Direct Sequence Analysis of the 14q+ and 18q- Chromosome Junctions in Follicular Lymphoma

By Finbarr Cotter, Christopher Price, Emanuele Zucca, and Bryan D. Young

Although the t(14;18) chromosome translocation has been demonstrated to be a highly consistent feature of follicular lymphomas, the underlying mechanism generating this fusion has remained uncertain. To examine this question further, a polymerase chain reaction strategy has been devised to permit the amplification and direct sequencing of the resultant 14q+ and 18q- reciprocal junctions. Direct sequence analysis of amplified 14q+ junctions established that 7 of 11 tumors contained a bcl-2 (mbr) sequence fused to an immunoglobulin Jh region (five were Jh and two were Jk). One of these junctions had an unusual configuration with the bcl-2 and Jh sequences separated by a recognizable Dh region. This finding suggests that at least some of the junctional sequences, previously thought of as N insertions, may be fragments of unrecognized Dh regions. It was also possible to amplify and sequence 18q- junctions using a primer based on the Dh recombination signal sequences. Several 18q- junctions were shown to consist of Dh/bcl-2 (either mbr or mcr) fusions. In two tumors the 14q+ and 18q- junctions were fully sequenced, and it was demonstrated that the bcl-2 sequence was conserved during mbr and mcr translocations. This contrasts with previous analyses that demonstrated either loss or duplication of several bases at the breakpoints in the bcl-2 gene.

APPROXIMATELY 85% of follicular lymphomas and 30% of diffuse large cell lymphomas have been demonstrated to carry the t(14;18)(q32.3;q21.3) chromosomal translocation by both cytogenetic and molecular techniques. The breakpoints on chromosome 18 occur at two sites, approximately 20 kilobases (kb) apart, within or near to a transcriptional unit called the bcl-2 gene. Approximately 60% of the breakpoints occur within 150 base pairs (bp) known as the “major breakpoint region” (mbr) in a 3' untranslated region of the bcl-2 gene. A further 25% have breakpoints at a site 20 kb 3' to the mbr sequence in a region called the “minor cluster region” (mcr), and these translocation events occur within 500 bp of each other, some clustering within 3 bp on chromosome 18. On chromosome 14, the breakpoints occur in the joining (Jh) region of the immunoglobulin IgH heavy chain gene. It has been postulated that the translocation occurs as an error during VDJ joining at a time when terminal deoxynucleotidyl transferase is active. At the junction on chromosome 14q+ short segments of unidentifiable nucleotides, up to 24 in length have been observed and postulated to represent “N” insertions normally found at the VD and DJ junctions after IgH rearrangement. In the few 18q- breakpoint cases studied, 7,10 it appears that Ig heavy chain diversity regions (Dh) are joined to the bcl-2 gene with loss of the 5' Jh region, suggesting a deletion between Dh and Jh in t(14;18) translocation in lymphomas. The translocation results in a hybrid bcl-2/IgH transcript, but a normal bcl-2 protein, as the coding region appears to be structurally unaltered. However, the quantity of protein is increased within the cells containing the t(14;18) translocation.11,12

The clustering of the majority of breakpoints on bcl-2 facilitates the use of the polymerase chain reaction (PCR) for amplification and analysis of the t(14;18) breakpoints. Bcl-2 oligonucleotide primers (either mbr or mcr) flanking the translocation have been used, with a consensus Jh sequence found at the 3' end of each Jh exon, to amplify the 14q+ junctions. We have extended this approach to the 18q- junctions using a primer based on part of the recombination signal sequences known to flank germline Dh sequences. Thus, it was possible to sequence junctions produced by translocations in either the mbr or mcr regions in a series of follicular lymphomas.

MATERIALS AND METHODS

Enzymatic amplification. DNA was extracted as previously described14 from 11 patients with histologically proven centroblastic/centrocytic follicular lymphoma. Enzymatic amplification was performed by the PCR procedure of Saiki et al15 using Thermus aquaticus (Taq) DNA polymerase and an automated Perkin-Elmer DNA Thermal Cycler (Cetus, Emeryville, CA). The final reaction volume included 1 μg of tumor DNA, oligonucleotide primers (1 mmol/L) (Boehringer Mannheim, FRG), 1.5 U of Taq DNA polymerase (Ampliaaq-Cetus), and gelatin (0.01% wt/vol) in 100 μL of Taq buffer (50 mmol/L KCl, 10 mmol/L Tris-C1 pH8.3, 1.5 mmol/L MgCl2). Amplification of the mbr-Jh junctions on chromosome 14q+ was performed with primers BC1 and JH1 and the Dh-mbr junctions on chromosome 18q- with primers DH1 and BC2 (see Table 1). The mcr-Jh junctions on chromosome 14q+ were amplified with primers BC5 and JH1 and the Dh-mcr junctions on chromosome 18q- with primers DH1 and BC6. An initial denaturing step of 10 minutes at 94°C was followed by 30 cycles of 15 seconds at 55°C (annealing), 1 minute at 70°C (extension), and 30 seconds at 94°C (denaturing). The final extension period was lengthened to 10 minutes. Taq polymerase (Cetus), 1.5 U was added to the reaction after the initial denaturing step. Aliquots (10 μL) from the reaction mixture were analyzed by electrophoresis in a 2% agarose gel in Tris borate electrophoresis (TBE) buffer and stained with ethidium bromide.

Direct sequence analysis. Direct nucleotide sequence analysis by the dideoxy chain termination method16 was performed on PCR products. DNA from the reaction mixture was purified using a Sephphex G20 column followed by ethanolic precipitation, freeze drying, and resuspension in distilled water. Sequencing was performed using a Big Dye chemistry kit (Bioviz, Foster City, CA) on an ABI-373A automated sequencer (Applied Biosystems, Foster City, CA). DNA from the reaction mixture was purified using a DNA purification kit (Promega, Madison, WI), and the cDNA from the reaction mixture was purified using a Sephphex G20 column followed by ethanolic precipitation, freeze drying, and resuspension in distilled water. Sequencing was performed using a Big Dye chemistry kit (Bioviz, Foster City, CA) on an ABI-373A automated sequencer (Applied Biosystems, Foster City, CA).

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Table 1. Position, Sequence, and Use of Synthetic Oligonucleotides for Analysis of t(14;18) Junctions

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Use</th>
<th>Position (strand)</th>
<th>Orientation (strand)</th>
</tr>
</thead>
<tbody>
<tr>
<td>JH1</td>
<td>5’ACCTGAGAGAGCGGTACC-3’</td>
<td>PCR</td>
<td>3’ Jγ consensus</td>
<td>–</td>
</tr>
<tr>
<td>DH1</td>
<td>5’GTGAGGCTGCTTGACTGCTG-3’</td>
<td>PCR</td>
<td>5’ flanking region</td>
<td>+</td>
</tr>
<tr>
<td>BC1</td>
<td>5’CCTTTAGAGAGGTGTTTACGTT-3’</td>
<td>PCR</td>
<td>3’ of mbr in bcl-2</td>
<td>–</td>
</tr>
<tr>
<td>BC2</td>
<td>5’ATATCCATATATCACTGTTTAGG-3’</td>
<td>PCR</td>
<td>3’ of mbr in bcl-2</td>
<td>–</td>
</tr>
<tr>
<td>BC3</td>
<td>5’CAGAGACCCACCAGGCCC-3’</td>
<td>Sequencing</td>
<td>5’ of mbr in bcl-2</td>
<td>+</td>
</tr>
<tr>
<td>BC4</td>
<td>5’GTCTGACTCTGCTTGCTCCTC-3’</td>
<td>Sequencing</td>
<td>5’ of mbr in bcl-2</td>
<td>+</td>
</tr>
<tr>
<td>BC5 (MC12)</td>
<td>5’GATGCGCTGGAGAGGTAT-3’</td>
<td>PCR</td>
<td>3’ of mbr in bcl-2</td>
<td>–</td>
</tr>
<tr>
<td>BC7 (MC7)</td>
<td>5’CTACGTCTCGGGAGAGTG-3’</td>
<td>Sequencing</td>
<td>5’ of mbr in bcl-2</td>
<td>–</td>
</tr>
<tr>
<td>BC8</td>
<td>5’TCAGCTCTCGGGAGAGTG-3’</td>
<td>Sequencing</td>
<td>3’ of mbr in bcl-2</td>
<td>–</td>
</tr>
</tbody>
</table>

Oligonucleotides BC5 and BC7 correspond to MC12 and MC7 used by Ngan et al. The heptamer recombination signal is underlined in oligonucleotide DH1.

formed using bcl-2 internal oligonucleotides as sequencing primers (Table 1) with the modified T7 DNA polymerase (Sequenase, USB, Cleveland, OH), a 5-minute labeling reaction at 15°C, and a 5-minute termination reaction at 37°C. The mbr-Jγ junctions were sequenced with kinase-labeled aliquots of primer BC3 and the Dγ-mbr junctions with primer BC4, the mcr-Jγ junctions with BC7, and the Dγ-mcr junctions with BC8.

RESULTS

The majority of breakpoints on chromosome 18 due to the t(14;18) translocation have been shown to occur in either of two regions within the bcl-2 gene (ie, the mbr or mcr). The corresponding breakpoints on chromosome 14 have been shown to occur at or close to members of the Jγ joining region genes of the Ig heavy chain locus. The high degree of clustering of both breakpoints renders the 14q+ junctions suitable for PCR amplification using either mbr or mcr oligonucleotides in combination with a Jγ consensus oligonucleotide. Similar amplification of the 18q- junction is more problematic, because in the few tumors analyzed a diversity region (Dγ) has been found fused to bcl-2 sequence. However, a common feature of diversity regions is that they are flanked by heptamer-nonamer recombination signal sequences. We have sought to use this feature to design an oligonucleotide (DH1) for amplification from the chromosome 14 side of the 18q- junction. The full set of primers is shown in Table 1.

Analysis of mbr junctions in bcl-2. DNA was prepared from a series of 11 follicular lymphoma samples. Enzymatic amplification using primers BC1 and JH1 was performed on each DNA sample, and electrophoresis showed specific products from 7 of 11 samples (data not shown). Under the conditions of amplification, normal human DNA yielded no fragments. Direct sequence analysis using primer BC3 showed the structure of these products, and the results are summarized in Fig 1. In all cases a fusion was demonstrated between a bcl-2 sequence and members of the Ig heavy chain joining region sequence of the chromosome 14 side of the 18q- junction. The full set of primers is shown in Table 1.

Fig 1. Sequences of the chromosome 14q+ junctions in seven follicular lymphomas. Bcl-2 sequence is shown in upper case and joining region sequence is in upper case italics, with the coding exons in bold type. Differences between the Jγ sequences and their germline equivalents are underlined. The intervening sequences between bcl-2 and Jγ are indicated in lowercase. The part of the intervening sequence that is identical to a previously identified Dγ region is underlined (patient E).
region exons (JH), confirming the presence of the t(14;18) translocation in these tumors. The breakpoint on bcl-2 fell within the range of previously determined7 breaks in the mbr region. There was a clear preponderance (5 of 7) for the JH member of the JH family. Most of the JH sequences had some single base differences when compared with their germline equivalents. This could be due to either polymorphic variation or somatic mutation, which is known to occur during D-J recombination. In contrast, the bcl-2 sequence showed no evidence of mutation. In every junction there was an intervening sequence between bcl-2 and JH, although in patient D this consisted of only a single base. In patient E there was a particularly large intervening sequence which, on further analysis, was found to contain a recognizable Dn region (Fig 1). The Dn (diversity) regions recombine with JH in normal Ig gene rearrangements; thus, this junction (bcl-2-Dn-JH) could result from translocation after Dn-JH recombination. Thus, this junction consisted of bcl-2 fused to Dn, which in turn was fused to JH. None of the other intervening sequences had significant homology to each other or to any other known Dn or putative N insertion sequences.

The primer DH1, in combination with the bcl-2 primer BC2, was used for amplification of the reciprocal junctions on chromosome 18q−. Although, on electrophoresis, bands were obtained from tumor samples, direct sequencing was successful from only a proportion of tumors. Thus, these bands may represent spurious products, unrelated to the IgH/bcl-2 junction on chromosome 18q. In every junction there was an interven-
ting sequence between bcl-2 and JH, although in patient D this consisted of only a single base. In patient E there was a particularly large intervening sequence which, on further

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**Fig 2.** The sequence of a chromosome 18q− junction in a follicular lymphoma. The mbr region of bcl-2 is fused to a sequence containing a region of homology (underlined) to a previously cloned Dn sequence.7 The vertical line indicates the junction between the two chromosomes.

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**Fig 3.** The sequences of the reciprocal 14q+ and 18q− junctions found in patient B are compared with the germline mbr region of bcl-2. Gaps in sequence have been introduced for clarity and the underlined region represents a previously unidentified Dn region. The vertical line indicates the position of the breakpoint in the mbr region.

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**DISCUSSION**

The use of the Dn-based primer (DH1) offers a means to amplify and analyze rapidly a proportion of the 18q− junctions. As a means of detecting the presence of the t(14;18), it appears to be less efficient than the equivalent strategy for the 14q+ junctions. Because this approach requires the presence of the 5′ recombination signals next to the junctional Dn exon, lack of amplification may be due to removal of the 5′ recombination signal sequence by a preceding Vn-Dn recombination. In addition, the Dn primer includes part of the intervening sequence between the heptamer and nonamer, which will tend to be less conserved. Although these features may tend to reduce the proportion of tumors in which the 18q− junctions can be successfully amplified, it is possible that other strategies, such as the use of a Vn consensus primer4,5, might be successful. Previous studies have shown that the 14q+ junctions are often characterized by short stretches of unrecognizable sequences, which, because of their position adjacent to a joining region, have been thought of as N-insertions. However, there are several features of these sequences that question this interpretation. Firstly, these translocation N-regions tend to be longer than normal N-region sequences, which rarely exceed 10 bases and are usually shorter.18 Secondly, the normal N-regions are GC rich whereas translocation N-regions are less so.18 Finally, Ngan et al.19 noted that two different tumors had an identical stretch of 11 bases in
their putative N-regions, and speculated that such regions could be derived instead from previously unrecognized D\textsubscript{n} sequences. The tumor from patient E (Fig 1) has been shown to contain a complete D\textsubscript{n} sequence within its putative N-region. This finding supports the idea that at least some of the putative N-regions could contain fragments of D\textsubscript{n} regions. Interestingly, this D\textsubscript{n} sequence is identical to one previously shown to be located 150 bases 5' to a D\textsubscript{n} involved in a follicular lymphoma translocation.\textsuperscript{7} Comparison with this sequence indicates that the germline recombination signals are missing from the 5' end of the D\textsubscript{n} sequence (Fig 1), suggesting that either the translocation has occurred as a mistake in V-D joining with a subsequent N insertion, or that V-D-J recombination had taken place before translocation. The former explanation has been invoked to explain a translocation in the Daudi Burkitt lymphoma cell line as a mistake in V-D joining with the D-J already rearranged.\textsuperscript{7} The latter course of events has been proposed\textsuperscript{20} to account for a t(8:22) translocation in Burkitt's lymphoma in which the Ig \lambda light chain complex was thought to be rearranged before translocation.

The underlying mechanism that generates the t(14;18) translocation has been the subject of some debate. It has been suggested that the translocation occurs as a result of mistakes in VDJ recombination. This is based on the presence of sequences on chromosome 18 close to some breakpoints, which bear a similarity to the heptamer-nonamer signal sequences mediating VDJ recombination.\textsuperscript{5} However, the homology is weak and breakpoints are not always associated with such sequences. Also, if this mechanism were operative the reciprocal set of recognition signals with a 23-bp spacer would be expected within mbr, and these have not been found.\textsuperscript{7} An alternative explanation for the breakage of bcl-2 that does not depend on the presence of heptamer-nonamer signal sequences has been proposed. Bakhshi et al\textsuperscript{17} analyzed the reciprocal junctions of a follicular lymphoma and noted a 3-bp duplication at the junction. It was proposed that this could be the result of a staggered double-stranded break of the type known to result in direct repeats flanking the insertion of foreign DNA. However, subsequent analysis of two tumors\textsuperscript{30} has shown small deletions, rather than duplications, of bcl-2 sequence at junctions. Moreover, in our study both tumors analyzed showed conservation of bcl-2 sequence, and therefore the duplication of junctional bcl-2 sequence is not a general feature of the t(14;18) translocation.

Interestingly, the breakpoint in mbr on chromosome 18 for patient B is at a location identical to that found in a tumor analyzed by Tsujimoto et al\textsuperscript{16} in which a deletion of 2bp had occurred. This effectively rules out the possibility that the gain or loss of bcl-2 sequence is, in some way, dependent on the position of the breakpoint. The analysis of the reciprocal junctions for the translocation in mcr (Fig 4) showed a similar structure to that found in mbr breakpoints. In particular, the fusion of a D\textsubscript{n} to mcr sequence forming the 18q– junction has the same configuration as that found in mbr translocations. This suggests that similar mechanisms are involved in mcr translocations. This breakpoint lies at exactly the position of two previously analyzed breaks in mcr\textsuperscript{8} which themselves were part of a tight cluster of five breaks within 4 bases of each other. An interesting feature of this cluster is that it is bounded by two direct repeats (CTGCAAAC) \textsuperscript{7} bases apart. Although the underlying mechanism for the t(14;18) translocation in follicular lymphoma remains unclear, similar factors appear to operate for breaks in both the mbr and mcr regions. Our sequence data suggest that, at least in some cases, it is possible that translocation has taken place after D-J rearrangement.

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