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*J Immunol* 2002; 169:3069-3075; doi: 10.4049/jimmunol.169.6.3069

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Supplementary Material

http://www.jimmunol.org/content/suppl/2002/08/29/169.6.3069.DC1

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Gene Conversion-Like Sequence Transfers Between Transgenic Antibody V Genes Are Independent of RAD54

Nicole D’Avirro,* David Truong, ‡ Michael Luong, ‡ Roland Kanaar, § and Erik Selsing*†‡

Homology-based Ig gene conversion is a major mechanism for Ab diversification in chickens and the Rad54 DNA repair protein plays an important role in this process. In mice, although gene conversion appears to be rare among endogenous Ig genes, Ab H chain transgenes undergo isotype switching and gene conversion-like sequence transfer processes that also appear to involve homologous recombination or gene conversion. Furthermore, homology-based DNA repair has been suggested to be important for somatic mutation of endogenous mouse Ig genes. To assess the role of Rad54 in these mouse B cell processes, we have analyzed H chain transgene isotype switching, sequence transfer, and somatic hypermutation in mice that lack Rad54. We find that Rad54 is not required for either transgene switching or transgene hypermutation. Furthermore, even transgene sequence transfers that are known to require homology-based recombinations are Rad54 independent. These results indicate that mouse B cells must use factors for promoting homologous recombination that are distinct from the Rad54 proteins important in homology-based chicken Ab gene recombinations. Our findings also suggest that mouse H chain transgene sequence transfers might be more closely related to an error-prone homology-based somatic hypermutational mechanism than to the hyperconversion mechanism that operates in chicken B cells. The Journal of Immunology, 2002, 169: 3069–3075.

During B cell maturation, Ab genes undergo a number of unique genetic changes. In early B cells, recombinations of V, D, and J segments are involved in determining the antigenic specificity of the differentiating cell (1). Subsequently, in mature B cells, antigenic stimulation induces isotype switch recombination (2) and further V region diversification by somatic hypermutation or gene conversion (3).

Ab transgenes have been used extensively to study the mechanisms and regulation of Ig gene diversification. H chain transgenes are capable of undergoing both somatic hypermutation and isotype switching, even though the transgenes are located at chromosomal sites outside of the \( IgH \) locus (4–11). Sequence transfers that resemble gene conversions have also been found between V gene segments in a mouse H chain transgene (12).

Analyses of isotype switching, hypermutation, and conversion-like sequence transfers in Ab transgenes might provide insights into the diversification mechanisms that act on endogenous Ig genes. Several studies of H chain transgenes have suggested that murine B cells might have specialized homologous recombination/gene conversion activities that could be involved in gene diversification. Conversion-like V gene sequence transfers in transgenes clearly require a form of homologous recombination (12), and analyses of some H chain transgenes have indicated that interchromosomal transgene isotype switching can involve homologous recombination or gene conversion events (4, 7, 8).

Studies have also indicated that homology-based sequence transfers might play a significant role in Ig gene somatic hypermutation. Transgene V gene sequence transfers have been found to be frequently associated with high levels of somatic hypermutation (12, 13), suggesting that homology-based recombination and somatic hypermutation might share some mechanistic steps (13, 14). Furthermore, DNA cleavages associated with somatic hypermutation of endogenous Ig genes are predominantly found at the \( G_s \) cell cycle stage; this observation is consistent with the hypothesis that homology-based repair might play a role in the hypermutational mechanism (15). The proteins that might be responsible for the various homology-based processes in mouse B cells are not known.

Homologous recombination through the RAD52 pathway is important in dsDNA break repair in Saccharomyces cerevisiae (16, 17). Mutants in this pathway display sensitivity to ionizing radiation because of a defect in repairing radiation-induced DNA double-stranded breaks. Rad51 and Rad54 are key proteins in the RAD52 dsDNA break repair pathway. Rad51 mediates the search for homologous DNA and DNA strand exchange (16, 17). In vertebrates a number of RAD51 paralogs exist, including XRCC2 and XRCC3 (18). Both of these genes are involved in homologous recombination in chicken as well as in rodent cells (19–22). Recently it has been shown that XRCC2 and XRCC3 influence Ig gene conversion in the DT40 chicken B cell line (23). Rad54 serves as an accessory protein to Rad51 in mediating homologous recombination (24–26). In mouse embryonic stem cells and in adult mice the RAD54 gene is involved in homologous recombination (27, 28). Specifically, in embryonic stem cells Rad54 is involved in gene conversion between sister chromatids (29). Furthermore, the chicken RAD54 gene is important in Ig gene conversion in the DT40 chicken B cell line (30).

Previous studies have shown that Rad54 does not appear to be involved in somatic hypermutation or isotype switching of endogenous mouse Ig genes (27, 31). However, given the role of Rad54 in DT40 gene conversion, we wished to investigate whether this
recombination protein might be involved in homology-based murine B cell recombination processes. We have analyzed the effects of mouse Rad54 on interchromosomal isotype switching, somatic hypermutation, and gene conversion-like sequence transfers in mice that lack Rad54 and carry the previously described VVC μ transgene (12). Rad54 does not appear to be required for transgene somatic hypermutation or for the homologous recombination processes that must be involved in transgene VDJ sequence transfers and that might be involved in interchromosomal isotype switching. Thus, at least one of the proteins that is important for homology-based gene hyperconversion in a chicken B cell line is not essential for the homology-based recombination processes that occur in mouse B cells. Because gene conversion-like sequence transgene transfers appear to be Rad54 independent, we suggest that they might be more closely related to somatic hypermutational mechanisms than to gene hyperconversion mechanisms.

Materials and Methods

Mouse strains and immunization

Previous reports describe the VVCμ transgenic mouse line (12) and the RAD54 knockout mouse (27). The two lines were crossed and genotyping of offspring was performed for the VVC transgene as described (12). Genotyping using the RAD54 mutation was done using 40 cycles of PCR amplification of the wild type and/or the targeted allele using wild-type (GCTCATTAGACGCATTGT), mutant (GCCCTTCTGACAGTCTTCC), and common (AAGATGGCAGTCAAGT) primers.

Intraperitoneal immunization of mice was initially done using 100 μg of p-azophenylarsonate (Ars)-keyhole limpet hemocyanin (KLH) in CFA. Mice were immunized three additional times, once 3 wk later and then every 2 wk after that, with 100 μgArs-KLH in IFA. Mice were boosted with 100 μg of Ars-KLH in 0.9% saline before the removal of spleens for analyses.

ELISA

Ab concentrations of mouse serum were determined by standard ELISA techniques using Immulon 1B microtiter plates (Dynex Technologies, Denkendorf, Germany) as described previously (11). Wells were coated with Ars-BSA or the rat mAB AD8 (32). Secondary Abs were either biotinylated rat anti-mouse IgG or IgM (Zymed Laboratories, San Francisco, CA). Development of the plates was accomplished using a streptavidin-alkaline phosphatase conjugate (Boehringer Mannheim, Indianapolis, IN) and the substrate para-nitrophenol (Sigma-Aldrich, St. Louis, MO). Quantitation of mouse Rad54 does not appear to cause any reduction in the extent of transgene isotype switching observed in the immunized VV5 animals.

The VVCμ transgene is known to be a substrate for the somatic hypermutation machinery (12). To determine whether the absence of Rad54 might affect somatic hypermutation in an H chain transgene, VV5:RAD54/− mice were immunized with Ars-KLH and titers of anti-Ars IgG were determined using ELISA analyses. As shown in Fig. 2, there was no detectable reduction in the levels of anti-Ars IgG in VV5: RAD54/− animals compared with both VV5:RAD54+/+ heterozygotes and parental VV5 mice. The ranges of titers and the average titers were quite similar for each group of transgenic animals regardless of the RAD54 genotype (Fig. 2). Thus, the absence of mouse Rad54 does not appear to cause any reduction in the extent of transgene isotype switching observed in the immunized VV5 animals.

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Results

To assess the role of the Rad54 protein in transgene isotype switching, somatic hypermutation, and transgene conversion, we crossed VV5 transgenic mice onto a RAD54 knockout background and genotyped offspring as described in Materials and Methods. VV5 transgenic mice have been described previously (12) and carry the VCμ H chain transgene that contains two closely linked VDJ segments (diagrammed in Fig. 1). The VDJ segments in VVCμ are highly homologous but differ at 17 nucleotide positions. The upstream VDJ segment in VVCμ is not transcribed because it lacks a promoter, whereas the downstream VDJ has a promoter and produces mRNA. In immunized VV5 mice, the μ transgene has been found to undergo isotype switching that results in the production of serum IgG derived from the transgene. Furthermore, some VV5 B cells from immunized mice exhibit sequence transfers between the VDJ segments that resemble gene conversions, and many transgene-expressing B cells in VV5 mice have been found to exhibit somatic hypermutation of the expressed transgene VDJ (12).

Previous reports have shown that Rad54 does not affect isotype switching of endogenous Ig H chain genes (27). To analyze the role of Rad54 in transgene isotype switching, VV5:RAD54/− mice were immunized with Ars-KLH and titers of anti-Ars IgG were determined using ELISA analyses. As shown in Fig. 2, there was no detectable reduction in the levels of anti-Ars IgG in VV5: RAD54/− mice as compared with both VV5:RAD54+/+ heterozygotes and parental VV5 mice. The ranges of titers and the average titers were quite similar for each group of transgenic animals regardless of the RAD54 genotype (Fig. 2). Thus, the absence of mouse Rad54 does not appear to cause any reduction in the extent of transgene isotype switching observed in the immunized VV5 animals.

To produce RT-PCR products for Southern blots splenic cDNA was amplified using nested PCR. The first round was amplified with the V gene leader specific primer mentioned above and a primer in Cγ (CCGAAACTTCACACACTGAT) and a Cy primer (GGAATTCGGGGCCATGGTAC) to produce RT-PCR products. Cloning was done using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA). Clones were sequenced at the Tufts University core facility using the leader specific primer mentioned above. Dot blot analyses of cloned PCR products were performed as described previously (13).

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transgene sequence transfers have occurred. This probe corresponds to the VDJ junction sequence present in both the R16.7 and 2B4 VDJ segments within the VVCμ transgene. A second probe is the R16.7 CDR2 oligonucleotide, which corresponds to the CDR2 of the transgene R16.7 VDJ segment and which is designed to detect VDJ segments that have not undergone transgene sequence transfers. Finally, the critical probe in this analysis is the 2B4 CDR2 oligonucleotide, which corresponds to the CDR2 of the 2B4 VDJ region in VVCμ and which is designed to detect transgene-derived mRNAs that have undergone sequence transfers.

The specificity of the RT-PCR/Southern blot assay for transgene conversion was confirmed by the analyses of control samples as shown in Fig. 4A. Four samples were used as controls; all of these were RT-PCR products from IgG-producing hybridomas derived from mice carrying the VVCμ transgene. The expressed VDJ regions in all of these hybrids were sequenced (data not shown). Three control samples (61E, 3A3, and 6A2) were from hybridomas in which the expressed VDJs exhibit gene conversion-like sequence transfers that encompass the CDR2, and these samples hybridized with the 2B4 CDR2 probe but not with the R16.7 CDR2 probe. The other control sample (8B2) was a hybridoma in which the expressed VDJ has not undergone any sequence transfer event, and this sample hybridized with the R16.7 CDR2 probe but not with the 2B4 CDR2 probe. All of the hybridomas hybridized with the TND probe. These controls demonstrate that the oligonucleotide probes have a high degree of specificity and that this RT-PCR/Southern blot approach can detect transgene sequence transfers.

The RT-PCR/Southern blot assay has been used to assess gene conversion-like sequence transfers in immunized VVCμ transgenic mice (13). Fig. 4A also shows a separate experiment for an individual animal (VV#5) and a control hybridoma. The hybridization band observed using the 2B4 CDR2 probe in the VV#5 sample indicated that transgene conversion had occurred in this immunized mouse. To demonstrate that the detected band does correspond to transgene conversion events, we sequenced 28 randomly selected cloned RT-PCR products from the VV#5 mouse. Fourteen of these clones contained inserts derived from the transgene VDJ segments and five of the fourteen clones displayed sequence transfers (Fig. 5) like those that have been previously reported for other immunized VVCμ mice (12, 13). All five of the RT-PCR products that had transgene conversion also had somatic mutations. These results are analogous to previous findings from sequences of hybridomas from VVCμ mice (12) that emphasize a correlation between the conversion and hypermutational processes in these transgenic animals.

The RT-PCR/Southern blot assay can clearly detect transgene sequence transfers in immunized VVCμ mice. However, previous studies have shown that somatic mutations in VDJ regions that have undergone sequence transfers can disrupt hybridization with the 2B4 CDR2 probe (13). Similar results were obtained when we used the 2B4 CDR2 probe to analyze dot blots of the clones described above; several of the sequenced clones that exhibited transgene sequence transfers did not hybridize with the 2B4 CDR2 probe (data not shown). However, Rad54 does not affect somatic hypermutation of endogenous Ig genes (31), and we have shown above that the same hypermutational levels and patterns are observed in VV5 mice that are either heterozygous or homozygous for the RAD54 mutation. This evidence indicates that mice with RAD54+/− and RAD54−/− backgrounds will exhibit similar frequencies of converted VDJ regions that are not detected in the PCR/blot assay. Therefore, using this assay to measure detectable sequence transfers allows a relative comparison of sequence transfer levels in groups of mice that differ only by the presence or absence of the RAD54 gene. Because transgene copy number or integration site might affect sequence transfer frequencies, we generated panels of transgenic RAD54+/− and RAD54−/− mice by breeding so that the copy numbers and integration sites were the same for the two types of mice.

The PCR/blot assay was used to analyze transgene conversion events in VV5 mice that were deficient for RAD54. Fig. 4B shows measurements of transgene sequence transfers in panels of immunized RAD54−/− and RAD54+/− VV5 mice as determined by the PCR/blot assay. Samples from immunized mice were used in the assays because it has been shown that sequence transfers occur at low frequency in VV5 mice and Ag selection provides for the most sensitive detection of these events (13). For the RAD54−/− VV5 mice (and also for the RAD54+/− VV5 mice), about half of the animals show sequence transfers in the assay, whereas the other half do not. The substantial percentage of mice showing no detectable sequence transfers (see Fig. 4B) suggests that only a few sequence transfers occur in those mice that do exhibit detectable transfers. Based on a Poisson analysis of the data sets, RAD54−/− VV5 mice show an average of 0.81 transfers in each mouse, whereas RAD54+/− VV5 mice show an average of 0.59 transfers; there is no significance to the difference between these calculated averages (p > 0.99 based on Fisher’s exact test). These findings suggest that RAD54 has little effect on sequence transfers in VVCμ mice.

To further assess sequence transfer events within these immunized mice, cloned PCR products were sequenced from animals 6,
Somatic hypermutation in RAD54<sup>−/−</sup> and RAD54<sup>+/−</sup> transgenic mice. A. Cloned PCR products derived from splenocyte IgG H chain mRNAs were sequenced and the number of somatic mutations in each clone were determined by comparison with the R16.7 VDJ sequence. Chain mRNAs were sequenced and the number of somatic mutations in each clone were determined by comparison with the R16.7 VDJ sequence.

**FIGURE 3.** Somatic hypermutation in RAD54<sup>−/−</sup> and RAD54<sup>+/−</sup> transgenic mice. A. Cloned PCR products derived from splenocyte IgG H chain mRNAs were sequenced and the number of somatic mutations in each clone were determined by comparison with the R16.7 VDJ sequence. Data sets are shown as spiral pie graphs with each segment representing the total number of clones represented in each graph. The types of nucleotide substitutions found in hypermutated VVC<sub>µ</sub> transgenes. Values represent frequencies of the total number of nucleotide changes found in sequences of cloned RT-PCR products obtained from the immunized mice as indicated.

9, and E in Fig. 4B. Shared patterns of somatic mutations among the clones (see supplemental material)<sup>4</sup> suggest that a single sequence transfer event had occurred in mouse E, two transfer events may have occurred in mouse 6, and three transfer events may have occurred in mouse 9. Thus, both the sequence analyses and PCR/Southern blot assays indicate only a small number of sequence transfer events in the individual immunized animals.

The PCR/Southern blot assays indicate average transfer frequencies that appear somewhat lower than the frequencies suggested by sequencing PCR products from individual animals. One possible explanation for this observation would be that some sequenced PCR products might come from sister cells within an animal yet show fewer or no shared mutations. Because VDJ junctions cannot be used to determine clonal relatedness in transgenic mice, relying on shared mutation patterns to indicate relatedness could lead to overestimating the number of sequence transfer events within an animal. It is also possible that, as we have shown previously (13), somatic mutations prevent some PCR products that have sequence transfers from being detected by the hybridization probes used in the Southern blots. We analyzed how undetected transfer events might impact our statistical consideration of the Southern blot/PCR assay. Somatic hypermutation rates are the same in RAD54<sup>−/−</sup> and RAD54<sup>+/−</sup> VV5 mice; therefore, transfer events undetectable due to mutations will occur at equal frequencies in these two types of mice. Overall, this will cause equal effects on the P(0) values for the data sets from each of the two types of mice; analyses indicate that adjusting for these effects would serve only to lessen differences between the average transfer frequencies calculated for the two strains. Because our data already indicate no significant differences between the average transfer frequencies in RAD54<sup>−/−</sup> and RAD54<sup>+/−</sup> VV5 mice, these calculations indicate that adjusting for undetected transfer events would not alter our conclusions about the similar sequence transfer frequencies in RAD54<sup>−/−</sup> and RAD54<sup>+/−</sup> mice.

To directly investigate whether some VVC<sub>µ</sub> mice having sequence transfers might be missed by the RT-PCR/Southern blot assay, we cloned and sequenced PCR products from three mice that did not exhibit a 2B4 hybridization band in Fig. 4B. For three animals (Fig. 4B, A, D, and G) we did not find any PCR products exhibiting sequence transfers (of 39, 17, and 41 total sequences, respectively). These data contrast with analyses of mice that showed 2B4 hybridization bands (Fig. 4B and supplemental material),<sup>4</sup> in which PCR products with sequence transfers were easily found (11 sequences with transfers of 35 total sequences for mouse 9; 7 of 35 for mouse E; and 4 of 41 for mouse 6). These results also indicate that undetected sequence transfers are not likely to have a significant impact on comparisons of sequence transfer frequencies in RAD54<sup>−/−</sup> and RAD54<sup>+/−</sup> mice.

Previous studies have shown that the lack of Rad54 in the chicken DT40 cell line reduces gene conversion frequencies by

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<sup>4</sup>The on-line version of this article contains supplemental material.
Probability that four of the nine RAD54 other mouse genes.

between chicken Ig genes or on homologous recombinations between frequencies would predict an average frequency of ~0.81 for RAD54−/− VV5 mice as determined above, an 8-fold reduction in sequence transfer frequencies would predict an average frequency of ~0.1 for RAD54−/− VV5 animals. Assuming this average frequency, the probability that four of the nine RAD54−/− VV5 mice analyzed in Fig. 4B would show sequence transfers is 0.0075. These results indicate that Rad54 has much less effect on sequence transfers within the mouse VVCμ transgene than on sequence transfers between chicken Ig genes or on homologous recombinations between other mouse genes.

Discussion

The Rad54 gene product is involved in homologous recombination and gene conversion in both yeast and higher species (17, 27, 29, 30). In vertebrates, Rad54 is important for the gene conversion processes that are central in the diversification of Abs in chicken B cells (30). Homologous recombination through gene conversion or other subpathways is important for transgene conversion and isotype switching of H chain Ab transgenes in mouse B lymphocytes and has been suggested to be important for somatic hypermutation. However, our results indicate that the Rad54 gene product is not required for transgene switching, hypermutation, or conversion-like sequence transfer, implying that these mouse B cell processes rely on the activity of other proteins involved in homology-based recombination or repair.

Transchromosomal isotype switching has been reported for a number of H chain transgenes (7, 8, 10, 33–36); however, the mechanisms involved in interchromosomal switching are not clear. Chromosomal translocations have been associated with transgene switching, and some transgene switch sites show features similar to normal class switch DNA recombinations (9). However, for some transgenes, interchromosomal switching shows features of homology-based recombination based on mechanisms that involve either gene conversion (8) or reciprocal strand exchange (7). Iso-

type switching of endogenous H chain genes does not appear to involve homologous recombination (2) and is not affected by the lack of Rad54 (27). This suggests that transgene switching might require two recombination events: one involving homology-based insertion of the transgene into the endogenous H chain locus and a second involving normal switch recombination to join the transgene VDJ region to a downstream CH region (7, 8, 14).

Our studies indicate that Rad54 is not required for VVCμ transgene isotype switching; this is consistent with the notion that interchromosomal switching might be mediated by the normal, Rad54-independent class switch mechanism. However, there is little evidence that the normal switching mechanism in mice might promote interchromosomal recombinations; only one such possible event has been reported (37), found in a transformed cell line that undergoes switching during cell culture. If, as seems more likely, transgene switching is a two-step process that begins with an interchromosomal homology-based recombination event, then our results indicate that this homologous recombination does not require Rad54.

Gene conversion is the dominant diversification mechanism for Ig genes in chicken B cells (38, 39). Ig gene conversion processes also occur in the chicken B cell line DT40 (40) and are dependent on Rad54 (30). Because gene conversion-like sequence transfers in VVCμ transgenic mice share some features of chicken Ig gene conversion (12), we and others (12, 41–43) have suggested that these processes might reflect a conserved mechanism that is present in all vertebrate B cells but that is used to greatly different extents in different species. However, based on the homology-based DNA repair activities that have recently been suggested to play a role in the somatic hypermutation process (15), transgene sequence transfers and somatic hypermutation might also be related. Because both transgene conversion and somatic hypermutation appear to be Rad54 independent, a relationship between these two processes may be more likely. However, we cannot rule out the possibility that the murine sequence transfer mechanism and the conversion process in chickens did arise from a common mechanism but that, in mammals, there may be redundant proteins to provide Rad54 function (44).

Based on the correlation between transgene conversion and somatic hypermutation in VVCμ transgenic mice, we have previously proposed that the somatic hypermutational mechanism might involve error-prone, homology-based sequence transfers between identical Ig gene sequences present on sister chromatids (14). Following a variety of recent findings, we propose refining this model to include the notion that the introduction of DNA breaks into Ab gene V(D)J segments (15, 45–48) will trigger processes that can repair these breaks and, at the same time, diversify Ab V regions. It also seems possible that more than one mechanism might be involved in the repair of these breaks.

Ab gene VDJ breaks might be repaired by a process that is based on gene conversion between homologous but nonidentical sequences and that involves the Rad54 protein. This type of mechanism appears to be important in the diversification of Ab genes by gene conversion in species such as chickens, rabbits, and cows. In contrast, VDJ breaks might also be resolved by homology-based DNA repair between identical sequences on sister chromatids. This hypothesis is an extension of our previous model and is supported by the finding that, in mice, the DNA breaks in VDJ regions are introduced during the G2 stage of the cell cycle (15), when DNA breaks are commonly repaired by homology-based mechanisms. If error-prone processes are part of this homology-based repair, then the “untemplated” mutations that are characteristic of somatic hypermutation could result, at least in part, from such a mechanism. Previous studies have indicated that somatic hypermutation is Rad54 independent (31) and thus would suggest that this hypothesized homology-based repair also would not require Rad54. In species where Ig gene conversions are rare, this error-prone, homology-based somatic hypermutation mechanism would dominate repair of VDJ DNA breaks. The regulation of the mechanisms used to repair VDJ DNA breaks might well reflect the developmental
timing of break induction and the levels of proteins available for the repair processes. This could explain species differences in Ab diversification mechanisms and is consistent with recent studies showing changes in Ig gene diversification mechanisms that depend on the particular DNA repair proteins present (23).

Finally, we propose that transgene conversion events in mice represent side reactions of an error-prone, homology-based somatic hypermutation machinery. We envisage that gene conversion-like transgene sequence transfers result from the repair of DNA breaks introduced into the transgene VDJ and that this repair mechanism uses essentially the same enzymes involved in somatic hypermutation. In general, these side reactions would be predicted to occur only infrequently because the interacting sequences are not identical. In VVCμ transgenic mice, we have found that such low-frequency events can be greatly amplified because of a favorable transgene structure together with preferential antigenic selection of the cells that have undergone sequence transfers (13).

Our proposed model does not imply that all untemplated somatic mutations in Ab VDJ regions are introduced by error-prone homology-based repair. This may be only one of several mechanisms that are involved in somatic hypermutation; previously reported studies have already suggested the possibility of more than one hypermutational mechanism (49–52). The capacity of VVCμ mice to provide sensitive detection of homology-based DNA sequence transfers suggests that additional genetic contexts with mice that lack other DNA repair proteins could help to define those factors that are important for both transgene conversion and, potentially, hypermutated DNA repair.

Acknowledgments

We thank Smita Ramanadham and Jess Janowski for help with sequence analyses and Dr. Naomi Rosenberg for critical reading of the manuscript.

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