mice, excluding the possibility of differential secondary structures in the template strand, a prerequisite for a comparison between transcription and somatic hypermutation. In these mice the frequency of hypermutation in the targeted V_HBl-8 gene correlated well with transcription, suggesting a role for transcription and/or a requirement for a single-stranded DNA substrate (in the form of a transcriptional 'bubble') in the hypermutation process.

Based on the finding that in human B cells deletions and duplications frequently accompany the introduction of point mutations, DNA strand breaks have been implicated in the mechanism of somatic hypermutation (Goossens *et al*. 1998). In addition, nucleotide insertions into the V but not C region have been revealed in a hypermutation-competent B-cell tumour line expressing a transfected terminal deoxynucleotidyl transferase (TdT) gene, suggesting the presence of either single or double-strand breaks as intermediates in somatically mutating Ig genes (Sale & Neuberger 1998).

Since non-homologous DNA double-strand break (DSB) repair is inherently associated with the generation of the primary B cell repertoire, the role of non-homologous DSB repair in secondary diversification of Ig genes can not be addressed directly in mice defective in this repair pathway. Therefore, we used the 'Ig insertion' mice described above to assess directly whether DSBs exist in Ig genes of GC B cells. The present report represents a summary of a full length article that will be published elsewhere (Bross *et al*. 2000).

2. METHODS

(a) *Mice*

The generation of pII^{inv} mice are described elsewhere (Bross *et al*. 2000). Targeted pII^{inv} El4.1 embryonic stem (ES) cells were injected into C57BL/6 blastocysts to generate chimeric mice. In these chimeras the ES cell-derived B cells can be identified using the Ly9.1-specific allotype marker. The pII and pΔ mice have been described elsewhere (Fukita *et al*. 1998).

(b) *Sorting of B-cell subsets*

The pΔ and pII mice (Fukita *et al*. 1998) were immunized intraperitoneally with 200 µl of a 10% sheep red blood cells (SRBC) suspension in phosphate-buffered saline. Six to ten days later splenic B cells were enriched by magnet activated cell sorting (MACS) using CD19-specific paramagnetic beads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). B cells were stained with fluorescein isothiocyanate-conjugated anti-CD45R (clone RA3-6B2) (PharMingen, Hamburg, Germany) and PNA-biotin (Vector, Burlingame, CA, USA). The latter was revealed indirectly with streptavidin-PE. For the cell sorting (MoFlo Cytomation, Fort Collins, CO, USA), B cells were gated electronically by means of forward scatter (FSC), side scatter (SSC) and collected as a $CD45R^+/PNA^{low}$ and a $CD45R^+/PNA^{high}$ fraction. Dead cells were excluded by propidium iodide (PI).

(c) *Preparation of genomic DNA*

Immediately after the sorting, high molecular weight genomic DNA was isolated from 1×10^6 sorted cells. Cells were

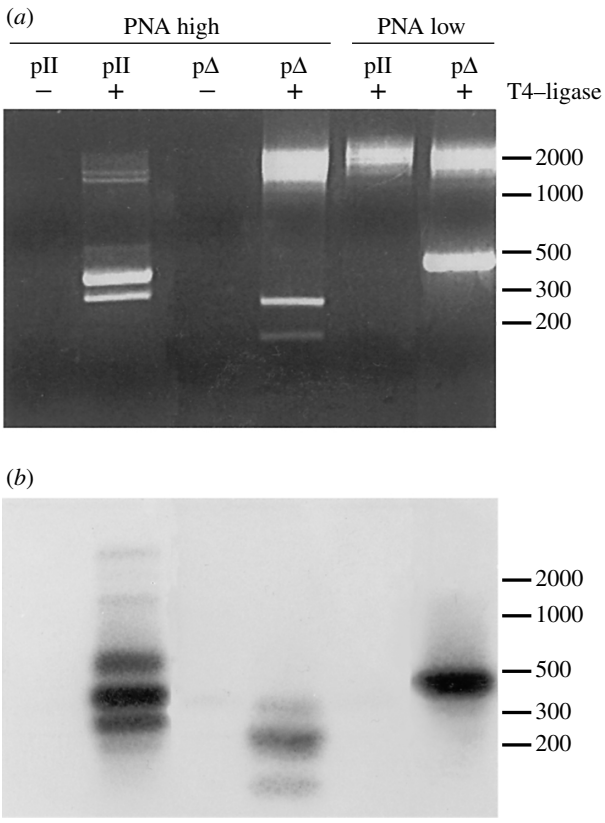

Figure 2. Detection of DNA double-strand breaks using ligation-mediated polymerase chain reaction (LMPCR). LMPCR products of 5′ break sites from 2000 cell equivalents of GC B cells (PNA^{high}) and 4000 cell equivalents non-GC B cells (PNA^{low}) from pΔ and pII mice were separated on a 2% (w/v) agarose gel and visualized with ethidium bromide under ultraviolet light (Eth. Br.) (*a*). Distinct bands ranging between 0.1 and 1.5 kb are found frequently in the V_HB1-8 gene of hypermutation competent PNA^{high} B cells of pII mice, and at a lower frequency in PNA^{high} B cells of pΔ mice. Southern blotting with a V_HB1-8-specific probe revealed the specificity of most products (*b*), suggesting the existence of DSBs within the targeted V_HB1-8 gene isolated from PNA^{high} and only rarely in PNA^{low} B cells. The finding of a DSB in pΔ but not in pII derived PNA^{low} B cells as seen in the present analysis is not typical (see also Bross *et al*. 2000). No specific PCR products were found in the mock-treated fractions, which lacked the T4-DNA ligase in the ligation step (–T4 ligase).

incubated overnight in 300 µl of 100 mM Tris-Cl ph 8.5, 5 mM EDTA, 0.2% SDS, 200 mM NaCl and 100 µg ml^{-1} proteinase K at 56 °C. Hereafter, 750 µl absolute ethanol was added and mixed by inverting the tube ten times. After centrifugation at 10 000 *g* for 10 min, the supernatant was carefully discarded. The pellet was washed with 1 ml of 70% ethanol and air-dried. The precipitate was dissolved by adding 10 mM Tris buffer pH 8.5 to a final concentration of $1–5 \times 10^3$ cell equivalents μl^{-1}. After an initial incubation for 20 min at 50 °C and gently flicking the tube from time to time the DNA was further dissolved overnight at 4 °C.

(d) *Generation and ligation of adapters*

For the detection of DSB, two oligonucleotides, 5′-CGAAGAGTAACCGTTGCTAGGAGAGACCGTGGCTGAATGGTGTCGACACTAGTGG-3′ and 5′-CCACTAGTGTCGACACCAGTCCTAATTTTTTTTTTCAAAAAAA-3′, were synthesized and duplexed to generate blunt ended, double-stranded splinkerettes (Devon *et al.* 1994). DNA from 2×10^4 cell equivalents were ligated to splinkerettes (0.1 pmol μl^{-1}) in a 40 µl volume with 2 U of T4-DNA ligase (Boehringer, Mannheim, Germany). Incubation was at 15 °C for 15 h and 20 °C for 1 h. Hereafter, the DNA was ethanol precipitated, air-dried and dissolved in 40 µl 10 mM Tris buffer at pH 8.5.

(e) *Amplification, cloning and sequencing of splinkerette-ligated V_HB1-8 genes*

Specific amplification of adapter-ligated V_HB1-8 genes from genomic DNA was achieved by using a nested PCR strategy. In the first round the external splinkerette primer (CGAAGAGTAACCGTTGCTAGGAGAGACC) (Devon *et al.* 1994), which specifically hybridizes to the long splinkerette-strand, was used in combination with either the external 5′ V_HB1-8 leader exon 1 primer (amplification of 5′ break end in pΔ and pII samples) or one of the 3′ external V_HB1-8 gene primers (amplification of 3′ break end): the 3′ JC intron 1 primer for pΔ samples and 3′ JC intron 2 primer for pII samples (Fukita *et al.* 1998). For the second round of amplification the internal splinkerette primer (GTGGCTGAATGAGACTGGTGTCGAC), which specifically hybridizes to the long splinkerette strand, was used either in combination with the nested 5′ V_HB1-8 leader exon 2 primer (amplification of 5′ break end in pΔ and pII samples) or the nested 3′ JC intron 3′ primer and the 3′ JC intron 1 primer for 3′ break ends from pII and pΔ samples, respectively. The gene-specific primers used bind specifically to the corresponding regions of the targeted V_HB1-8 allele (Fukita *et al.* 1998). To detect any V_HB1-8-specific ligation products, we used the same PCR conditions as described for the amplification of the targeted V_HB1-8 gene from single cells (Fukita *et al.* 1998). Each amplification step consisted of 30 cycles. PCR products were separated from primers on a 2% Tris acetate EDTA (TAE) agarose gel and visualized under long wave UV light. DNA was isolated from agarose gel slices using QiaQuick matrix (Qiagen, Hilden, Germany) and cloned into the TopoTA vector (Invitrogen, Groningen, The Netherlands). For sequencing the DyeDeoxy Terminator Cycle Sequencing (Applied Biosystems, Warrington, UK) was used in combination with either of the nested primers.

(f) *Analysis of V_HB1-8 mutations in memory B cells of pII^{inv} mice*

ES cell-derived B cells in pIIinv mice were identified using a Ly9.1-specific monoclonal antibody. Single memory B cells ($Ly9.1^+$, $V\lambda1^+$, $B220^+$, IgD^-, $CD11B^-$, $Thy1.2^-$) were sorted from the spleen and used for the specific amplification of the targeted V_HB1-8 gene (Fukita *et al.* 1998). PCR products were isolated from 1% TAE agarose gels using Spin-X columns (Costar, Cambridge, MA, USA). Isolated PCR products were directly sequenced by Dyedeoxy Terminator Cycle Sequencing (Applied Biosystems) as described previously (Fukita *et al.* 1998).

3. RESULTS AND DISCUSSION

(a) *Double-strand breaks in hypermutating V_HB1-8 genes*

B cells activated in T-dependent antibody responses expand in histological structures called germinal centres (GCs), which can be revealed histologically in lymphatic

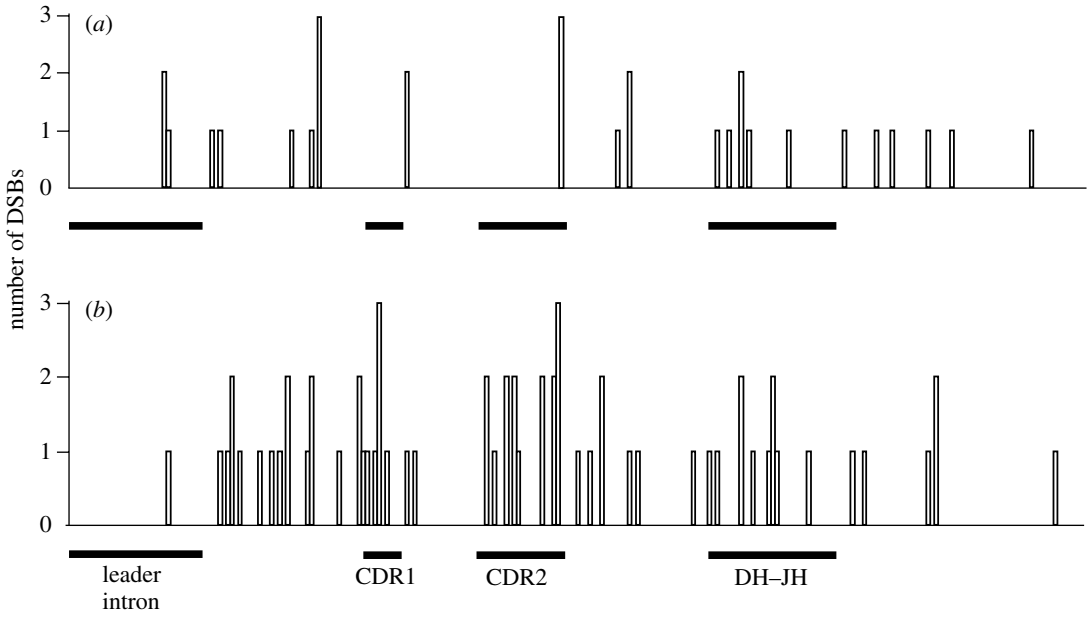

Figure 3. (*a*) Distribution of DSB in the targeted V_HB1-8 gene. Starting at the first base pair of the leader intron, the location of each DSB found by LMPCR in B cells of pII and pΔ mice is plotted as the number of DSBs versus one the following 612 nucleotides. (*b*) Distribution of DEs in the targeted V_HB1-8 gene. Starting at the first base pair of the leader intron, the location of each DE, as identified by single-cell PCR and sequencing in memory B cells pII^{inv} mice, is plotted as the number of DEs versus one of the following 612 nucleotides. Bars below each histogram indicate the position of the leader intron, CDR1 and CDR2 and the D_HJ_H region.

tissues by their intense staining with peanut agglutinin (PNA) (MacLennan 1994). This also allows GC B cells (PNA^{high}) to be distinguished cytometrically from non-GC B cells (PNA^{low}). The GC is the microenvironment that promotes secondary diversification of Ig genes through somatic hypermutation (Jacob *et al.* 1991).

To address the question whether DSBs appear as potential reaction intermediates of somatic hypermutation, non-GC (PNA^{low}) and GC (PNA^{high}) $CD19^+$, $CD45R^+$ B lymphocytes were sorted from pΔ and pII 'V_HB1-8 IgH knock-in' mice and high molecular weight genomic DNA was prepared from these fractions. Since all B cells carry the same VH-passenger allele, albeit under different promoters, the use of pΔ and pII mouse strains simplified the experimental design and allowed a direct comparison between the occurrence of double-strand breaks and the transcriptional activity in the targeted V_HB1-8 gene (figure 1*a*,*b*).

Aliquots from these DNA samples were ligated to splinkerettes, i.e. special blunt-ended DNA adapters (Devon *et al.* 1994), and used for ligation-mediated polymerase chain reaction (LMPCR). The primer sets used in each round hybridize specifically to the inserted V_HB1-8 gene and the long strand of the splinkerette, respectively. If DSBs exist in the V_HB1-8 region of hypermutating B cells, the PCR products from the amplification of the ligated 5′-end should occur in a size range of the hypermutation domain. Indeed, distinct PCR products ranging between 0.1 and 1.5 kb were found frequently in the V_HB1-8 gene of hypermutation competent PNA^{high} cells of pII mice and at a lower frequency of pΔ mice (see figure 2; Bross *et al.* 2000). Southern blot analysis with a radiolabelled V_HB1-8 probe and sequencing revealed the specificity of nearly all PCR products (figure 2), suggesting the existence of DSBs within the targeted V_HB1-8 gene isolated from PNA^{high} and only rarely in PNA^{low} B cells. No specific PCR products were found in the mock treated fractions, which lacked the T4-DNA ligase in the ligation step (figure 2). The amplification of 5′ break sites in the V_HB1-8 gene from GC B cells of pII and pΔ is more efficient than 3′ break (Bross *et al.* 2000). This 'ligation protection phenomenon' might relate to the presence of a transient DNA hairpin or, alternatively, a covalently bound peptide, which renders the 3′ break site inert to the ligation of an adapter molecule.

Table 1. *Preference and occurrence of DNA double-strand breaks at RGYW tetramers in V_HB1-8*

(Break-points at the RGYW motif are defined as position 1–5. The number and location of independently amplified DSBs is compared to the eight possible tetrameric sequences with an RGYW–WCRY consensus motif. Numbers in brackets relate to the unique situation of finding DSBs in the palindromic AGCT motif, which does not allow to discriminate positions one from five and two from four.)

RGYW–WRCY	1 (R) ↓	2 (G) ↓	3 (Y) ↓	4 (W) ↓	5 ↓
AGCA/TGCT	—	7	—	4	—
AGCT/TCGA	3 (4)	1 (2)	—	2 (1)	4 (3)
AGTA/TACT	—	2	—	2	—
AGTT/AACT	—	—	—	—	1
GGCT/AGCC	1	3	—	—	—
GGTA/TACC	1	1	—	1	—
GGCA/TGCC	—	—	—	—	1
GGTT/AACC	—	—	—	1	—

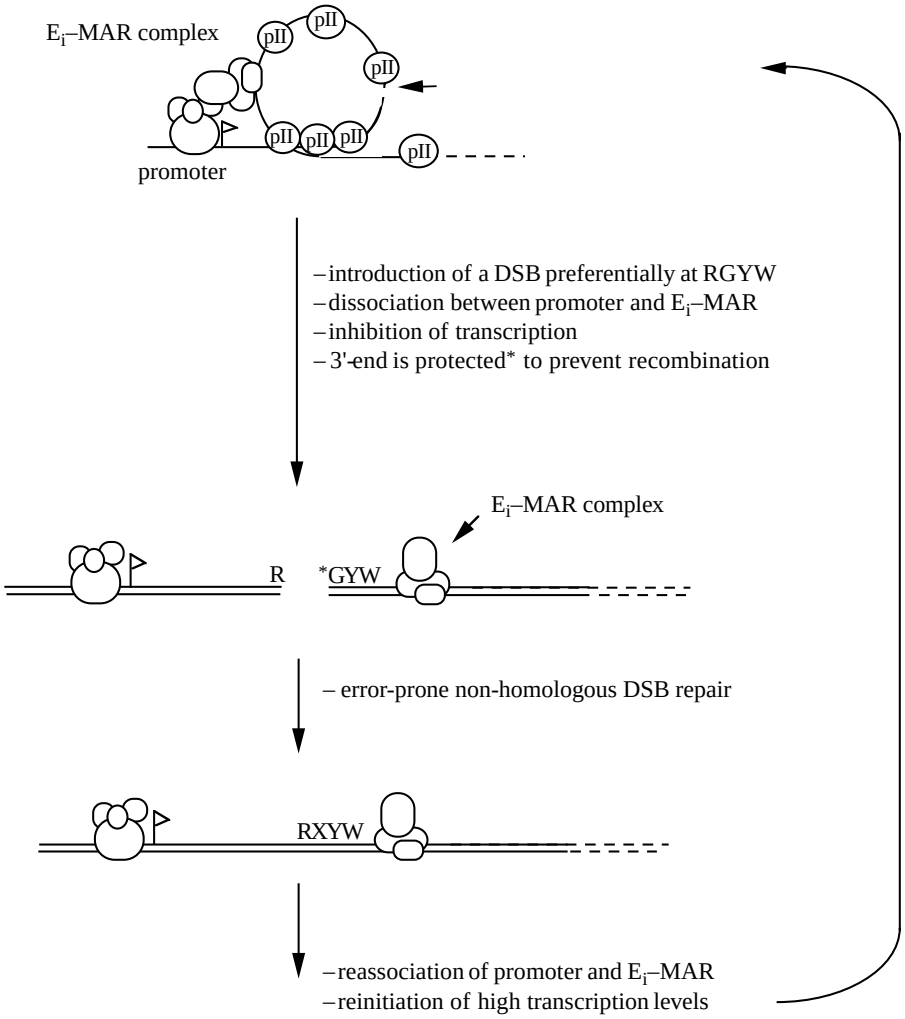

Figure 4. Model of DSB-mediated hypermutation (see § 3(b)).

(b) *Characterization of the double-strand breaks in targeted V_HB1-8 genes*

Cloning and sequencing of the LMPCR products allowed us to map the DSBs in the V_HB1-8 gene of living GC B cells. Similar to the distribution of mutations in rearranged Ig genes (Lebecque & Gearhart 1990; Peters & Storb 1996; Rothenfluh *et al.* 1993; Weber *et al.* 1991), DSBs are found in an area of 1.3 kb downstream of the promoter and peak around CDR2 and CDR3 (not shown, see Bross *et al.* 2000). The distribution of DSBs (figure 3*a*) along the targeted V_HB1-8 gene is remarkably similar to that of the somatic point mutations (Fukita *et al.* 1998). Consistent with the higher transcription level and mutation frequency of pII as compared to pΔ V_H B1-8 genes (Fukita *et al.* 1998), the number of LMPCR products derived from the same number of GC B cells from pII mice is higher as compared with those from pΔ (Bross *et al.* 2000). This suggests that transcription favours the generation of DSBs.

Like hot spots of somatically mutated Ig genes, the DSBs do not occur randomly but preferentially locate at RGYW motifs (table 1) (Rogozin & Kolchanov 1992). Considering the 95 RGYW motifs in the hypermutation domain of the V_HB1-8 gene, 32% [(5 × 95) 0.475 kb/1.5 kb] of randomly distributed DSBs are expected to occur at one of the 95 RGYWs. However, similar to somatic point mutations, 62% (32 out of 52) of all DSBs are found at RGYWs. Of those, 38% (20 out of 52) locate at AGCWs, which on a random basis cover only 8% (25 AGCWs 5′ positions ⇒125 positions/1500 positions) in V_H B1-8. Even within the RGYW motif these breaks do not occur randomly (table 1), but preferentially (25 out of 34 breaks) 5′ of the G and R residues of the RGYW motif, referred to as position two and one in table 1. This observation might relate to the previously observed differential mutability of the four residues within the RGYW motif (G > R > Y > W; Rogozin & Kolchanov 1992).

Nucleotides in codons 31^{II}, 38^{III}, 65^{II}, 100^{II}, 100^{AII} and III and 105^{II} (numbering according to Kabat & Wu 1991) have been described as mutational hot spots of the V_HB1-8 gene (Fukita *et al.* 1998; Schittek & Rajewsky 1992; Weiss & Rajewsky 1990). From a total of 52 breaks, two breaks occurred 5′ of 38^{III}, five breaks 5′ of 65^{II} and one break 5′ of $100A^{III}$. Thus, 15% (8 out of 52) of DSBs locate directly 5′ of three hot spots of somatic hypermutation, suggesting an error-prone repair pathway at the protected 3′ break side. This would explain the preference of finding mutations in G (position two) and R (position one) more frequently than mutations in Y (position three) or W (position four) within the RGYW motif. Note that both strands of the V gene are hypermutation targets (Dorner *et al.* 1998; Milstein *et al.* 1998). Therefore, the breaks at

positions four and five of the unique palindromic hot spot AGCT can be considered as positions two and one, respectively (table 1).

These data favour a model in which early in somatic hypermutation a DSB is introduced by an as yet unknown endonuclease with an intrinsic preference for RGYW motifs (see figure 4; Bross *et al.* 2000). While our assay detects only blunt-ended DSBs, they may initially be introduced as staggered DSBs, which would explain the generation of small duplications (fill-in reaction by a DNA polymerase) or deletions (exonuclease activity) in some mutated $V_HD_HJ_H$ gene segments. An error-prone fill-in process of the 3′ overhang might introduce point mutations at this stage of DSB repair already. As a consequence of the DSB, the intronic enhancer–matrix attachment region (E_i–MAR) complex is transiently uncoupled from the promoter complex, thereby possibly blocking transcription and subsequent deleterious DSBs. The formation of a hairpin by transesterfication or the covalent binding of a protein at the 3′ break side may render this end inert to translocations–recombinations, explaining the inefficient detection of the 3′ sides of DSBs in our assay. Subsequently, an error-prone repair process might act at the break sites and introduce a mutation. As soon as the DSB is religated by a non-homologous DSB repair system, the enhancer–promoter complex reassembles and transcription is reinitiated.

(c) *Large deletions in V_HB1-8 of 'IgH promoter–V_HB1-8 inversion mice'*

One feature of the proposed model is the introduction of a DSB and the subsequent dissociation of IgH promoter–E_i–MAR enhancer complexes. Thus, consecutive promoter-proximal DSBs would be expected in a situation in which the IgH promoter and E_i–MAR enhancer region cannot dissociate after the initial DSB. Deleterious deletions in the hypermutating V_H gene would result. For functional Ig genes, this would lead to a failure of the B cell to produce surface Ig and cause the extinction of this cell by apoptosis (Lam *et al.* 1997).

To test this concept, 'IgH promoter–V_HBl-8 inversion mice' were derived by gene targeting, which hereafter are referred to as pII^{inv} mice. In pII^{inv} mice the IgH promoter–V_HBl-8 transcription unit is inverted such that the IgH promoter locates proximal to the E_i–MAR region, pointing away from the E_i–MAR region (figure 1*c*). Like pII mice, the pII^{inv} mice carry the same targeted non-functional V_HBl-8 IgH passenger allele under the transcriptional control of its own Ig heavy chain promoter.

Three chimeric-pII^{inv}mice were used for the analysis of somatic hypermutation of the targeted, inverted *V_HB1-8* gene. Single, pII^{inv}-ES cell derived ($Ly9.1^+$), isotype-switched ($V\lambda1^+$, $B220^+$, IgM^-IgD^-) memory B cells were sorted and 60 transgene-specific V_HBl-8 PCR products were sequenced. 73% (44 out of 60) of pII^{inv} memory B cells were mutated and 479 point mutations were found in these mutated samples, resulting in a mutation frequency of 1.8% (Bross *et al.* 2000). These results indicate that the orientation of the IgH promoter–V_HBl-8 unit relative to the E_i–MAR element is not essential for the mechanism underlying somatic hypermutation.

Table 2. *Preference and occurrence of deletion ends at RGYW tetramers in V_HB1-8 of pII^{inv} memory B cells*

(DEs at the RGYW motif are defined as position 1–5. The number and location of independently amplified DEs is compared to the eight possible tetrameric sequences with an RGYW–WCRY consensus motif. Numbers in brackets relate to the unique situation of finding DEs in the palindromic AGCT motif, which does not allow discrimination of positions one from five and two from four.)

RGYW–WRCY	1 (R) ↓	2 (G) ↓	3 (Y) ↓	4 (W) ↓	5 ↓
AGCA/TGCT	2	3	4	—	1
AGCT/TCGA	3 (1)	2 (2)	2	2 (2)	1 (3)
AGTA/TACT	1	1	2	—	—
AGTT/AACT	—	1	2	1	1
GGCT/TACC	2	1	—	1	1
GGTA/TACC	—	—	—	—	—
GGCA/TGCC	—	—	—	—	—
GGTT/AACC	—	—	—	—	—

Therefore, the critical role of the E_i–MAR in somatic hypermutation of rearranged V genes appears to relate primarily to transcription, i.e. the efficient loading of RNA polymerases at the IgH promoter and not, for instance, a transcriptional pausing site.

Thirty-four deletions and two duplications were found in 495 PCR products (7.3%) from V_HBl-8 genes. Compared with deletions in pII mice, which range between 4 and 32 bp, deletions in memory B cells of pII^{inv} mice range between 7 and 338 bp and are frequently longer than 100 bp (Bross *et al.* 2000). Thus, as predicted from the model (figure 4), positioning the E_i–MAR upstream of the V_H gene promoter in the pII^{inv} system results in an increased frequency of large deletions within the rearranged $V_HD_HJ_H$ element.

(d) *Mapping the 5′- and 3′-ends of deletions*

Deletions found in the targeted V_HBl-8 gene of pII^{inv} mice are probably introduced by successive DSBs. Therefore, the distribution of deletion ends (DEs) should reflect the distribution of DSBs and point mutations in the targeted V_HBl-8 gene of GC and memory B cells, respectively. Indeed, the distribution of DEs along the targeted V_HBl-8 gene (figure 3*b*) is very similar to that of DSBs (figure 3*a*) as well as of somatic point mutations (Bross *et al.* 2000). In addition, 53% (34 out 64) of all 5′ and 3′ DEs map to position 1–5 of RGYW motifs (table 2). Therefore, like the DSBs in the V_HBl-8 genes of pII and pΔ B cells, deletions in pII^{inv} mice are not introduced randomly but like hot spots of somatically mutated Ig genes preferentially at RGYW motifs. Furthermore, 31% (20 out of 64) of all DEs, or 59% (20 out of 34) of all DEs at RGYW, map at AGCW, the latter value being identical to that obtained in LMPCR based analysis of pII and pΔ mice, where 20 out of the 34 DSBs mapped to AGCW (table 2). Excluding the DEs at the unique palindromic hot spot AGCT, 11 out of 24 DEs map at position one or two of RGYW, eight at position three and five at position four or five, indicating again a preference for DSBs to occur at position one or two rather than four and five (table 2).

4. CONCLUDING REMARKS

The occurrence and location of DSBs and DEs in the targeted V_HBl-8 passenger transgenes of B cells derived from pΔ, pII and pII^{inv} mice follow the established rules of somatic hypermutation. Therefore, although we cannot exclude that the DSBs we identify represent repair intermediates following the initial introduction of staggered DSBs, somatic hypermutation may relate to an error-prone non-homologous DSB repair pathway, active in B cells of the GC.

The introduction of DSBs and subsequent non-homologous DNA end-joining would then not only be part of the mechanisms creating the primary Ig repertoire through V(D)J recombination and changing Ig effector functions through Ig class switching, but also the formation of the secondary Ig repertoire through somatic hypermutation. Thus, the introduction of a DSB into Ig genes may be used repeatedly to diversify the Ig repertoire and Ig effector functions, leaving a potential for oncogenic translocations at early and late stages of B-cell development.

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