Transcription-Dependent Somatic Hypermutation Occurs at Similar Levels on Functional and Nonfunctional Rearranged IgH Alleles

Laurent Delpy, Christophe Sirac, Caroline Le Morvan and Michel Cogné

*J Immunol* 2004; 173:1842-1848;
doi: 10.4049/jimmunol.173.3.1842
http://www.jimmunol.org/content/173/3/1842

References

This article cites 29 articles, 8 of which you can access for free at:
http://www.jimmunol.org/content/173/3/1842.full#ref-list-1

Subscription

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Transcription-Dependent Somatic Hypermutation Occurs at Similar Levels on Functional and Nonfunctional Rearranged IgH Alleles

Laurent Delpy, Christophe Sirac, Caroline Le Morvan, and Michel Cogné

Allelic exclusion of IgH chain expression is stringently established before or during early B cell maturation. It likely relies both on cellular mechanisms, selecting those cells in which a single receptor allows the best possible Ag response, and on molecular restrictions of gene accessibility to rearrangement. The extent to which transcriptional control may be involved is unclear. Transcripts arising from nonfunctional alleles would undergo nonsense-mediated degradation and their virtual absence in mature cells cannot ensure that transcription per se is down-regulated. By contrast, somatic hypermutation may provide an estimate of primary transcription in Ag-activated cells since both processes are directly correlated. For coding regions, the rate and nature of mutations also depend upon Ag binding constraints. By sequencing intronic sequence downstream mouse VDJ genes, we could show in the absence of such constraints that somatic hypermutation intrinsically targets nonfunctional alleles at a frequency approaching that of functional alleles, suggesting that transcription also proceeds on both alleles at a similar rate. By contrast and confirming the strong dependency of somatic hypermutation upon transcription, we show that artificial blockade of transcription on the nonfunctional allele by a knock-in neomycin resistance cassette keeps the VDJ region unmutated even when its promoter is intact and when it is fully rearranged.

Received for publication August 12, 2003. Accepted for publication April 26, 2004.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by grants from Réseau SWITCH, Ligue Nationale Contre le Cancer, and Conseil Régional du Limousin.

2 L.D. and C.S. contributed equally to this work.

3 Address correspondence and reprint requests to Prof. Michel Cogné, Centre National de la Recherche Unité Mixte de Recherche 6101, Laboratoire d’Immunologie, 2, rue du Dr. Marcland, 87025 Limoges, Cedex, France. E-mail address: cogné@unilim.fr

4 Abbreviations used in this paper: SHM, somatic hypermutation; FR, framework region; PP, Peyer patch; PNA, peanut agglutinin; AID, activation-induced cytidine deaminase.

Copyright © 2004 by The American Association of Immunologists, Inc.
IgH locus, a linear correlation between expression and mutability has been reported (17–19).

**V(D)J rearrangement and presence of a V promoter are known as mandatory for high-level SHM (18, 20). Although unarranged V segments and incomplete DJ rearrangements are transcribed at low levels in immature B cells, V to DJ approximation boosts transcription by positioning one V_{JH} promoter under the control of the Eμ enhancer (reviewed in Ref. 21). Regarding allelic exclusion, the relative transcription rate of Ig loci in wild-type B cells carrying two VDJ rearrangements has not been studied; by contrast, the presence of somatic mutations within out-of-frame rearranged V genes has been documented (22, 23). Since functional and nonfunctional Ig sequences are strongly related and cannot be individually studied by the means of hybridization techniques, we thus decided to use the accumulation of mutations for the quantitative evaluation of the relative transcription rates of both alleles in primary B cells that carry double VDJ rearrangements. Mutations are known to freely accumulate in the framework regions of non-functional allele (24).

Materials and Methods

Mice

C57BL/6 wild-type mice (wt “b” allotype) were mated either with 129/Ola wild-type mice (wt “a” allotype), providing the control wild-type a/b mice. C57BL/6 mice were also mated with the B cell-deficient mouse line J_{Pa}, Neo-Eμ, which carries a homozygous disrupted a allotype IgH locus. The J_{Pa}, Neo-Eμ mutation features the insertion of a neomycin resistance cassette at the Nael site immediately downstream of J_{Pa} (Fig. 1A). This mutation is known both to preserve the occurrence of V(D)J recombination and to result in a complete transcriptional silencing of rearranged V(D)J segments (25). Heterozygous wtJ_{Pa}, Neo-Eμ mice were selected, in which all B cells carried a functional b allotype IgH locus and a nonfunctional a allotype IgH locus.

Cell cytometry and sorting procedures

Peyer patches (PP) were recovered by dissection from the small intestine. Cell suspension was prepared by pressing PP through a nylon mesh and cells were washed three times in cold DMEM containing 10% FCS (In-vitrogen, Gaithersburg, MD). Cell suspensions were adjusted at 10^6 cells/ml after elimination of clumps of dead cells. Cells were incubated with anti-B220-SPRD for 30 min at 4°C. After two washes with PBS containing 10% FCS, FITC-conjugated peanut agglutinin (PNA) was added and incubated on ice for an additional 30 min, before washing and resuspension in medium. Purification of B220^+ PNA^{b/b} and B220^-PNA^{a/a} cells was realized on a FACSVantage (BD Biosciences, Franklin Lakes, NJ). The purification rates of these cell populations were constantly above 95% (Fig. 1B).

**DNA extraction and amplification**

Genomic DNA was extracted from PNA^{b/b} B cells using the QIAamp Tissue kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. DNA was amplified by PCR in 50 μl of Taq 1× PCR buffer containing 200 μM of each dNTP, 100 ng of each primer, and 1 U Taq polymerase (Pharmacia, Piscataway, NJ), with the following program: 1 cycle at 94°C for 5 min, 35 cycles (94°C for 45 s, 58°C for 45 s, and 72°C for 45 s), and 1 cycle at 72°C for 7 min. The following primers were used: forward V_{H183}, 5'-CGGTACCAAAGAASAMCCTGTWWCCCTGCAAATG - 3', corresponding to an intronic sequence between the J_{H2} and J_{H3} segments; 3', Nael, 5'-TCTCTAAAGGCTCTGAGATCCC-3', corresponding to an intronic sequence downstream of rearranged VDJ junctions were amplified using a forward V_{H183} family

![FIGURE 1. Strategy for the analysis of mutations in PP lymphocytes. A, PCR strategy for the analysis of V(D)J rearrangements involving V_{H183} family gene segments and the J_{H2} or J_{H3} segment in wild-type mice (V_{H183},-3'J_{H2} PCR) and involving the various J_{H} segments in g_{H,Neo-Eμ} b^- mice (V_{H183},-3' Nael PCR). B, Sorting of PP germinal center B cells after staining with B220-SPRD and PNA-FITC. Cells from the B220^-/ PNA^- gate enriched in centroblasts were sorted and used for PCR cloning of V(D)J genes.](http://www.jimmunol.org/)

**Results**

Functionally and nonfunctionally rearranged alleles accumulate mutations at close rates in B cells from wild-type mice

PP PNA^{b/b} B cells from wt a/b mice were sorted and checked for hypermutation. To appreciate SHM in these cells regardless of their Ag specificity, intronic sequences downstream of rearranged VDJ junctions were amplified using a forward V_{H183} family

![Downloaded from http://www.jimmunol.org/ by guest on April 29, 2017](http://www.jimmunol.org/)
FIGURE 2. Mutations in functional vs nonfunctional rearrangements from wild-type and from e^{HNeo-E} mice. A. Intron sequences directly flanking the \( J_H \) segment of \( V_H DJ_H \) genes cloned from wild-type B cells were classified into two groups depending on whether they came from genes with a functional or a nonfunctional \( V(D)J \) segment. B. Intron sequences flanking the \( J_H \) segment of rearranged \( V_H DJ_H \) genes cloned from e^{HNeo-E} B cells in which sequences from the wild-type allele are functional while sequences from the knock-in allele are allelically excluded. JH1GL, JH2GL, and JH3GL represent the germline unmutated sequences; exon sequences are in bold.
### Functional Sequences

<table>
<thead>
<tr>
<th>V190</th>
<th>V191</th>
<th>V192</th>
<th>V193</th>
<th>V194</th>
<th>V195</th>
<th>V196</th>
</tr>
</thead>
<tbody>
<tr>
<td>G<strong>A</strong></td>
<td>C<strong>A</strong></td>
<td>C<strong>G</strong></td>
<td>A<strong>G</strong></td>
<td>C<strong>A</strong></td>
<td>G<strong>T</strong></td>
<td>C<strong>G</strong></td>
</tr>
</tbody>
</table>

### Non-Functional Sequences

<table>
<thead>
<tr>
<th>V901</th>
<th>V902</th>
<th>V903</th>
<th>V904</th>
<th>V905</th>
<th>V906</th>
<th>V907</th>
</tr>
</thead>
<tbody>
<tr>
<td>G<strong>C</strong></td>
<td>A<strong>G</strong></td>
<td>C<strong>A</strong></td>
<td>C<strong>G</strong></td>
<td>G<strong>A</strong></td>
<td>A<strong>G</strong></td>
<td>C<strong>G</strong></td>
</tr>
</tbody>
</table>

*FIGURE 2. (Continued)*
framework region (FR) 3 consensus primer along with a backward primer located in the intron downstream of J_{H2}

Sequence analysis focused on the rearranged J_{H2} segment and the following intronic 180-bp sequence, a region known to be strongly targeted by the SHM process (8). This sequencing strategy also avoided ambiguities for the assignment of V sequences to their germline counterparts. Mutations were counted either only in the intronic sequence or in the complete sequence including the J_{H} encoded fourth FR (FR4). Sequencing also allowed us to check VDJ junction sequences for functionality and to classify them into two groups: functional sequences featured an in-frame rearrangement with no stop codon created at the VD or DJ junction and nonfunctional sequences either carried out-of-frame junctions or included premature termination codons within the D segment.

Of 50 functional and 23 nonfunctional sequences analyzed from PP PNA^{high} B cells, somatic mutations were readily observed on both classes of rearranged alleles in wild-type animals (Figs. 2A and 3). SHM reached the level of 13.1 mutations/1000 bp for functionally rearranged genes and 11.9 mutations/1000 bp for nonfunctionally rearranged alleles (Table I). This difference was not statistically significant. Since our sequencing interval included the J_{H} region encoding the V domain FR (FR4) for which substitutions might be counterselected in expressed functional genes, we also restricted the comparison to intronic sequences flanking the rearranged J_{H}. This comparison again indicated similar mutation frequencies of 13.6 and 11.1%, respectively, for the functional and the nonfunctional sequences. In both cases, the frequency was not significantly lower for nonfunctional genes (Student’s t test, p > 0.05).

Untranscribed rearranged VDJ gene segments do not undergo SHM on the targeted allele of heterozygous wt/J_{H}-Neo-Eμ germinal center B cells

Although preserving V(D)J recombination to J_{H1}, J_{H2}, and J_{H3}, the J_{H}-Neo-Eμ mutation blocks transcription of both germline J_{H} and rearranged DJ_{H} or VDJ_{H} segments (24). Although B cell development is arrested in homozygous J_{H}-Neo-Eμ mutant mice (24), using heterozygous mice with a single knock-in IgH allele allows normal B cell maturation and warrants that all functionally expressed sequences originate from the wild-type allele. Since the J_{H}-Neo-Eμ knock-in features the insertion of a neomycin resistance cassette at a Nαel site located ~100 bp downstream of J_{H4}, it could be used as a “tag” allowing to easily distinguish sequences from the knock-in allele or from the functional allele with a wild-type Nαel site.

As for wild-type mice, PP PNA^{high} B cells from heterozygous wt/J_{H}-Neo-Eμ mice were sorted and checked for hypermutation. Although the disrupted IgH locus was from the a allotype, heterozygous mice obtained after breeding with C57/B6 mice provided a wide-type b allotype IgH locus. By force, B cells of such mice all carry a functionally rearranged b allotype J_{H} region and an allelically excluded a allotype locus. Since a number of B cells carry double complete VDJ rearrangements, it was readily possible from these cells, to amplify rearranged sequences originating from both alleles. To that goal, a unique V_{7710}5V consensus forward primer was used along with a backward primer either specific for the knock-in neo gene downstream of J_{H4} or specific for the wild-type J_{H} 3′ flanking intron. In addition, the use of heterozygous IgH a/b allotype mice in which known allelic differences are located in the J_{H} germline region provided a further control of the origin of cloned sequences. The respective mutation frequencies on both a functional b wt allele and a rearranged but untranscribed a allele carrying the J_{H}-Neo-Eμ mutation could finally be appreciated in identical conditions and in the very same PNA^{high} B cell samples (Fig. 2B).

A complete lack of somatic mutation of rearranged J_{H} sequences was noticed on the untranscribed neo knock-in allele, reaching 1.1 mutations/1000 bp (i.e., close to the range of mutations introduced by the Taq polymerase enzyme in PCR experiments) instead of a simultaneous 30 mutations/1000 bp on the wild-type b control allele (Table I). The J_{H}-Neo-Eμ knock-in thus significantly blocked SHM of nonfunctional alleles carrying VDJ rearrangements (Student’s t test, p < 0.02).

Discussion

In transgenic models, the hypermutation mechanism is known to act only on gene copies that are demethylated as well as transcribed (15–19, 26). Although endogenous alleles of Ig loci may show some asymmetry with regard to accessibility, DNA methylation, and pattern of early replication, it has not been clearly ascertained whether the processes of primary transcription and of SHM preferentially target the actively expressed allele. Mutations were previously observed in FRs of nonfunctional rearranged Vλ genes but their frequency could not be compared with that of functional genes in which FR mutations are negatively selected (22).

**FIGURE 3.** Comparison of hypermutation in functional and nonfunctional IgH alleles from wild-type (upper panel) and from J_{H}-Neo-Eμ mice (lower panel). The data are presented as pie charts and indicate the proportion of sequences that contain 0, 1, 2, etc., mutations in the rearranged I_{H} segment and the succeeding intronic 180-bp sequence.
By analyzing noncoding sequences which are not subjected to Ag-driven pressure and cell selection, we studied whether the SHM machinery by itself may similarly target sequences appropriately located close to a VDJ junction on both the functional and the allelically excluded alleles of the IgH locus.

The herein reported data show that in a given B cell population, SHM indeed targets both alleles at levels that are roughly similar. These data thus provide indirect but clear evidence that the numerous B cells which spontaneously carry double VDJ rearrangements in wild-type animals also transcribe both alleles at comparable rates in mature B cells, at least in those cells that are undergoing the SHM process. In addition and in agreement with the clear links previously demonstrated by other studies between the rate of transcription and the level of SHM of Ig genes, we show that a mutation blocking V(D)J transcription on the excluded allele also prevents the recruitment of the SHM machinery on that allele. Although a similar reduction in SHM was previously observed upon deletion of the pVH promoter (18), our data show that not only the presence of the promoter upstream of a rearranged V gene, but also active transcription from this promoter is required for SHM. These results also fall in agreement with recent in vitro observations showing that deamination of cytosines by the AID enzyme needs transcription of its DNA substrate (13, 14).

It may be difficult to reconcile these observations with the previously published data about the early asymmetry of Ig alleles which appears to be established even before commitment to the B cell lineage (2, 3). However despite their asymmetry, κ loci were recently shown to be germline transcribed on both alleles (27). Our data suggest that the asymmetry in replication pattern and nuclear localization that has been observed for IgH alleles is either an inconstant feature of Ig loci or does not result in a significant blockade of transcription of nonfunctionally rearranged alleles. Cells carrying complete VDJ rearrangements of both IgH loci thus show allelic inclusion at the transcriptional level, at least up to the stage of germinal center B cells undergoing SHM. It thus seems that approximation of a Vκ promoter to the intronic Eκ enhancer is sufficient for transcription to occur and be maintained, whatever the allele involved, then overriding the potential extinction previously imposed by late replication or nuclear localization allelic asymmetry. Alternatively, it is also conceivable that only a certain proportion of B cells display asymmetric accessibility of Ig genes, while others would undergo symmetric activation of Ig loci. Dual complete VDJ rearrangement would then only occur in those cells that have escaped the rule of allelic asymmetry. Consequently, only the latter cells could have the ability to fully rearrange both IgH loci and to display allelic inclusion at the transcriptional level. However, the observation that knock-in mice carrying two functional VDJ rearrangements express dual BCR on most B cells argues against the idea that allelic asymmetry could definitely retain expression on one allele in a significant proportion of mature or activated B cells (28). Rather, asymmetry could be restricted to the pro-B/pre-B cell stage and only account for the preferential rearrangement of one allele. Both our data and those from dual BCR-expressing mice with a double knock-in of functionally rearranged genes (28) clearly indicate that for IgH genes, double rearrangements result in allelic inclusion at the transcriptional level and that allelic asymmetry is either unable to completely block transcription or that this blockade is relieved by VDJ rearrangement. Importantly and further indicating transcriptional competence of both IgH alleles in mature B cells, germline transcripts from constant genes that precede class switching were also shown to arise from both the expressed and the “allelically excluded” IgH locus (29).

The major conclusion of the present work is that whether they are functional or not, rearranged VDJ genes undergo SHM at roughly similar rates. By contrast, preventing transcription of an allelically excluded VDJ blocks its accessibility to SHM. It can be inferred from these observations that allelic exclusion of IgH expression in normal B cells is not effected at the transcriptional level for the numerous mature cells carrying double VDJ rearrangements. The early asymmetry in the replication pattern or nuclear localization observed for Ig loci could thus have limited effects, acting only by down-regulating V to DJ rearrangement in pro-B cells. Later on and in a number of selected B cells, allelic asymmetry could also be strongly reinforced secondarily to VDJ rearrangement, as a consequence of the asymmetric activation of a single Vκ promoter in those cells with a single V to DJ rearrangement. Both our data and those showing allelic inclusion in mice with double knock-in of functional VDJ segments favor the hypothesis that allelic exclusion is mostly effected by the stochastic process of VDJ rearrangement. Since in-frame rearrangements encoding a functional H chain able to pair with its L chain partner constitute rare events, they are highly unlikely to occur on both alleles and to be simultaneously expressed at the protein level. Our findings that nonfunctionally rearranged alleles are not silenced at the level of VDJ transcription further demonstrates that, by contrast to cytokine genes, transcriptional asymmetry plays little role in the allelic exclusion of Ig gene expression. Downstream of transcription, these findings underline the importance of RNA surveillance pathways that degrade nonfunctional mature Ig mRNAs, thus preventing the “wastage” that would result from their translation into truncated Ig chains. They also confirm that in the course of B cell malignancies, both Ig loci may be accessible to mutations, recombinations, and translocations.

Acknowledgments
We thank Vincent Denis and Claire Carrion for their kind technical assistance.

References


1848 HYPERMUTATION OF ALLELICALLY EXCLUDED V GENES