B cell biology and the development of mature B cell lymphomas
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Chapter 14

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 (V\textsubscript{H}) 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 V\textsubscript{H} gene transcripts were found in which a sequence stretch of 116 bp between the framework region 1 (FR1) and complementarity determining region 2 (CDR2) had been deleted. We provide evidence for this alternative Ig\textsubscript{V} \textsubscript{H} 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 Ig\textsubscript{V} \textsubscript{H} 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 Ig\textsubscript{V} \textsubscript{H} processing in B cell malignancies and as a potential mechanism of somatic Ig diversification is discussed.

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.\textsuperscript{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,\textsuperscript{3,4} not only involves single nucleotide exchanges but also, to a significant extent, deletions and insertions of different size.\textsuperscript{5,6} 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.\textsuperscript{7–11} Although most examples of receptor revision involved Ig\textsubscript{V} \textsubscript{H} genes, it has also recently been demonstrated in human Ig\textsubscript{V} \textsubscript{L} genes.\textsuperscript{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.\textsuperscript{14} In follicular lymphoma (FL), the cytological and architectural characteristics as well as the immunophenotype are all highly compatible with a GC derivation.\textsuperscript{15,16} B-CLL was initially considered to be derived from naive B cells.\textsuperscript{17,18} However, Fais \textit{et al}\textsuperscript{19} 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 Ig\textsubscript{V} \textsubscript{H} genes. Moreover, similar to normal (post) GC B cells\textsuperscript{20,21} and (post) GC-derived B cell malignancies,\textsuperscript{22,23} the BCL-6 gene was found to be somatically mutated in a fraction of the Ig\textsubscript{V} \textsubscript{H} mutated B-CLLs.\textsuperscript{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 Ig\textsubscript{V} \textsubscript{H} genes have a significantly better prognosis than those with unmutated Ig\textsubscript{V} \textsubscript{H} genes.\textsuperscript{25,26}

Here, we present Ig\textsubscript{V} \textsubscript{H} gene analyses of three distinct GC-derived B cell malignancies in which we observed alternative processing of the rearranged and somatically mutated V4–34 Ig\textsubscript{V} \textsubscript{H} gene segment. We provide evidence that the internal Ig\textsubscript{V} \textsubscript{H} 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 Ig\textsubscript{V} \textsubscript{H} 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.\textsuperscript{27} The third patient (DLBCL4) was a 77-year-old female 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 TRIZOL 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.27,28

**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, Cα, Cγ, or Cδ. For the VH family-specific PCR, the reactions were performed with VH family-specific leader primers combined with the appropriate reverse primer, either JH, Cα, Cδ 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;27,28 B-CLL FR3 5’-CGGCCAGTCACAGAGCCTCAA-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 with an ABI sequencer (Perkin Elmer Corporation, Norwalk, CT, USA) using the big dye-terminator cycle-primed method into TOP10 bacteria, according to the manufacturer’s instructions. PCR products were cloned into pTOPO vectors and transformed into TOP10 bacteria, according to the manufacturer’s instructions (Invitrogen, Groningen, The Netherlands). The two parts were purified and cultured in IMDM complete medium 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 reversed the somatic mutation in codon 64 to the germline nucleotide. The V4–34 of the B-CLL was amplified in two separate parts: the 5’ part with the VH 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’-GAGTCACCA-TATCGAGGAC-3’; B-CLL-R 5’-GACTCTTGAAGCAGGGGTTG-3’. The underlined nucleotide is a mismatch which reverts the somatic mutation in codon 64.

**Results**

**Assessment of clonality, IgH chain isotype expression and V, constant 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)8 as well as by a CDR3-specific PCR. This PCR makes use of clone-dependent length differences of the CDR3 regions in the Vγ, 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 Cα). 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 provided by Dr 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 × 10⁵ SP2/0 myeloma cells in Iccove’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).
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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<sub>H</sub>-family and CDR3-specific PCRs: L, leader sequence; V<sub>H</sub>, variable gene segment; D, diversity gene segment; J<sub>H</sub>, joining gene segment; C<sub>H</sub>, 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<sub>H</sub>, C<sub>H</sub>/H9262, C<sub>H</sub>/H9253, C<sub>H</sub>/H9251 and C<sub>H</sub>/H9254 downstream primers. Indicated are the clonal products of 297 bp, 184 bp and 112 bp using the J<sub>H</sub> 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<sub>H</sub>, C<sub>H</sub>/H9262 and C<sub>H</sub>/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<sub>H</sub> primer.

combinations, a sharp band was only observed with the C<sub>µ</sub> and C<sub>δ</sub> primers, which was in accordance with the immunohistochemically demonstrated co-expression of IgM and IgD. With both the Cy and Cx 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<sub>µ</sub> or Vc leader primers in combination with the J<sub>H</sub>, C<sub>µ</sub> and C<sub>δ</sub> or J<sub>c</sub> and C<sub>c</sub> downstream primers, respectively. On genomic DNA, only J<sub>H</sub> or J<sub>c</sub> downstream primers were used in view of the presence of J<sub>H</sub>–CH<sub>δ</sub> and J<sub>c</sub>–CH<sub>δ</sub> intronic sequences. The V<sub>H</sub> 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<sub>H</sub> 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<sub>µ</sub> downstream primer, a 473 bp product and a weaker 357 bp product were visible, whereas with the C<sub>δ</sub> primer only one product was detectable (Figure 1c). The general length difference between the IgV<sub>µ</sub> family PCR products generated from either genomic DNA or cDNA is explained by the 83 bp leader intron present in genomic DNA only.

By Vc family-specific PCR and sequencing, it was determined that the B-CLL expresses a Vc1 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<sub>µ</sub> region of the B-CLL displayed highest homology with the V4–34, D4 and JH3b germline gene segments, respectively (Figure 2).<sup>29,34</sup> In the V<sub>µ</sub> gene segment, a total of 13 somatic mutations were identified. The CDR1 and CDR2 contained five somatic mutations with a replacement (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 JH, C<sub>µ</sub> and C<sub>δ</sub> downstream primers, respectively. Nested CDR3-PCR on these cloned VH4 RT-PCR products was performed using the VH4 family-specific primers and J<sub>H</sub> 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<sub>H</sub> primer.

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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

Figure 2 Nucleotide sequence of the functionally rearranged VH locus of the B-CLL. (a) The upper line represents the germline VH, D and J segments and the lower the sequence as found in the B-CLL. Only nucleotide differences are indicated. Indicated regions: FR, frame work region; CDR, complementarity determining region; N, non-templated nucleotide additions; 5' splice site around codon 26; 3' splice site around codon 64; Δ, somatic mutation resulting in closer homology to the 3' splice consensus sequence; ΔΣ, 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; Δ, nucleotide identical to the N-region; Δ, nucleotide not identical to the N-region.
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 different lengths were sequenced. We did not detect intracopalional point mutation differences between the cloned IgV sequences, which is in accordance with a previous study on B-CLL. Also, the rearranged Vx1 gene harbored somatic point mutations (four in the CDRs and six in the FRs) compared to the most homologous germline Vx1 gene segment, DPK3/L11 (data not shown).

**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 ↓ G T 3′) whereas the 3′ site at codon 64 displayed homology for the 3′ splice site consensus sequence (5′ (Y) N Y AG ↓ G 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 IgV4+, 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 Igλ 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

<table>
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<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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<td>VH4–JH</td>
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<td>4</td>
<td>3</td>
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<tr>
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<td>3</td>
<td>0</td>
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<td>112 bp</td>
<td>8</td>
<td>3</td>
</tr>
<tr>
<td>VH4–Cδ</td>
<td>112 bp</td>
<td>3</td>
<td>0</td>
</tr>
</tbody>
</table>

**Table 1** Summary of plasmid clones generated from RT-PCR

![Figure 3](image)

**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.

![Figure 4](image)

**Figure 4** Schematic overview of the VH4+ transcripts identified in the B-CLL. L, leader sequence; VH4, 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 and CDR2, nine somatic mutations were present with a replacement vs silent (R/S) mutation ratio of 2.0. The FRs contained 26 somatic mutations with an R/S ratio of 1.0. The intraclonal variation, which is calculated as the mean number of nucleotide differences for each molecular clone compared to the consensus sequence was 6.3 nucleotides/clone, which is rather high according to previous studies on DLBCLs and FLs.27,28 The 180 bp product represented an alternatively processed IgH transcript in which the cryptic 3' splice donor site at codon 26 was directly spliced to the CH1-C4 exon. In this case, as in FL13, no somatic mutations were found in the cryptic 5' splice donor site. This mode of splicing yields an in-frame truncated heavy chain and has been described previously in two VH4-expressing Burkitt's lymphoma cell lines.38,39

Internal V4–34 splicing depends on a single nucleotide substitution

To exclude that the alternative V4–34 RNA processing was due to an aberrant splicing machinery of tumor B cells, we transfected SP2/0 myeloma cells with the B-CLL IgH. The B-CLL-derived V4–34 genes with either one JH3b (1J) or two JH3b (2J) gene copies were introduced into the pIgH(y3)-2 transfectant, which expresses the V4–34 RNA variant with one JH3b gene segment coupled to Cy3. Theoretical transcripts representing the V4–34 splice variant with one JH3b gene segments coupled to Cy3, with a predicted length of 414 bp, were not detected. In the PCR on the pIgH(y3)-1J transfectant, we detected a dominant 458 bp product and a very weak product of 342 bp, representing the unspliced and spliced 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 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 V4–34 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 and c, lanes 3 and 4). We therefore conclude that the alternative processing of V4–34 RNA is not invoked by a disturbed splicing machinery of the B-CLL but is V4–34-encoded and critically depends on one single point mutation.

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 germline 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} family germline 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 genes (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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Leukemia

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–Cγ PCRs on cDNA of SP2/0 transfected with B-CLL-derived Vγ, D-2γ and Vγ, D-1γ with (lanes 1 and 2) or without the somatic mutation in codon 64 (lanes 3 and 4). The 530 bp product (lanes 1 and 3) represents Vγ, D-2γ coupled to Cγ. The 458 bp product (lanes 1–4) and 342 bp product (lanes 1 and 2) represent unspliced and spliced Vγ, D-1γ, 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 Vγ 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 Vγ germ line genes see the Vbase database on internet (http://www.mrc.cpe.cam.ac.uk/).29,30 M = Ao or C, R = Ao 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 JH3b gene segment, which included parts of the JH3-JH4 intron sequence. Directly upstream of the JH3b-II and the JH3b-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 JH3b segment (Figure 2b). This suggests that the JH3b 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 JH3b-I and JH3b-III segments. Assuming that the JH3b-I segment served as a template, it is difficult to envisage why this mutation was not shared by the JH3b-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’ 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 DLBCL4, aberrant in-frame IgH transcripts were found in which the cryptic 5’ splice donor site had been directly spliced to the CH1-Cμ 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-
Alternative splicing of heavy chain variable region

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