

# Hypermutation of Immunoglobulin Genes in Memory B Cells of DNA Repair-deficient Mice

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## Summary

To investigate the possible involvement of DNA repair in the process of somatic hypermutation of rearranged immunoglobulin variable (V) region genes, we have analyzed the occurrence, frequency, distribution, and pattern of mutations in rearranged V $\lambda$ 1 light chain genes from naive and memory B cells in DNA repair-deficient mutant mouse strains. Hypermutation was found unaffected in mice carrying mutations in either of the following DNA repair genes: xeroderma pigmentosum complementation group (XPA) and XPD, Cockayne syndrome complementation group B (CSB), mutS homologue 2 (MSH2), radiation sensitivity 54 (RAD54), poly (ADP-ribose) polymerase (PARP), and 3-alkyladenine DNA-glycosylase (AAG). These results indicate that both subpathways of nucleotide excision repair, global genome repair, and transcription-coupled repair are not required for somatic hypermutation. This appears also to be true for mismatch repair, RAD54-dependent double-strand-break repair, and AAG-mediated base excision repair.

Key words: DNA repair • DNA repair-deficient mice • memory B cells • naive B cells • somatic mutations

In humans and mice, the primary Ig repertoire is generated by V(D)J recombination in B cell progenitors of the embryonic liver and adult bone marrow. Rearranged Ig genes of antigen-specific B cells can be further diversified by hypermutation in germinal center B cells, which develop in secondary lymphatic tissues during immune responses (for review see reference 1). The mutations are mainly point mutations, with rare deletions and insertions (2). The mutation rate has been estimated to be as high as  $10^{-3}$  base pairs/generation, which is approximately six orders of magnitude higher than the spontaneous mutation rate (3–5).

Notwithstanding the extensive knowledge of the immunobiology (1), the molecular mechanism underlying somatic hypermutation remains speculative (6–9). Several models have been proposed: (a) “gratuitous” transcription-

coupled repair (TCR<sup>1</sup>; 10, 11), (b) site-specific nicking and repair of DNA via an error prone DNA polymerase (12, 13), (c) gene conversion (14), (d) local DNA hyperreplication (15), (e) cDNA retrotransposition of reverse transcribed Ig mRNA (16), (f) altered DNA replication (17), and (g) inhibition of mismatch repair (8).

<sup>1</sup>Abbreviations used in this paper: AAG, 3-alkyladenine DNA-glycosylase or 3-methyladenine DNA-glycosylase; AP, apurinic or apyrimidinic; BER, base-excision repair; CS, Cockayne syndrome; DSB, double-strand-break repair; GGR, global genome repair; MMR, mismatch repair; MSH, mut S homologue; NER, nucleotide-excision repair; PARP, poly (ADP-ribose) polymerase; PI, propidium iodide; PMS, postmeiotic segregation; RAD, radiation sensitivity; TCR, transcription-coupled repair; Thy1, CD90; TTD, trichothiodystrophy; XP, xeroderma pigmentosum complementation group.

Recent advances in cloning, characterizing, and targeting eukaryotic DNA repair genes (18) has provided mouse models that allow us to test present concepts on the mechanism of hypermutation. The molecular mechanism underlying hypermutation is unlikely to be controlled by a single gene product and most likely has evolved from preexisting components involved in DNA modification. We speculate that mutations are introduced by a "site-specific mutator" that depends on other molecules involved in DNA repair and/or DNA synthesis, a scenario that is reminiscent of V(D)J-recombination, which uses the site-specific recombination activating gene (RAG) recombinase as well as components of DNA repair such as DNA-dependent protein kinase and x ray cross-complementing (*XRCC*)4 (19).

Therefore, seven mouse strains with predefined mutations in either one of the DNA repair genes xeroderma pigmentosum complementation group (*XP*)A (20), *XP*D (20a), Cockayne syndrome (*CS*)B (21), mutS homologue 2 (*MSH2*; 22), radiation sensitivity 54 (*RAD54*; 23), poly (ADP-ribose) polymerase (*PARP*; reference 24), and 3-alkyladenine DNA-glycosylase or 3-methyladenine DNA-glycosylase (*AAG*; 24a) were tested for the occurrence of somatic mutations in naive and memory B cells. The different repair pathways where XPA, XPD, CSB, MSH2, RAD54, PARP, and AAG are involved are briefly outlined below. For further details the reader is referred to Friedberg et al. (25).

The XPA protein is an essential component of the nucleotide-excision repair (NER) pathway. XPA protein functions in a preincision step of NER, i.e., the recognition of DNA damage and controls both NER pathways, global genome repair (GGR) and TCR. It recruits the basal transcription factor (TF)IIH, which among other downstream components is common to GGR and TCR (26). *XP*-deficient mice are highly susceptible to UV-B- or chemical-carcinogen-induced skin and eye tumors (20, 27).

XPD and XPB are components of the basal transcription factor TFIIH and both display helicase activity. Specific mutations in the *XP*D gene and in the *XP*B gene can cause the multisystemic disorder trichothiodystrophy (TTD) (28). Clinically, the defining feature of TTD is sulfur-deficient brittle hair, which is due to a reduced cysteine content. TTD is also associated with reduced size, mental retardation, unusual facial appearance, and ichthyosis. These and other findings led to the hypothesis that the clinical features underlying TTD relate to defective transcription, i.e., a repair/transcription syndrome concept (29).

Unlike *XP*D and *XP*B, the *CS*B gene is not essential for transcription but appears to play a key role in coupling transcription and NER. *CS*B is thought to control the functional transition of a RNA-polymerase II complex into a "repairosome" as soon as the polymerase becomes stalled at a DNA lesion. Alternatively, repairosomes might continuously be active in scanning-transcribed DNA for the occurrence of DNA damage. *CS* is a multisystemic disorder. *CS* patients are deficient in TCR. TCR is a sub-pathway of NER that accomplishes the fast and efficient repair of certain types of lesions from the transcribed strand

of active genes (30). Mutations in either the *CS*A or *CS*B gene give rise to the classical form of *CS*. Specific mutations in the *XP*D, *XP*B, or *XP*G genes can also cause *CS* symptoms in combination with *XP* (28, 29). Consistent with a lack in TCR, *CS*B mutant mice are UV sensitive, show a selective inactivation of TCR but unaffected GGR, and are unable to resume RNA synthesis after UV exposure (21).

*MSH2* is the eukaryotic homologue of the bacterial *mutS* gene. Like *mutS*, the *MSH2* protein plays a central role in mismatch repair (MMR) and blocks recombination between nonhomologous DNA strands, i.e., antirecombination activity of MMR (22). *MSH2* binds together with *MSH6* to base mispairs and to extrahelical loops that occur during homologous recombination and replication. In contrast, the *MSH3/MSH6* heterodimer (*MSH3* is another *mutS* homologue) recognizes preferentially extrahelical loops (31–33). Upon recognition of the mismatch, the *MSH2*-containing heterodimer recruits other components of the mismatch repair system, i.e., the *MLH1-PMS2* heterodimer (*MLH*, *mutL* homologue; *PMS*, postmeiotic segregation). This complex triggers the excision of the mismatched nucleotide by removal of a tract of single-stranded DNA that contains the mismatched nucleotide. Resynthesis of the excised DNA strand and ligation of the remaining nick completes the repair process.

*RAD54* is a key factor in the *RAD52* DNA repair pathway. *RAD54*, *RAD51*, and *RAD52* belong to the *Saccharomyces cerevisiae* *RAD52* epistasis group (*RAD50-RAD57*, *RAD24*, *RAD59*, meiotic recombination [*MRE*]11, and x-ray sensitivity [*XRS*]2). Mutants show a wide range of recombination deficiencies including a defect in double-strand-break repair (DSBR) through homologous recombination. Consistent with a defect in DSBR, *RAD54* double-knockout embryonic stem cells display a reduced frequency in homologous recombination and are sensitive to ionizing radiation, mitomycin C, and the alkylating agent methyl methane-sulfonate, but not to UV light (23). *RAD51* and its mouse counterpart are functional homologues of the *Escherichia coli* recombination protein *RecA*, which forms nucleoprotein filaments with single-stranded DNA and mediates homologous DNA pairing and strand exchange. *RAD54* is likely to be involved in this process since it was found to physically interact with *RAD51* in *S. cerevisiae* (34).

*PARP*, a zinc-finger DNA binding protein, is involved in single-stranded break detection. In response to these breaks, the catalytic domain of *PARP* catalyses the covalent attachment of poly-ADP ribose to itself and to nuclear proteins. Its catalytic activity requires free DNA ends. Upon binding, *PARP* is thought to recruit DNA-repair components. A subsequent automodification of the enzyme negatively interferes with DNA binding. Because of these features, *PARP* appears to act as a "nick sensor". This function is consistent with the increased sensitivity of *PARP*-deficient mice to  $\gamma$ -irradiation and *MNU* treatment (24).

The *AAG* is like other DNA-glycosylases involved in

the initial step of base-excision repair (BER), the detection and processing of a modified base (25). Removal of the damaged base generates an AP (apurinic or apyrimidinic) site. The AP site is processed by the sequential action of AP-endonuclease/deoxyribosephosphodiesterase or AP-lyase/3' repair diesterase, leaving a single-stranded gap, which in mammalian cells is filled by the DNA polymerase  $\beta$  (35). Alternatively, proliferating cell nuclear antigen-dependent longer patch repair synthesis may contribute to the BER pathway (36). Ligation of the remaining nick is thought to be performed by DNA ligase II. AAG mutant mice are viable and do not show an overt phenotype.

## Materials and Methods

**Mice.** The background, genotyping, and characterization of *XPA*, *CSB*, *MSH2*, *RAD54*, *PARP*, and *AAG* (our manuscript submitted) mutant mice have been published elsewhere (20–24a). Since all mice had to be imported from outside laboratories, there was an infection risk for our animal facility at the University of Cologne (Cologne, Germany). Given this problem and the limited space in our quarantine facility, the availability of the mice was limited and they had to be analyzed soon after arrival. Non-immunized wild-type and *CSB*, *MSH2*, *XPA*, and *PARP* mutant mice were analyzed at an age of 10–30 wk. *RAD54* and *AAG* mutant mice were immunized with 50  $\mu$ g NP-CG alum in 100  $\mu$ l PBS 10 d before analysis and killed at an age of 10 and 11 wk, respectively. For the analysis of hypermutation in *XPD*<sup>TTDIBEL</sup> mutant mice, which carry the same mutation in *XPD* as the TTD<sup>IBEL</sup> patient (20a), an immunized mouse (10 wk) and a non-immunized mouse (11 wk) were used. Data from immunized or nonimmunized mice are presented together, since immunization did not affect the frequency and pattern of  $\lambda$ 1 mutations in memory B cells.

**Isolation of Single B220<sup>+</sup>, Ig $\mu$ <sup>+</sup>, Ig $\delta$ <sup>+</sup>,  $\lambda$ 1<sup>+</sup> Naive B Cells and B220<sup>+</sup>, Ig $\mu$ <sup>-</sup>, Ig $\delta$ <sup>-</sup>,  $\lambda$ 1<sup>+</sup> Memory B Cells from Spleen.** Single cell suspensions were obtained by mincing the spleen through a nylon mesh (cell strainer; Falcon; Becton Dickinson, Franklin Lakes, NJ). Upon lysis of the erythrocytes, white blood cells were washed twice with 10 ml PBA (PBS, 0.5% BSA, and 0.05% sodium azide). After washing, memory B cells were enriched by means of magnetic cell depletion using IgM<sup>-</sup>, thymidine (Thy)-1<sup>-</sup> or CD5<sup>-</sup>, and MAC-1-specific beads according to the procedure of the manufacturer (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). The memory B cell-enriched cell population was stained with saturating amounts of anti-IgM FITC (clone R33-24-12), anti-IgD FITC (clone 1.3), anti-Thy1.2 FITC (clone CFO-1), anti-MAC-1 FITC (clone M1/70.15.11), anti-B220 APC (clone RA3-6B2), and anti- $\lambda$ 1 PE (clone LS136) as described (37). After washing twice in PBA, lymphocytes were sorted in the presence of propidium iodide (PI) on a FACStar<sup>®</sup> Plus flow cytometer (Becton Dickinson, Mountain View, CA). Cells were gated electronically by means of forward scatter, side scatter, B220<sup>+</sup> (APC<sup>+</sup>), and Ig $\mu$ <sup>-</sup>, Ig $\delta$ <sup>-</sup>, Thy-1<sup>-</sup>, Mac-1<sup>-</sup> (FITC<sup>-</sup>) staining. From those single, viable (PI<sup>-</sup>),  $\lambda$ 1<sup>+</sup> (PE<sup>+</sup>) cells were sorted into PCR tubes (Roth, Karlsruhe, Germany) containing 20  $\mu$ l Taq buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl, and 2.5 mM MgCl<sub>2</sub>; GIBCO BRL, Gaithersburg, MD) and 33 ng/ $\mu$ l 5S *E. coli* rRNA (Boehringer Mannheim, Indianapolis, IN). Since  $\lambda$ 1<sup>+</sup> memory B cells are very infrequent (~0.01% of all splenic B cells), no statements on the numbers of memory B

cells present in the various experimental animals can be made. Naive B cells were sorted by gating on viable B220<sup>+</sup>, Ig $\mu$ <sup>+</sup>,  $\delta$ <sup>+</sup>,  $\lambda$ 1<sup>+</sup> cells. Cells were put immediately on dry ice and stored at -80°C.

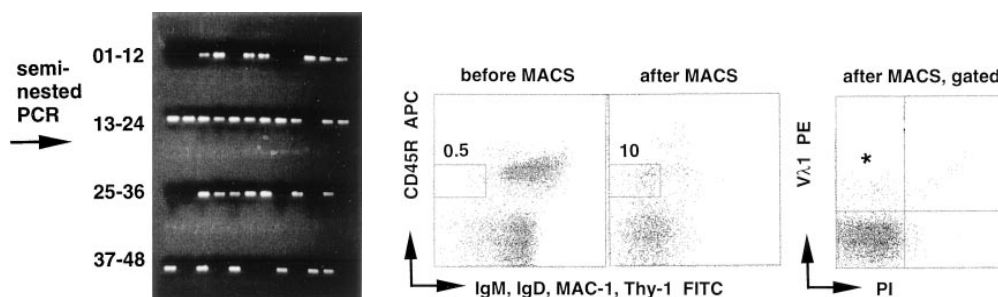
**Amplification of Rearranged  $\lambda$ 1 Genes from Single Cells.** Single cells in 20  $\mu$ l of Taq buffer were incubated with 0.5  $\mu$ g/1  $\mu$ l proteinase K (Boehringer Mannheim) for 40 min at 55°C. After heat inactivation of the protease (8 min at 95°C), specific amplification of rearranged  $\lambda$ 1 genes was achieved by using a seminested PCR strategy. The first round of amplification was carried out in the same reaction tube in a 50- $\mu$ l volume containing 20 mM Tris-HCl, pH 8.4; 50 mM KCl; 2.5 mM MgCl<sub>2</sub>; 400  $\mu$ M each dATP, dGTP, dCTP, and dTTP (Pharmacia Biotech, Piscataway, NJ), 40 nM  $\lambda$ 1 primer (5'-GGGTATGCAACAATGCGCATCT-TGTC-3'), 40 nM  $\lambda$ 1 primer (5'-CACGGACAGGATCCT-AGGACAGTCAG TTTGGT-3'), and 5U Taq DNA polymerase. The polymerase was added after the first denaturation step. The temperature cycle program consisted of one cycle at 95°C for 2 min, 88°C for 3 min, 60°C for 2 min, 72°C for 2 min, followed by 28 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 90 s. The final extension time was 10 min, and thereafter the reaction was cooled to 15°C.

Of the first round of PCR, a 1.5  $\mu$ l sample was used for the second, seminested amplification in a 50- $\mu$ l volume using the same buffer but containing 140 nM of a nested  $\lambda$ 1 primer (5'-GCG-AAGAGAAGCTTGTGACTCAGGAATCTGCA-3'), 140 nM of the  $\lambda$ 1 primer used in the first round of PCR, and 5 U of Taq DNA polymerase (GIBCO BRL). The cycle program consisted of one step at 92°C for 1 min, followed by 30 cycles of 92°C for 1 min, 63°C for 1 min, and 72°C for 90 s. Again, the final extension time was 10 min and thereafter the reaction was cooled to 15°C.

**Sequencing of  $\lambda$ 1 PCR Products.** PCR products were separated from primers on a 1% TAE agarose gel (SeaKem GTG agarose; Biozym, Hessisch Oldendorf, Germany) and visualized under long wave UV light. DNA was isolated from agarose gel slices by centrifugation through a 0.22- $\mu$ m molecular filter (SpinX tubes; Costar Corp., Cambridge, MA). Ethanol-precipitated DNA was resolved in 25  $\mu$ l water and 50–150 ng of template was used for dye-terminated automatic sequencing (PE Applied Biosystems, Foster City, CA) in combination with either the nested  $\lambda$ 1 primer or the  $\lambda$ 1 primer.

## Results and Discussion

**Ex vivo Analysis of Hypermutation by Single Cell PCR.** To analyze somatic hypermutation efficiently in any mouse strain, we established a seminested PCR approach to amplify and sequence functionally rearranged  $\lambda$ 1 genes from single cells. The method is based on the availability of the  $\lambda$ 1-specific monoclonal Ab LS136 (38) that allows the identification and isolation of single B cells expressing a functionally rearranged  $\lambda$ 1 gene (Fig. 1). An equivalent monoclonal Ab specific for the translational product of a unique V gene in both IgH allotypes is not available. In addition, gene conversion, which might to some extent contribute to the diversification of rearranged VH genes (39), does not occur in  $\lambda$ 1 genes (40). Therefore, the analysis of hypermutation in rearranged  $\lambda$ 1 genes will focus on the primary molecular mechanism of somatic hypermuta-



sorted on a FACStar<sup>®</sup>. PCR products were run on a 1% TAE gel; lanes 1 and 2 are negative controls (non-B cells), Vλ1<sup>+</sup> B cells, and lanes 11–48 are the products of the Vλ1<sup>+</sup> memory B cells. Numbers above the gates indicate percentages. The analysis from a wild-type mouse is shown.

**Figure 1.** Enrichment and sorting of single Vλ1<sup>+</sup> memory B cells for amplification and sequencing of the rearranged Vλ1 gene. Cells, before and after magnetic activated cell sorting (MACS) were stained as described in the Materials and Methods section. The enriched memory B cells (CD45R<sup>+</sup>, IgM<sup>-</sup>, IgD<sup>-</sup>) were gated. From those, single, viable (PI<sup>-</sup>), Vλ1<sup>+</sup> memory B cells (\*, the population) were

tion, the introduction of untemplated point mutations into rearranged Ig heavy and light chain gene segments.

The single cell PCR approach omits cloning of the PCR products and thus excludes the potential formation of recombination products between PCR intermediates generated during amplification of genomic DNA or cDNA from sorted populations. It is also essentially not obscured by errors of the Taq polymerase since the amplicates are directly sequenced. Using this *ex vivo* approach it is possible to efficiently analyze hypermutation parameters like mutation frequency, base exchange pattern, ratio of transitions versus transversions, and distribution in any mouse strain.

**Frequency, Distribution, and Base Exchange Pattern of Somatic Hypermutation in DNA Repair-deficient Mouse Strains.** A possible role of NER in somatic hypermutation was analyzed in *XPA*, *XPD*, and *CSB* mutant mice. Consistent with the role of CSB in TCR, *CSB*-deficient mice lack TCR activity (21) and accordingly form an ideal system to test the gratuitous TCR-based hypermutation model (6, 10, 11). This model proposes a mutator that is loaded during transcription initiation and carried into the gene by the elongating transcription complex. Next, mutator-mediated stalling of RNA polymerase II, in the absence of DNA lesions (41), is thought to recruit factors involved in NER. A small single-stranded DNA fragment of the transcribed strand is excised by the NER machinery, and the fill in of the single stranded gap by an unknown error-prone DNA polymerase is thought to introduce the mutation. The mutation rate is determined by the error rate of the DNA polymerase.

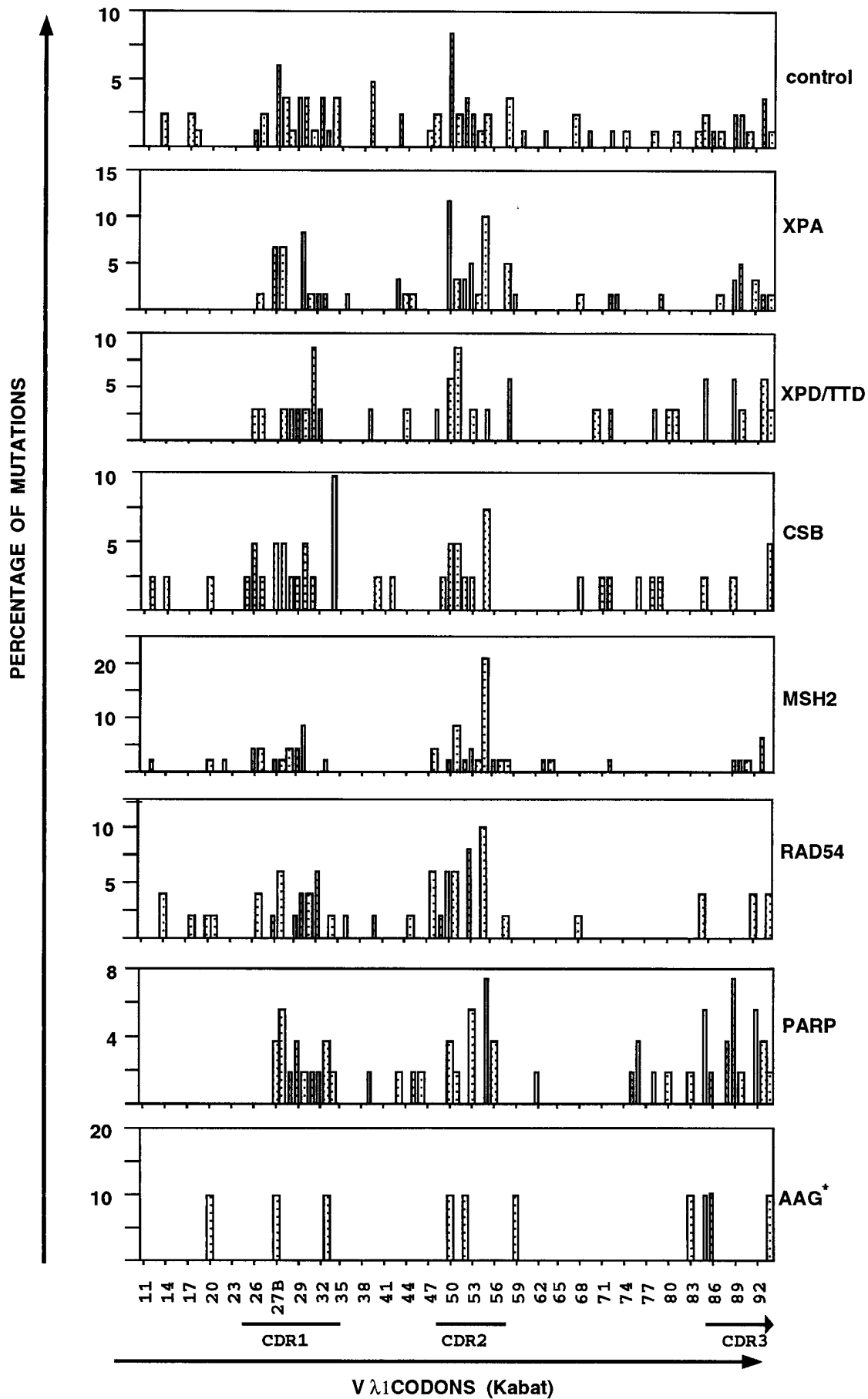
In line with these considerations, triplex-forming oligonucleotides were found to inhibit transcription and lead to mutations 5' of a transcriptional block in mammalian cells. Mutagenesis was dependent on NER since it was not detectable in *XPA*- or *CSB*-deficient cells, respectively (42). The mechanism by which the triplex-mediated transcrip-

tion inhibition not only triggers repair but also induces mutagenesis was extrapolated from the model of Hanawalt (41), which proposes that the TCR pathway of a cell may trigger "gratuitous repair" when transcription stalls at natural pause sites, even in the absence of a classical DNA lesion. These data raise the possibility that a transcriptional block might initiate the hypermutation process. A transcriptional block, for example in the V gene intron, mediated by an as yet unknown cis motif(s), could explain the reduced mRNA and protein levels of Ig in germinal center B cells (43, 44). In the frame of these speculations, hypermutation should not occur in *XPA*-, *XPD*-, or *CSB*-deficient mice.

However, with regard to distribution of point mutations along the rearranged gene (Fig. 2), strand bias of hypermutation, as reflected by a preferential mutation of A, G, and C rather than T bases (Fig. 3), frequency and ratio of transitions versus transversions (Table 1), the pattern of hypermutation appears to be normal in memory B cells of *XPA*-, *XPD*-, and *CSB*-mutant mice and hypermutation is absent in naive B cells (0/7 naive B cells carrying mutations in *XPA*-mutant mice, 0/5 in *XPD*-mutant mice, 1/28 in *CSB*-mutant mice, and 2/61 in wild-type mice; data not shown). Naive B cells were included in this analysis because of the possibility that the mutator is constitutively expressed throughout B cell development is efficiently counteracted by a specific DNA repair pathway in naive B cells and becomes only apparent in germinal center B cells. The slightly decreased mutation frequency in *XPD*<sup>TTDIBEL</sup> mice (0.7% in *XPD* versus 1.0–1.6% in other mice) might relate to the fact that relatively young mice were analyzed in this case (Table 1), since both the proportion of B cells that express mutated V-genes and the average number of nucleotide substitutions carried by the mutant V genes can increase considerably with age (2). Hypermutation thus occurs in the absence of NER, in accord with the finding of nor-

**Figure 2.** Distribution of point mutations found in memory B cells from *XPA*, *XPD*<sup>TTDIBEL</sup>, *CSB*, *MSH2*, *RAD54*, *PARP*, and *AAG* mutant mice. The point mutations found in the Vλ1 locus of memory B cells are indicated as percentage of mutations versus 87 Vλ1 codons (11–94, numbering according to Kabat, reference 56). \*, a small data base of point mutations in the *AAG*-mutant mice. Irrespective of the mouse strain (*right*), a typical clustering of mutations within the CDR regions was found. The published hot spots in Vλ1 (57): Ser30 II, Asn31 III, Gly50 II, Ala55 I ανδ II, and Leu90 III were confirmed in this study. Additional hot spots are at position Ala27B I, Val27C III, Gly50 II, Thr51 I, Ala89 II, and Ser93 II and III. The Roman numbers of the hot spots indicate the nucleotide position in the codons.

Distribution of Somatic Mutations in  $V\lambda 1+B220+$  IgM/D- memory B cells



wt n=85		to				%
		A	G	C	T	
from	A		15	9	10	40
	G	20		9	1	35
	C	3	1		9	15
	T	-	3	5		10

XPA n=61		to				%
		A	G	C	T	
from	A		11	3	5	32
	G	16		3	3	35
	C	0	5		7	20
	T	3	2	3		13

XPD n=35		to				%
		A	G	C	T	
from	A		2	5	7	40
	G	6		2	-	23
	C	1	1		8	28
	T	1	0	2		9

CSB n=42		to				%
		A	G	C	T	
from	A		8	5	4	41
	G	6		2	3	26
	C	-	2		9	26
	T	-	1	2		3

MSH2 n=48		to				%
		A	G	C	T	
from	A		6	3	2	23
	G	8		7	1	33
	C	3	6		10	39
	T	-	-	2		4

RAD54 n=52		to				%
		A	G	C	T	
from	A		7	4	9	38
	G	5		2	5	23
	C	2	1		12	29
	T	-	1	4		10

PARP n=54		to				%
		A	G	C	T	
from	A		8	4	7	35
	G	10		4	2	30
	C	2	3		7	22
	T	2	-	5		13

AAG n=11		to				%
		A	G	C	T	
from	A		1	1	1	28
	G	2		1	1	36
	C	1	-		3	36
	T	-	-	-		0

**Figure 3.** Base exchange patterns in the  $\lambda$ 1 genes of memory B cells from *XPA*<sup>-</sup>, *XPD*<sup>TTDIBEL</sup><sup>-</sup>, *CSB*<sup>-</sup>, *MSH2*<sup>-</sup>, *RAD54*<sup>-</sup>, *PARP*<sup>-</sup>, and *AAG*-mutant mice. Note that the typical strand bias of hypermutation, which is defined by a preferential mutation of A, G, and C rather than T bases (AGC > T) is evident in all mice. *n*, the number of mutations found.

mal hypermutation in XPB, XPD, XPV, and CSA patients (45, 46). This strongly argues against the gratuitous TCR-based model, but does not exclude a physical link between hypermutation and transcription. The latter is based on the findings that hypermutation is most efficient in the presence of both Ig transcriptional enhancers (47), displays a strand bias that is based on the preference to mutate A, G, or C rather than T (2), is restricted to a 1.5-kb window downstream of the leader intron (48–51), and can be reinitiated by placing an additional Ig $\kappa$  promoter immediately 5' to the constant portion of the Ig $\kappa$  chain gene, which leads to hypermutation in the rearranged VJ region and the C $\kappa$  region but not in the spacer region between these elements (11). However, the apparent dependency of the mutator on an active locus might merely reflect the accessibility of the rearranged Ig locus for the mutator. This possibility calls for the analysis of other transcription-independent DNA repair pathways in hypermutation.

The *MSH2*-deficient mouse strain allowed us to test the

MMR-based model of somatic hypermutation (8). A temporal and/or local inhibition of MMR would increase the mutation rate. First, because of an increased frequency of recombination between identical and related sequences, and second, because of a defective repair of mismatches that occur during DNA replication or DNA repair. This model has an interesting evolutionary aspect. Both gene conversion and hypermutation, two processes known to diversify the Ig repertoire in chickens (conversion), sheep (hypermutation), and rabbits (conversion and hypermutation) (52–54) would be increased in the absence of MMR. In addition, a temporal and/or local lack of MMR could explain how a mismatch that has been introduced by the mutator can be maintained until segregation of the daughter cells. Alternatively, the mutator might “abuse” mismatch recognition proteins to fix the introduced mismatches by a temporal and/or local inhibition of subsequent MMR steps. However, in the absence of *MSH2*-mediated MMR throughout B cell development, the mutation frequency in memory B cells of *MSH2*-deficient mice was neither increased (as expected from the MMR inhibition model) nor decreased (as expected from the MMR abuse model), and the distribution of mutations along the rearranged locus were normal (Fig. 2 and Table 1). An excess of mutations at germline G-C rather than A-T pairs was found (Fig. 3). This is in accord with Phung et al., who suggested that MMR, although not required for the occurrence of somatic mutations, may influence its pattern (55). In another study, high levels of mutations were also found in variable genes from mice deficient for the mismatch repair protein PMS2. However, genes from PMS2-deficient mice showed a higher frequency of tandem mutations (Gearhart, P., personal communication). In *MSH2*-deficient memory B cells, the frequency of doublet mutations appears normal, i.e., one doublet mutation out of 54 mutations as compared with one triplet and two doublet mutations out of 85 mutations in wild-type memory B cells. The difference between the two mutants might relate to the fact that PMS2 lies downstream of not only *MSH2* but also *MSH3*, which is known to bind preferentially to extrahelical loops. Considering a constitutively active mutator that is efficiently counteracted by MMR in naive B cells, a lack of MMR throughout B cell development might have caused hypermutation in naive B cells. However, seven out of seven naive B cells analyzed were unmutated (data not shown).

A possible role of *RAD54* in somatic hypermutation was addressed in *RAD54*-mutant mice. The *RAD54* mutants are deficient in DSB repair through homologous recombination (23). One could imagine that pairing of homologous Ig alleles can take place in germinal center B cells. Based on the unique DNA sequence at the V(D)J recombination site, which lacks a homologous stretch of DNA on the other allele, pairing would be interrupted at this site. To resolve the Holiday junction(s), a resolvase would have to introduce nick(s) into the V(D)J region. A subsequent error-prone repair process, which might involve a nuclease activity or displacement synthesis, would introduce mutations in the recombinant strand. However, a lack of *RAD54* did not

**Table 1.** Mutation Analysis of V21 Genes from Memory B Cells from XPA-, XPD<sup>TTDIBEL-</sup>, CSB-, MSH2-, RAD54-, PARP-, and AAG-mutant Mice

Genotype	Deficiency	Remark	Age	No. of mutated sequences/total no. sequences	Mutation Frequency	Mutation range	Mutation frequency in mutated cells	Transition/transversions
Wild type	—	—	<i>wk</i>					
XPA	NER (GGR)	Complete knockout	12, 20	27/42 (64%)	0.78% (85/10,962)	1–11	1.2% (85/7,047)	1.4
XPD	NER	XPD mutation of transcription	30	16/30 (53%)	0.78% (61/7,830)	1–8	1.4% (61/4,176)	1.5
CSB	NER (TCR)	K337 stop*	10, 11	19/40 (48%)	0.33% (35/10,440)	1–3	0.7% (35/4,959)	1.1
MSH2	MMR	Complete knockout	30	16/20 (80%)	0.80% (42/5,220)	1–5	1.0% (42/4,176)	1.5
RAD54	DSBR	Complete knockout	20	20/30 (66%)	0.66% (52/7,830)	1–6	1.0% (52/5,220)	1.2
PARP	Nick sensing	Complete knockout	10	13/22 (59%)	0.91% (53/5,742)	1–7	1.6% (53/3,393)	1.2
AAG*	BER	Complete knockout	12	16/33 (48%)	0.65% (56/8,613)	1–7	1.3% (56/4,176)	1.3
Naive B cells from all mice		Internal PCR control in each experiment	11	06/14 (43%)	0.46% (17/3,654)	1–5	1.0% (17/1,599)	1.0 <sup>‡</sup>
				3/130 (2%)	0.03% (11/33,930)	3–4		

\*The CSB protein is not detectable.

<sup>‡</sup>A small data base.

influence hypermutation in memory B cells (Figs. 2 and 3, Table 1) and was absent in naive B cells (data not shown). Also V(D)J recombination and class switching were found to be normal in RAD54-mutant mice (23). Additional experiments are required to verify, whether other components of the *S. cerevisiae* RAD52 epistasis group (RAD50–57, RAD24, and x-ray sensitivity [XRS]2) or other recombinational repair genes are involved in the molecular mechanism underlying somatic hypermutation.

Hypermutation is characterized by nucleotide exchanges, which likely involve “nicks” of the DNA. Nicks exist as intermediates in DNA repair and DNA recombination, are intermediates in most hypermutation models, and were proposed to be actively introduced to initiate somatic hypermutation (12). To test whether the “nick-sensing activity” of PARP might be functionally involved in hypermutation, e.g., by recruiting the mutator, we analyzed hypermutation in PARP-deficient mice. Again, hypermutation occurred normally in memory B cells from PARP-deficient mice (Figs. 2 and 3, Table 1) and did not occur in the 13 naive B cells analyzed (data not shown).

In the course of this study, AAG-deficient mice became available and were included in the analysis, as AAG is one of the enzymes involved in BER, another mechanism of

DNA repair that could potentially be involved in somatic hypermutation. Hypermutation appears to be unaffected in AAG-deficient memory B (Figs. 2 and 3, Table 1) and naive B cells (5/5 unmutated). Although our data base comprises only 11 mutations (six clonally related mutations were excluded), the base exchange patterns are within the expected range. In addition, hypermutation has been found to be normal in DNA polymerase  $\beta$ -deficient memory B cells (Texido, G., and K. Rajewsky, unpublished data). These data argue against a connection between BER and hypermutation.

In conclusion, single B220<sup>+</sup>, Ig $\mu$ <sup>+</sup>, Ig $\delta$ <sup>+</sup>, V $\lambda$ 1<sup>+</sup> naive B cells and B220<sup>+</sup>, Ig $\mu$ <sup>-</sup>, Ig $\delta$ <sup>-</sup>, V $\lambda$ 1<sup>+</sup> memory B cells were isolated ex vivo from XPA, XPD, CSB, MSH2, RAD54, PARP, and AAG mutant mice and used to amplify and sequence rearranged Ig $\lambda$ 1 genes. The results indicate that NER does not control somatic hypermutation, strongly arguing against the gratuitous TCR-based models of somatic hypermutation. Furthermore MMR, as well as RAD54-dependent DSBR- and AAG-mediated BER are not required for the hypermutation process. Although not addressed in detail, the presence of V $\lambda$ 1<sup>+</sup>, Ig $\mu$ <sup>-</sup>, Ig $\delta$ <sup>-</sup> memory B cells in all the mutant mice analyzed in addition indicates, that Ig class switching takes place in these animals.

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