B cell biology and the development of mature B cell lymphomas
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Immunoglobulin diversification in B cell malignancies: internal splicing of heavy chain variable region as a by-product of somatic hypermutation

Richard J. Bende, Wilhelmina M. Aarts, Steven T. Pals and Carel J.M. van Noesel

In this study we describe alternative splicing of somatically mutated immunoglobulin (ig) variable heavy chain (VH) genes in three distinct primary B cell non-Hodgkin’s lymphomas (B-NHL). In two V4–34 expressing lymphomas, ie a post-germinal center type B cell chronic lymphocytic leukemia (B-CLL) and a follicular lymphoma (FL), internally spliced VH gene transcripts were found in which a sequence stretch of 116 bp between the framework region 1 (FR1) and an alternatively spliced region 2 (CDR2) had been deleted. We provide evidence that for this alternative IgVH mRNA processing a known cryptic 5’ splice donor site and a previously unidentified cryptic 3’ splice acceptor site were used. Site-directed mutagenesis showed that the cryptic 3’ splice acceptor site had been activated by specific somatic point mutations. The B-CLL further harbored a triplication of the rearranged JH3 gene segment including the putative N region and part of the JH3-JH4 intron sequence. This triplication probably took place via a repeated mechanism of DNA double strand break followed by homologous recombination, a mechanism which was recently proposed also involved in the somatic hypermutation process and is compatible with the post-germinal center derivation of this B-CLL. Finally, in a V4–34 expressing diffuse large B cell lymphoma, we observed alternative IgVH mRNA processing using the same cryptic 5’ splice donor site and the normal splice acceptor site of the CH1-C2 exon. The significance of alternative IgVH processing in B cell malignancies and as a potential mechanism of somatic ig diversification is discussed.

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Keywords: Ig gene; somatic hypermutation; alternative splicing; B cell; lymphoma

Introduction

During early B cell development in the bone marrow, IgV regions of both heavy (H) and light (L) chain genes are formed by recombination of V, (D) and J gene segments. The potential recombinatorial repertoire, that includes the ‘random’ pairing of IgH and IgL chains, is significantly enlarged due to imprecise joining of the different gene segments. This junctional diversity depends largely on the enzyme terminal deoxynucleotidyl transferase (TdT), that is responsible for the addition of non-templated so called N-nucleotides to the V(D)J junctions.1,2 In mature B cells, the IgV regions can be further diversified, particularly in the course of T helper cell-dependent antigen-specific responses in germinal centers (GC). Here, the Ig loci may be altered by somatic hypermutation and IgH chain class-switch recombination. It has recently been reported that the somatic hypermutation process, that is believed to occur in the GC environment exclusively,5,6 not only involves single nucleotide exchanges but also, to a significant extent, deletions and insertions of different size.7,8 Furthermore, as the RAG genes may be expressed during the GC stage, it has been proposed that secondary Ig rearrangements may rescue GC B cells with unfavorable somatic mutations, a process also designated as receptor revision. Indeed, in both mice and humans recombination intermediates have been detected in GC B cells.9–11 Although most examples of receptor revision involved IgVL genes, it has also recently been demonstrated in human IgVH genes.12,13 It is currently believed that, due to the occurrence of these complex genetic alterations, the GC reaction plays a role in lymphomagenesis. This notion is supported by the fact that the majority of non-precursor B-NHL entities express somatically mutated V genes, compatible with a GC or post-GC derivation.14 In follicular lymphoma (FL), the cytological and architectural characteristics as well as the immunophenotype are all highly compatible with a GC derivation.15,16 B-CLL was initially considered to be derived from naive B cells.17,18 However, Fais et al19 recently reported that, within a large panel of B-CLL, approximately 50% of the IgM+ B-CLL and 75% of the non-IgM+ B-CLL cells exhibit somatic mutations in their IgVH genes. Moreover, similar to normal (post) GC B cells20,21 and (post) GC-derived B cell malignancies,22,23 the BCL-6 gene was found to be somatically mutated in a fraction of the IgVH mutated B-CLLs.24 These data also indicate that a substantial proportion of B-CLL derive from GC-experienced B cells. Furthermore, these findings suggest that within the clinicopathological group of B-CLL, at least two separate entities are included. In accordance, two recent studies indicated that patients with somatically mutated B-CLL IgV genes have a significantly better prognosis than those with unmutated IgV genes.25,26 Here, we present IgVH gene analyses of three distinct GC-derived B cell malignancies in which we observed alternative processing of the rearranged and somatically mutated V4–34 IgVH gene segment. We provide evidence that the internal IgVH mRNA splicing observed in a B-CLL and in a FL was not due to a defect in the splicing machinery of the tumor cells but caused by specific somatic nucleotide substitutions which activated a previously unidentified cryptic 3’ splice acceptor site present in some germline IgVH gene segments.

Materials and methods

Patient material

One patient was a 76-year-old female who suffered from B-CLL accompanied by severe auto-immune hemolytic anemia (AIHA), for which she was splenectomized. Fresh frozen spleen tissue was obtained from the Department of Pathology, the Westeinde Hospital, The Hague, The Netherlands. The second patient (FL13) was a 78-year-old male who suffered from lymphadenopathy; a supraclavicular lymph node was removed on which a follicular lymphoma was diagnosed.27 The third patient (DLBCL4) was a 77-year-old male who suffered from diffuse large B cell lymphoma which was diag-
nosed on a groin lymph node. The second and the third patient were from our hospital.

DNA, RNA isolation and cDNA synthesis

DNA and RNA was isolated using the DNAZOL or the TRI-ZOL reagent, respectively (Life Technologies, Breda, The Netherlands) according to the manufacturer’s instructions. cDNA was synthesized with Pd(N)6 random primers (Pharmacia Biotech, Roosendaal, The Netherlands) or for some experiments with an oligo-dT primer (Promega, Leiden, The Netherlands) as described.

PCR reactions

The complementary determining region 3 (CDR3) was amplified using a forward primer specific for the framework region 3 (FR3) in combination with one of the different downstream primers specific for JH, Cμ, Cγ, Ca or Cd. For the VH family-specific PCR, the reactions were performed with VH family-specific leader primers combined with the appropriate reverse primer, either JH, Cμ, Cd or the B-CLL specific FR3 reverse primer. The different PCRs were performed with 1 μl of cDNA or 500 ng genomic DNA in a volume of 25 μl. Primer sequences were as described; B-CLL FR3 5′-CGGCCGGTCACACAGAGCCTAA-3′

Cloning and sequencing

PCR products were cloned into pTOPO vectors and transformed into TOP10 bacteria, according to the manufacturer’s instructions (Invitrogen, Groningen, The Netherlands). The colonies were sequenced on both strands. Sequencing was performed on an ABI sequencer (Perkin Elmer Corporation, Norwalk, CT, USA) using the big dye-terminator cycle-sequencing kit. The sequences found were compared to published germline sequences, using the VBase database and for some experiments with an oligo-dT primer (Promega, Leiden, The Netherlands) as described.

Expression vectors

The IgH(y3) expression vector was produced by modifying the pRTM1 vector. The IgH expression vector consists of a rearranged V(D)J gene flanked by Clal and SpeI restriction sites, which are used to introduce the IgVH gene of interest. Downstream, the Cγ3 constant gene is located. The expression of the recombinant IgH is regulated by normal human IgH promoter and enhancer sequences. For selection in mammalian cells the neomycin phosphotransferase gene is present, which allows selection of stably transfected cells with geneticin. The IgL(κ) expression vector was produced starting from the pSVG-Vx3 vector. The IgL(κ) expression vector consists of a rearranged Vλ gene flanked by Clal and NcoI restriction sites. These restriction sites are used to introduce the IgVL gene of interest. The constant gene is located downstream. The expression of the recombinant Igκ is regulated by normal human Igκ promoter and enhancer sequences. Detailed description of the generation of these immunoglobulin expression vectors is available on request.

The pRTM1 and the pSVG-Vx3 vectors were kindly provided by Dr J Kipps (University of California, San Diego, CA, USA). The IgH(y3) and IgL(κ) expression vectors were kindly provided by Drs J van Es and T Logtenberg (University Hospital, Utrecht, The Netherlands).

Generation of transfectants in SP2/0 cells

For transfection, 10 μg IgH(y3) and 10 μg IgL(κ) were linearized with PvuI and mixed with 10 × 10⁶ SP2/0 myeloma cells in Iscove’s modified Dulbecco’s medium (IMDM) containing 10% FCS (Hyclone, Logan, UT, USA), 100 IU/ml penicillin and 100 μg/ml streptomycin. The suspension was electroporated in a 0.4 cm gap cuvette using a Biorad gene pulser with capacity extender at 960 μF and 250 V. The cells were selected and cultured in IMDM complete medium containing 400 μg/ml geneticin (Life Technologies).

Nucleotide reversion of the somatic mutation in codon 64 of the B-CLL

Primers were designed that matched around codon 64, one primer included one mismatch which reverses the somatic mutation in codon 64 to the germline nucleotide. The V4–34 fragment of the B-CLL was amplified in two separate parts: the 5′ part with the VH4 family-specific primer in combination with B-CLL-R containing the mismatch that reversed the somatic mutation; the 3′ part was amplified with the B-CLL-F primer combined with the JH primer. These PCRs were performed according to the protocol used for the VH family-specific PCR except that Pfu polymerase was used (Stratagene, La Jolla, CA, USA). The two parts were purified, combined and used as template in a VH4-specific PCR. Bacterial clones were generated and sequenced to confirm the reversion of the somatic mutation in codon 64. Next, this B-CLL V4–34 fragment was cloned into the IgH(y3) expression vector as described above. Primer sequences were as follows: B-CLL-F 5′-GAGTCACTATTACGTTGGAC-3′; B-CLL-R 5′-GACCTCTGAGCGAGCGGTTG-3′. The underlined nucleotide is a mismatch which reverts the somatic mutation in codon 64.

Results

Assessment of clonality, IgH chain isotype expression and Vμ, Vγ, and Vκ gene family usage of the B-CLL

Clonality of the B-CLL was demonstrated immunohistochemically and confirmed by Southern blot, using a JH probe (data not shown) as well as by a CDR3-specific PCR. This PCR makes use of clone-dependent length differences of the CDR3 regions in the Vμ chains. A CDR3-PCR of a polyclonal B cell population yields products of variable size, visible as multiple bands or a smear pattern on agarose gel, whereas a clonal B cell population yields a single band. The CDR3 region of the rearranged immunoglobulin IGH locus was amplified from both genomic DNA and complementary DNA (cDNA), using a combination of the FR3 and the JH primer. To determine the constant IgH chain (CH) isotype of the B-CLL, cDNA was also amplified using the FR3 primer in combination with different CH-specific primers (Cμ, Cδ, Cγ and Ca). Unexpectedly, with the FR3–JH primer combination, from both cDNA and genomic DNA three clonal products of 112 bp, 184 bp and 297 bp were amplified (Figure 1b). With FR3–CH primer
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Figure 1  CDR3 and VH4 family PCRs on the B-CLL. (a) Schematic representation of the IgH locus and the primers used for the V\text{\textsubscript{H}}-family and CDR3-specific PCRs: L, leader sequence; V\text{\textsubscript{H}}, variable gene segment; D, diversity gene segment; J\text{\textsubscript{H}}, joining gene segment; C\text{\textsubscript{H}}, constant gene segment; N, non-templated nucleotide additions. (b) Results of CDR3 PCR on cDNA and genomic DNA, using the FR3 upstream primer and J\text{\textsubscript{H}}, C\text{\textsubscript{H}}/H9262, C\text{\textsubscript{H}}/H9253, C\text{\textsubscript{H}}/H9251 and C\text{\textsubscript{H}}/H9254 downstream primers. Indicated are the clonal products of 297 bp, 184 bp and 112 bp using the J\text{\textsubscript{H}} primer on cDNA and genomic DNA (lanes 1 and 6). (c) Results of VH4 family PCR on cDNA and genomic DNA using the upstream VH4 leader primer and J\text{\textsubscript{H}}, C\text{\textsubscript{H}}/H9262 and C\text{\textsubscript{H}}/H9254 downstream primers. Indicated are the four different PCR products of 516 bp, 588 bp, 701 bp and 1071 bp obtained on genomic DNA with the J\text{\textsubscript{H}} primer.

combinations, a sharp band was only observed with the C\text{\textsubscript{H}} and C\text{\textsubscript{\textalpha}} primers, which was in accordance with the immunohistochemically demonstrated co-expression of IgM and IgD. With both the C\text{\textalpha} and C\text{\textalpha}\text{\textgamma} primers, a smear pattern was obtained, most likely derived from polyclonal non-neoplastic B cells and/or plasma cells. Formally, however, the presence of a minor subpopulation of class-switched tumor cells cannot be ruled out (Figure 1b). Subsequently, the IgV genes were amplified with family-specific V\text{\textsubscript{T1}} or V\text{\textalpha} leader primers in combination with the J\text{\textsubscript{H}}, C\text{\textsubscript{\textmu}} and C\text{\textalpha} or J\text{\textsubscript{\textkappa}} and C\text{\textkappa} downstream primers, respectively. On genomic DNA, only J\text{\textsubscript{H}} or J\text{\textsubscript{\textkappa}} downstream primers were used in view of the presence of J\text{\textsubscript{H}}-CH and J\text{\textsubscript{\textkappa}}-C\text{\textsubscript{\textkappa}} intronic sequences. The V\text{\textsubscript{\textmu}} family-specific PCRs revealed that the B-CLL expressed a VH4 family gene segment (data not shown). The PCR on genomic DNA with the VH4 leader primer in combination with the J\text{\textsubscript{H}} primer yielded three clear products of 516, 588 and 701 bp, respectively, and a larger, albeit weaker product of 1071 bp (Figure 1c). The same PCR on cDNA also resulted in at least three clear bands. With the C\text{\textsubscript{\textmu}} downstream primer, a 473 bp product and a weaker 357 bp product were visible, whereas with the C\text{\textalpha} primer only one product was detectable (Figure 1c). The general length difference between the IgV\text{\textsubscript{T1}} family PCR products generated from either genomic DNA or cDNA is explained by the 83 bp leader intron present in genomic DNA only.

By V\text{\textalpha} family-specific PCR and sequencing, it was determined that the B-CLL expresses a V\text{\textalpha}1 family gene segment (data not shown).

The IGH-chain locus of the B-CLL
To clarify the structure of the IGH locus of the B-CLL, the product obtained by the VH4-family specific PCR on genomic DNA was cloned. By CDR3 PCR, we identified clones with CDR3 products of 112, 184 and 297 bp, thus identical to the products found in the CDR3 PCR on genomic DNA (Figure 1b). One of the plasmid clones contained an extra long 667 bp CDR3 product. A VH4 family-specific PCR on this particular bacterial clone yielded a 1071 bp product, comparable to the largest product obtained in the VH4 family-specific PCR on total genomic DNA (Figure 1c). Clones, representative of the four different CDR3 lengths found, were sequenced. The V\text{\textsubscript{\textkappa}} region of the B-CLL displayed highest homology with the V4–34, D4 and JH3b germline gene segments, respectively (Figure 2).29,34 In the V\text{\textsubscript{\textkappa}} gene segment, a total of 13 somatic mutations were identified. The CDR1 and CDR2 carried five somatic mutations with a replacement vs silent (R/S) mutation ratio of 1.5. The FRs contained eight somatic mutations with an R/S ratio of 0.3.

Interestingly, we found that the IGH locus of the B-CLL contained a triplication of the JH3b gene segment. The second JH3b (JH3b-II) gene segment was located 21 bp downstream of JH3b-I, in the JH3–JH4 intron and the third JH3b gene segment (JH3b-III) was located 55 bp downstream of JH3b-II, again in the JH3–JH4 intron. The somatic mutations in the three successive JH3b gene segments, with parts of the JH3–JH4 intronic sequence, were all unique except for one mutation that was shared by the JH3b-I and JH3b-III gene segment (Figure 2).

Since in each of the three JH3b copies the 5’ splice donor site was preserved, potentially VH4 transcripts can be produced coupling one, two or three JH3b copies to CH gene segments. To investigate whether this was the case, bacterial clones were produced from the VH4 RT-PCR products obtained with the J\text{\textsubscript{H}}, C\text{\textsubscript{\textmu}} and C\text{\textalpha} downstream primers, respectively. Nested CDR3-PCR on these cloned VH4 RT-PCR...
Figure 2  Nucleotide sequence of the functionally rearranged VH locus of the B-CLL. (a) The upper line represents the germline VH, D and JH sequences and the lower the sequence as found in the B-CLL. Only nucleotide differences are indicated. Indicated regions: FR, framework region; CDR, complementarity determining region; N, non-templated nucleotide additions; 5' splice site around codon 26; 3' splice site around codon 64; O, somatic mutation resulting in closer homology to the 3' splice consensus sequence; X, the only mutation shared by JH3bI and JH3bIII. This nucleotide sequence has been deposited at the GenBank database (accession No. AF417291). (b) Significant homology of the different 'N' regions proximal to the second and third JH3b gene segment for the N region proximal to the first JH3b gene segment; G, nucleotide identical to the N-region; x, nucleotide not identical to the N-region.

products, using the JH downstream primer, again yielded products with one, two or three JH3b copies, of 112, 184 and 297 bp, respectively. However, the clones derived from VH4 PCR products generated with the CH1-Cμ and CH1-Cδ downstream primers contained the short CDR3 length of 112 bp only. Also, when a CH3-Cμ downstream primer was used, only amplimers with one JH3b segment were found (data not shown), excluding the possibility that the two- and/or three-JH3b containing transcripts had been spliced to the second or third Cμ exon (CH2, CH3). It thus seems that the majority of μ and δ transcripts contain a functional IgVH region with a single JH3b segment. Moreover, it is noteworthy that from the
putative RNA message containing all three JH3b gene segments, no protein product is to be expected due to a frameshift directly upstream of the third JH3b gene segment.

A total of 20 cDNA-derived PCR clones of the B-CLL a 116 bp deletion (between codons 26 and 64) within the V4–34 gene segment (Table 1, Figures 2 and 3a). Detailed analyses of the sequences flanking this deletion indicated that the 5' site at codon 26 (FR1) was strongly homologous with the mammalian 5' splice site consensus sequence (5' A/C A G T 3') whereas the 3' site at codon 64 displayed homology for the 3' splice site consensus sequence (5' Y, N Y A/G A T 3'). In these splicer acceptor and donor sites the underlined nucleotides are highly conserved whereas the doubly underlined nucleotides are absolutely necessary for RNA splicing. Interestingly, a nucleotide substitution in codon 64 (A → C) had caused a closer homology to the consensus 3' splice acceptor site at a critical position. In addition, the mammalian branch-point consensus sequence, which maps 18–38 nucleotides upstream of the 3' splice site (5' T N C T R A C 3'), was present in the mutated V4–34 and here also a specific somatic mutation had contributed to a closer homology (Figure 3a). The clones with this deletion were all devoid of the VH4 leader intron, proving the RNA derivation of the cloned fragments. This, and the fact that we did not isolate genomic DNA-derived clones with the V4–34 deletion, indicates that this deletion was the result of alternative splicing. It must be noted that the alternative V4–34 splicing is accompanied by a shift of the reading frame and the putative messenger RNA thus cannot be translated. In Figure 4, a summary is provided of the different transcripts found in the B-CLL. With the information obtained, the smaller band of 357 bp in the VH4-family specific PCR with the Cμ primer (Figure 1c), is now explained as an alternatively spliced V4–34 gene.

Based on these findings we analyzed the IgVH transcripts of three previously studied IgM+ V4–34-expressing FLs, Nos 13, 15 and 67 and an IgM+, V4–34-expressing diffuse large B cell lymphoma (DLBCL4). Figure 5 shows that in a VH4–Cμ RT-PCR, a small product of approximately 350 bp was not only present in the B-CLL but also in FL13. In contrast, in FLs Nos.15, 67 and in DLBCL4 this particular product was not detected. Instead, in DLBCL4 a 180 bp product was visible. FL13 co-expressed μ and δ IgH chains, harboring nine somatic mutations compared to the V4–34 germline in association with an A IgL chain. Surprisingly, in this case a nucleotide substitution in codon 64 (A → G) of V4–34 was found as well that, although at a position different from that in the B-CLL, also gave rise to a closer homology with the consensus 3' splice acceptor site (Figure 3b). Sequencing confirmed that the 350 bp product had been generated by similar V4–34 splicing which again was accompanied by a frameshift. In the

## Alternative splicing of V4–34 gene transcripts

In addition to the described transcripts with differences in the number of JH3b copies included, we found in eight of the 20 cDNA-derived PCR clones of the B-CLL a 116 bp deletion (between codons 26 and 64) within the V4–34 gene segment (Table 1, Figures 2 and 3a). Detailed analyses of the sequences flanking this deletion indicated that the 5' site at codon 26 (FR1) was strongly homologous with the mammalian 5' splice site consensus sequence (5' A/C A G T 3') whereas the 3' site at codon 64 displayed homology for the 3' splice site consensus sequence (5' Y, N Y A/G A T 3'). In these splicer acceptor and donor sites the underlined nucleotides are highly conserved whereas the doubly underlined nucleotides are absolutely necessary for RNA splicing. Interestingly, a nucleotide substitution in codon 64 (A → C) had caused a closer homology to the consensus 3' splice acceptor site at a critical position. In addition, the mammalian branch-point consensus sequence, which maps 18–38 nucleotides upstream of the 3' splice site (5' T N C T R A C 3'), was present in the mutated V4–34 and here also a specific somatic mutation had contributed to a closer homology (Figure 3a). The clones with this deletion were all devoid of the VH4 leader intron, proving the RNA derivation of the cloned fragments. This, and the fact that we did not isolate genomic DNA-derived clones with the V4–34 deletion, indicates that this deletion was the result of alternative splicing. It must be noted that the alternative V4–34 splicing is accompanied by a shift of the reading frame and the putative messenger RNA thus cannot be translated. In Figure 4, a summary is provided of the different transcripts found in the B-CLL. With the information obtained, the smaller band of 357 bp in the VH4-family specific PCR with the Cμ primer (Figure 1c), is now explained as an alternatively spliced V4–34 gene.

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### Table 1: Summary of plasmid clones generated from RT-PCR

<table>
<thead>
<tr>
<th>PCR</th>
<th>CDR3 length</th>
<th>No. of clones sequenced</th>
<th>No. of clones with a deletion in V4–34</th>
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</thead>
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<tr>
<td>VH4–JH</td>
<td>112 bp</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>VH4–JH</td>
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<td>3</td>
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<tr>
<td>VH4–C8</td>
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<td>3</td>
<td>0</td>
</tr>
</tbody>
</table>

### Figure 3

**Comparison of the V4–34 expressed by the B-CLL (a) and FL13 (b) with 5' and 3' consensus splice sites.** FR1, frame work region 1; CDR2 complementarity determining region; * mutation that resulted in a closer homology with the consensus splicing sequence; x, nucleotide not matching the consensus sequence; +, mutation not resulting in a closer homology with the splicing consensus sequence. The somatic mutations in codon 64, which resulted in closer homologies to the 3' splice acceptor site are marked by arrows. The underlined nucleotides are necessary for RNA splicing. M = A or C, R = A or G, N = any nucleotide and Y = T or C.

### Figure 4

**Schematic overview of V4–34 transcripts identified in the B-CLL.** L, leader sequence; V4–34, variable gene segment; D, diversity gene segment; JH, joining gene segment; I, intron; Cμ, constant μ sequence; Cδ, constant δ sequence.
mutated V4–34 sequences of FLs 15 and 67 no somatic mutations in the ‘cryptic’ 3′ splice acceptor site were found (data not shown). DLBCL4 expressed a V4–34 with 35 somatic mutations, as assessed by sequencing of three plasmid clones. In the CDR1/H9253 JH3b (2J) gene copies were introduced into the pIgH(H9260/H9253) expression vector. The transfectants were selected with geniticin. On cDNA derived from these transfectants, we suspected that the A→C mutation in codon 64 was pivotal by causing a closer homology to the consensus 3′ splice acceptor site (Figure 3a), we also transfected cells with 1J and 2J constructs in which this mutation had been reversed. By RT-PCR, we indeed found that in neither the 1J nor the 2J revertants were alternatively spliced V4–34 forms detectable (Figure 6b, lane 2). With the B-CLL-specific FR3 downstream primer, the V4–34 splice variant was also detectable in the 2J and the 1J transfectants (Figure 6c, lanes 1 and 2). Here, no difference is visible between products with one JH3b or with two JH3bgene segments since in this PCR the downstream primer anneals 5′ of the JH region. It is remarkable that in the 2J transfectant, the ratio of spliced:unspliced transcripts is significantly higher than in the 1J transfectant. This suggests that the abnormal size and structure of V4–D–2 favors the use of the cryptic splice sites in V4–34.

As we suspected that the A→C mutation in codon 64 was pivotal by causing a closer homology to the consensus 3′ splice acceptor site (Figure 3a), we also transfected cells with 1J and 2J constructs in which this mutation had been reversed. By RT-PCR, we indeed found that in neither the 1J nor the 2J revertants were alternatively spliced V4–34 forms detectable (Figure 6b, lane 2). With the B-CLL-specific FR3 downstream primer, the V4–34 splice variant was also detectable in the 2J and the 1J transfectants (Figure 6c, lanes 1 and 2). Here, no difference is visible between products with one JH3b or with two JH3b gene segments since in this PCR the downstream primer anneals 5′ of the JH region. It is remarkable that in the 2J transfectant, the ratio of spliced:unspliced transcripts is significantly higher than in the 1J transfectant. This suggests that the abnormal size and structure of V4–D–2 favors the use of the cryptic splice sites in V4–34.

**Comparison of germline V\textsubscript{H} family genes with the consensus splice site sequences**

Alignment analyses indicated that several of the germline V\textsubscript{H} genes bear, in the regions mentioned above, homology with the 5′ and 3′ splice consensus sequences. Although the extent of homology is variable among the different V\textsubscript{H} genes it is likely that, due to superimposed nucleotide substitutions, internal V\textsubscript{H} mRNA splicing is not confined to a single V\textsubscript{H} species. Of all V\textsubscript{H} family genes (codons 25–28), however, the germline sequences of the VH4 family members display the highest degree of homology with the consensus 5′ splice site sequence. Except for the VH4 family genes and the V1–18 germine gene, none of the other germline V\textsubscript{H} genes contain the essential GT in codon 26 (Figure 7 and data not shown). In addition, only V4–34 harbors the highly conserved guanine at the third position of codon 27 (Figure 7). Comparison of codons 56–65 of the V\textsubscript{H}1 family germine genes with the consensus 3′ splice site sequence indicated that, although only the VH1 family members contain the essential YAG at codon 64, the overall homology was again most obvious for the VH4 family segments (Figure 7). Although the V1–18 gene, in germline configuration, also potentially fulfills the requirements for the internal splicing we were unable to detect this phenomenon in two V1–18 expressing B cell lymphomas (data not shown).
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Figure 6  V4–34 splicing in Ig-transfected SP2/0 cells. (a) Schematic representation of the PCRs performed on cDNA of SP2/0 transfected with B-CLL-derived V4–34. (b) Results of VH4-Cy PCRs on cDNA of SP2/0 transfected with B-CLL-derived V_{4,D-2} and V_{4,D-1} with (lanes 1 and 2) or without the somatic mutation in codon 64 (lanes 3 and 4). The 330 bp product (lanes 1 and 3) represents V_{4,D-2} coupled to Cy. The 458 bp product (lanes 1–4) and 342 bp product (lanes 1 and 2) represent unspliced and spliced V_{4,D-1}, respectively. (c) Results of VH4–FR3 PCRs on the same samples. The internal splice variants (visible in lanes 1 and 2) are not detected in the codon 64 revertants (lanes 3 and 4).

Figure 7  Alignment of some germline VH genes with the 5'/H11032 and 3'/H11032 consensus splice site sequences. The underlined nucleotides are highly conserved and the boxed nucleotides are essential for RNA splicing. For a complete alignment of all VH germ line genes see the Vbase database on internet (http://www.mrc.cpe.cam.ac.uk.). M = A or C, R = A or G, N = any nucleotide and Y = T or C.

Discussion

In this report, we describe three B-NHLs of different types in which, apart from normal IgH gene transcripts, aberrant transcripts were detected due to an extraordinary make-up of the IgH chain locus and/or alternative processing of IgH mRNA.

The IgH locus of a B-CLL contained a triplication of the J_{H3b} gene segment, which included parts of the J_{H3-JH4} intron sequence. Directly upstream of the J_{H3b-II} and the J_{H3b-III} segment, stretches of 5 bp and 13 bp respectively were present, each of which contained 4 bp homologous to putative N nucleotides directly upstream of the first J_{H3b} segment (Figure 2b). This suggests that the J_{H3b} triplication took place in an already rearranged IgH locus. Moreover, this N-region may have served as the target region in the triplication process that most likely took place in replicating cells, possibly via a repeated mechanism of DNA double strand break followed by homologous recombination. These alterations may have occurred in dividing precursors in the bone marrow after the initial IgH rearrangement had been completed. Alternatively, the finding that this B-CLL exhibited somatically mutated IgV genes, indicates that the neoplastic cells were derived from a (post) GC B cell, as has been described for approximately 50% of IgM-expressing B-CLLs. As the somatic hypermutation process involves DNA double strand breaks possibly followed by repair via homologous recombination, the triplication could also have occurred during the hypermutation process. An important argument that duplications occur during the GC reaction is the finding of both unique as well as shared point mutations in duplicated regions. In this B-CLL we found one mutation shared by the J_{H3b-I} and J_{H3b-II} segments. Assuming that the J_{H3b-I} segment served as a template, it is difficult to envisage why this mutation was not shared by the J_{H3b-II} segment.

In three of the B cell lymphomas studied, we observed alternative processing of IgH mRNA using a previously described cryptic splice donor site present in the FR1 of VH4 genes. In two of these cases, ie the somatically mutated B-CLL and FL13, we observed an identical mode of internal splicing of the V4–34 gene transcripts involving a so far unidentified cryptic 3'/H11032 splice acceptor site (Figures 3 and 4, Table 1). Moreover, by transfection studies in combination with the site-directed mutagenesis, it was established that in the B-CLL this cryptic splice acceptor site had been activated due to a specific nucleotide substitution in codon 64 (CTC AAG → CTC CAG) (Figure 6). Strikingly, FL13 carried a nucleotide substitution in codon 64 as well (CTC AAG → CTC AGG) although at a different position (Figure 3). In both cases, however, a CAG sequence was generated which apparently was sufficient for the creation of a functional 3' splice acceptor site (Figures 3 and 4, Table 1). Moreover, by transfection studies in combination with the site-directed mutagenesis, it was established that in the B-CLL this cryptic splice acceptor site had been activated due to a specific nucleotide substitution in codon 64 (CTC AAG → CTC CAG) (Figure 6). Strikingly, FL13 carried a nucleotide substitution in codon 64 as well (CTC AAG → CTC AGG) although at a different position (Figure 3). In both cases, however, a CAG sequence was generated which apparently was sufficient for the creation of a functional 3' splice acceptor site. This cryptic 3' splice acceptor site in V4–34 consists of a 26 bp stretch of which at least 18 bp are identical to consensus branchpoint and 3' splice acceptor sequences (Figures 3 and 7). In DLBCL, aberrant in-frame IgH transcripts were found in which the cryptic 5' splice donor site had been directly spliced to the CH1-C4 exon. This mode of IgH mRNA processing has been documented before in two Burkitt's lymphoma cell lines and leads to production of truncated, so-
called μ heavy chain disease (μHCD) proteins, which are secreted without associated IgL chain.\textsuperscript{34,39} In these cases, alternative splicing rescued the cells harboring non-functional V\textsubscript{H} genes with stop codons and/or framenshifts due to deletions or insertions. As we found high intracranal variation in the V\textsubscript{H} gene of DLBCL\textsubscript{4} it can be conceived that a fraction of the tumor cells contain disabled V\textsubscript{H} genes due to nonsense mutations and/or deletions/insertions and that these subclones use alternative V\textsubscript{H} splicing to survive. We can, however, not discriminate whether alternative splice forms are indeed produced by a subfraction of tumor cells or whether all tumor cells produce both normally and aberrantly spliced μ transcripts. In γ- and α-HCD, which is associated with a variety of B cell malignancies, IgH splicing is even more extensive and usually involves part or all of the V\textsubscript{H} region and the whole CH1 domain. In most of these cases, the V\textsubscript{H} leader, with or without a small part of the V\textsubscript{H} region, is directly spliced to the hinge region.\textsuperscript{41}

The V\textsubscript{4}-34 gene segment has been found in virtually all cases of ‘cold’ agglutinin disease in which the autoantibodies have specificity for the i/i antigen present on erythrocytes.\textsuperscript{42,43} It is presently unclear whether the mutated V\textsubscript{4}-34 IgM/D expressed by our B-CLL has any causative role in the accompanying severe auto-immune hemolytic anemia (AIHA). In general, AIHA observed in B-CLL is considered to be caused by polyclonal, ‘warm’ reactive IgG antibodies unrelated to antibodies expressed by the tumor cells.\textsuperscript{44,45}

Of all germline V\textsubscript{H} gene segments, codons 25–28 of FR1 of V\textsubscript{4}-34 have the highest homology to the consensus 5’ splice site sequence (Figure 7). This may imply that V\textsubscript{4}-34-expressing B cells are more likely to produce HCD-like proteins, as we also detected in DLBCL\textsubscript{4} on the transcriptional level. If true, this may be of relevance for the reported, overrepresentation of V\textsubscript{4}-34 usage in IgM/D of DLBCL\textsubscript{4} it can be conceived that a fraction of the tumor cells contain disabled V\textsubscript{H} genes due to nonsense mutations and/or deletions/insertions and that these subclones use alternative V\textsubscript{H} splicing to survive. We can, however, not discriminate whether alternative splice forms are indeed produced by a subfraction of tumor cells or whether all tumor cells produce both normally and aberrantly spliced μ transcripts. In γ- and α-HCD, which is associated with a variety of B cell malignancies, IgH splicing is even more extensive and usually involves part or all of the V\textsubscript{H} region and the whole CH1 domain. In most of these cases, the V\textsubscript{H} leader, with or without a small part of the V\textsubscript{H} region, is directly spliced to the hinge region.\textsuperscript{41}

The internal IgV\textsubscript{H} splicing observed in this B-CLL and in FL13, which to our knowledge has not been observed before, may have significance as an additional mechanism of somatic hypermutation, like some HCD proteins,\textsuperscript{49,50} they could be instrumental in cellular transformation.

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