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 Jκ region (five were Jκ and two were Jλ). One of these junctions had an unusual configuration with the bcl-2 and Jκ sequences separated by a recognizable Dκ region. This finding suggests that at least some of the junctional sequences, previously thought of as N insertions, may be fragments of unrecognized Dκ regions. It was also possible to amplify and sequence 18q− junctions using a primer based on the Dκ recombination signal sequences. Several 18q− junctions were shown to consist of Dκ/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 bcl-2 sequence in a region called the “minor cluster region” (mcr), and these translocations occur within 500 bp of each other, some clustering within 3 bp on chromosome 18. On chromosome 14, the breakpoints occur in the joining (Jκ) region of the immunoglobulin (Ig) 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− chromosomal breakpoints studied, it appears that Ig heavy chain diversity regions (Dκ) are joined to the bcl-2 gene with loss of the 5' Jκ region, suggesting a deletion between Dκ and Jκ 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.

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 Jκ sequence found at the 3' end of each Jκ 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 Dκ 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 described from 11 patients with histologically proven centroblastic/centrocytic follicular lymphoma. Enzymatic amplification was performed by the PCR procedure of Saiki et al 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 (AmpliTaq-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-Dκ junctions on chromosome 14q+ was performed with primers BC1 and JH1 and the Dκ-mbr junctions on chromosome 18q− with primers DH1 and BC2 (see Table 1). The mer-Jκ junctions on chromosome 14q+ were amplified with primers BC5 and JH1 and the Dκ-mer 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 method was performed on PCR products. DNA from the reaction mixture was purified using a Sephadex G50 column followed by ethanol precipitation, freeze drying, and resuspension in distilled water. Sequencing was performed using a primer based on the Dκ recombination signal sequences known to flank germline Dκ sequences. Several 18q− junctions were shown to consist of Dκ/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.

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From the Imperial Cancer Research Fund, Medical Oncology Unit, St Bartholomew’s Hospital, London, UK; and Medical Oncology Department, Ospedale San Giovanni, Bellinzona, Switzerland. Submitted November 20, 1989; accepted March 6, 1990.

Supported by a grant from the Caroline Lawson Trust for Children’s Cancer Research.

Address reprint requests to Finbarr E. Cotter, MD, ICRF Medical Oncology Unit, St. Bartholomew’s Hospital, London EC1, UK.

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0006-4971/90/7601-0022$3.00/0

Blood, Vol 76, No 1 (July 1), 1990; pp 131-135

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

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Use</th>
<th>Position (strand)</th>
<th>Orientation</th>
</tr>
</thead>
<tbody>
<tr>
<td>JH1</td>
<td>5'ACCTGAGGAGGCTGACC-3'</td>
<td>PCR</td>
<td>3' JH consensus</td>
<td>-</td>
</tr>
<tr>
<td>DH1</td>
<td>5'-GTGAGGTCTGTGCTACCTTG-3'</td>
<td>PCR</td>
<td>5' flanking region</td>
<td>+</td>
</tr>
<tr>
<td>BC1</td>
<td>5'-CCTTCTAGGAGAGCTGTTAC-3'</td>
<td>PCR</td>
<td>5' of mbr in bcl-2</td>
<td>+</td>
</tr>
<tr>
<td>BC2</td>
<td>5'-ATATTATTCTATCCATCAAGT-3'</td>
<td>PCR</td>
<td>3' of mbr in bcl-2</td>
<td>-</td>
</tr>
<tr>
<td>BC3</td>
<td>5'-CACAGAGCAACCCAGACCC-3'</td>
<td>Sequencing</td>
<td>5' of mbr in bcl-2</td>
<td>+</td>
</tr>
<tr>
<td>BC4</td>
<td>5'-GTCCTGCAATGTCATTCCCT-3'</td>
<td>Sequencing</td>
<td>3' of mbr in bcl-2</td>
<td>-</td>
</tr>
<tr>
<td>BC5 (MC12)</td>
<td>5'-GATGGCAGCTGAGAGGTAT-3'</td>
<td>PCR</td>
<td>5' of mcr in bcl-2</td>
<td>+</td>
</tr>
<tr>
<td>BC7 (MC7)</td>
<td>5'-TCAGTCTCGGAGAGGAGTGG-3'</td>
<td>Sequencing</td>
<td>5' of mcr in bcl-2</td>
<td>+</td>
</tr>
<tr>
<td>BC6</td>
<td>5'-TTATTGAGTCTGTGCTTTC-3'</td>
<td>PCR</td>
<td>3' of mcr in bcl-2</td>
<td>-</td>
</tr>
<tr>
<td>BC8</td>
<td>5'-TCAGTCCTCGGGAGAGGTGG-3'</td>
<td>Sequencing</td>
<td>3' of mcr in bcl-2</td>
<td>-</td>
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</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.

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 JH 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 mbrI3 or mcr primer oligonucleotides in combination with a JH consensus oligonucleotide. Similar amplification of the 18q- junction is more problematic, because in the few tumors analyzed7,10 a diversity region (Dn) 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.

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 JH sequences and their germline equivalents are underlined. The intervening sequences between bcl-2 and JH are indicated in lowercase. The part of the intervening sequence that is identical to a previously identified Dn region is underlined (patient E).
**SEQUENCE ANALYSIS t(14;18) IN FOLLICULAR LYMPHOMA**

<table>
<thead>
<tr>
<th>CHROMOSOME 14</th>
<th>CHROMOSOME 18</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCCCCGGTTTCGGTATGGGGAATTACGTATGAACCGGTGGCCTGAGA</td>
<td>CCGGGGTTTTCCTATGGCTGTTCCTTCCAGGTTT</td>
</tr>
</tbody>
</table>

**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. The vertical line indicates the junction between the two chromosomes.

region exons (Jn), confirming the presence of the t(14;18) translocation in these tumors. The breakpoint on bcl-2 fell within the range of previously determined breakpoints in the mbr region. There was a clear preponderance (5 of 7) for the Jn member of the Jn family. Most of the Jn 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 Jn, 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 Jn in normal Ig gene rearrangement; thus, this junction (bcl-2-Dn-Jn) could result from translocation after Dn-Jn recombination. Thus, this junction consisted of bcl-2 fused to Dn, which in turn was fused to Jn. 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 18. The sequence of an 18q- junction is shown in Fig 2, in which a putative Dn region is fused to the mbr region of bcl-2. The sequence of an 18q- junction is shown in Fig 2, in which a putative Dn region is fused to the mbr region of bcl-2. In the sample from patient B, direct sequencing also showed the structure of the 18q- junction, and this has been compared with the 14q+ and normal chromosome 18 sequence in the same tumor in Fig 3. It is clear that during breakage and rejoicing, the bcl-2 gene has been conserved without any of the short duplications or deletions noted by others.

**Analysis of mcr junctions in bcl-2.** A similar analysis was performed using oligonucleotide primers for the mcr region of bcl-2. In a particular patient it was possible to sequence both the 14q+ and 18q- junctions, and the results are shown in Fig 4. The 14q+ junction is similar to those reported by others, with mcr sequence fused to a member of the Jn family. Because the junction is well within the Jn exon, it is not possible to identify uniquely which Jn gene is involved. There is very little (at most a single base) sequence that could be called an N-insertion, and the breakpoint in mcr is precisely at a position which has been broken in two previously analyzed tumors. The sequence of the reciprocal 18q- junction is also shown in Fig 4, and it is clear, as in Fig 3, that there has been no net loss or gain of bcl-2 sequence. Furthermore, as with mbr translocations, this junction is composed of a Dn sequence fused to bcl-2.

**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 primer, 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. Secondly, the normal N-regions are GC rich whereas translocation N-regions are less so. Finally, Ngan et al noted that two different tumors had an identical stretch of 11 bases in

**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.
their putative N-regions, and speculated that such regions could be derived instead from previously unrecognized D_{H} sequences. The tumor from patient E (Fig 1) has been shown to contain a complete D_{H} 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_{H} regions. Interestingly, this D_{H} sequence is identical to one previously shown to be located 150 bases 5' to a D_{H} involved in a follicular lymphoma translocation.\(^7\) Comparison with this sequence indicates that the germline recombination signals are missing from the 5' end of the D_{H} 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.\(^7\) The latter course of events has been proposed\(^9\) to account for a t(8;22) translocation in Burkitt's lymphoma in which the Ig 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.\(^5\) 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.\(^7\) An alternative explanation for the breaking of tcl-2 that does not depend on the presence of heptamer-nonamer signal sequences has been proposed. Bakhshi et al\(^7\) 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\(^1\) has shown small deletions, rather than duplications, of tcl-2 sequence at junctions. Moreover, in our study both tumors analyzed showed conservation of tcl-2 sequence, and therefore the duplication of junctional tcl-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\(^1\) in which a deletion of 2bp had occurred. This effectively rules out the possibility that the gain or loss of tcl-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_{H} 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\(^1\) 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) 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.

**ACKNOWLEDGMENT**

The authors gratefully acknowledge the advice of G. Phear in performing direct sequencing; the synthesis of oligonucleotides by I. Goldsmith; and the clinical support of T.A. Lister.

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Direct sequence analysis of the 14q+ and 18q- chromosome junctions in follicular lymphoma

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