Application of Long-Distance Polymerase Chain Reaction to Detection of Junctional Sequences Created by Chromosomal Translocation in Mature B-Cell Neoplasms

By Takashi Akasaka, Mikiko Muramatsu, Hitoshi Ohno, Ikuo Miura, Eiji Tatsumi, Shirou Fukuhara, Toru Mori, and Minoru Okuma

Junctional sequences created by chromosomal translocations in mature B-cell neoplasms, which involve immunoglobulin gene loci (IG) and putative proto-oncogenes on reciprocal partner chromosomes, are unique to neoplastic cells characterized by particular histological and immunological phenotypes. To establish a rapid and sensitive method to detect neoplastic cells carrying a specific chromosomal translocation, we have developed a novel strategy based on long-distance polymerase chain reaction (LD-PCR) amplification. Genomic DNA was extracted from tumor cells carrying t(14; 19)(q32; q13), t(8; 14)(q24; q32), t(3; 22)(q11; q11), t(2; 3)(p12; q27), or t(3; 14)(q27; q32). Thirty-two to 35-mer oligonucleotide primer pairs were designed to be complementary to exons or flanking sequences of the BCL2, BCL3, c-MYC and BCL6 oncogenes, and to IG constant region genes. LD-PCR with a newly available Taq polymerase for longer product synthesis successfully amplified fragments representing BCL3/Cα junctional sequences for t(14;19); c-MYC/Cμ, c-MYC/Cγ, and c-MYC/Cα for t(8;14); BCL6/Cα for t(3;22); BCL6/Cc for t(2;3); 5′-BCL6/Cμ, and 5′-BCL6/Cγ for t(3;14).

Human leukemias and lymphomas are often associated with specific chromosomal translocations that correlate closely with particular histological and immunological phenotypes. Many of the genes located on the breakpoints of these recurring translocations have been cloned, and it has been shown that chromosomal translocation leads to the formation of fusion genes encoding chimeric proteins with unique biochemical properties or to alterations in expression of the genes. Because the junctional sequences created by the fusion of two genes are specific to particular subtypes of leukemias and lymphomas, detection of these sequences provides valuable information for diagnosis and subsequent management of hematological neoplasms. Translocations in acute leukemias and chronic myelocytic leukemia result in production of fusion transcripts that contain information from two genes involved in the translocation. These fusion transcripts can be easily detected by reverse transcriptase (RT)-mediated polymerase chain reaction (PCR) amplification, although the amplified fragments are not unique to individual patients.

In contrast, many of the translocations in mature B-cell neoplasms involve immunoglobulin gene (IG) loci and, in general, coding regions of the putative oncogenes on partner chromosomes are not interrupted by the translocation. The overall consequence of these translocations appears to be inappropriate levels of oncogene product compared with normal lymphocytes at the equivalent stage of B-cell differentiation. Thus, targets for PCR amplification in B-cell neoplasms are single-copy junctional sequences of translocations within complex genomic DNA. A generally applicable example of PCR detection of junctional sequences is t(14; 18)(q32; q21) translocation. Tight clustering of the breakpoints at two main regions of the BCL2 gene and the availability of consensus sequences of the J segments of IG heavy chain gene (IGH) makes this a particularly favorable target for PCR amplification to detect lymphoma cells containing the translocation. However, PCR amplification in other B-cell tumor-associated translocations has not yet been established, as the breakpoint on the partner chromosome can be distributed over a large region, such that the region enclosed by the primers is too large to yield a PCR product by standard PCR techniques, and because breakage of the IGH gene sometimes occurs in switch regions composed of repeat sequences, making it difficult to design unique primers immediately adjacent to breakpoints.

Recently, Taq DNA polymerase for PCR amplification has been improved for longer product synthesis, and effective amplification of long targets from human genomic DNA has been described. In this study, we designed specific oligonucleotide primer pairs complementary to exons or flanking sequences of the c-MYC, BCL2, BCL3, and BCL6 oncogenes and to constant region genes of the IG genes. We present here a novel strategy for detection of the junctional sequences of translocations in mature B-cell neoplasms, based on long-distance polymerase chain reaction.

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Submitted November 13, 1995; accepted March 21, 1996.

Supported by Grants-in-Aid for Cancer Research from the Ministry of Health and Welfare (7-30), and from the Ministry of Education, Science and Culture of Japan (07671196).

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distance (LD)-PCR amplification using a newly available Taq DNA polymerase.

MATERIALS AND METHODS

Tumor materials and cell lines. Involved lymph node tissues or other tumor samples from patients with lymphoid neoplasms were studied by routine cytological and histological review, immunohistochemical analysis, immunological phenotyping, and antigen receptor gene rearrangement analysis to determine B-cell origin. Cell lines used in this study were as follows: Burkitt's lymphoma/leukemia (BL) cell lines Ramos, Manca, Raji, Daoud, and P3HR-1 were characterized previously by other investigators\(^1\); KS-Bu3, DL-4, Kobayashi, L-1091, ITO,\(^2\) Kimura, KPB-L2,\(^3\) Middle91, Tree92, and Black93 lines were established and characterized in our laboratory; HBL-4 and HBL-5\(^4\) were provided by Dr M. Abe (Fukushima Pre- fectural University of Medicine, Fukushima, Japan); BW-1 was ob- tained from Dr T. Tsuda (Wakayama Prefectural University of Med- cine, Wakayama, Japan); and MD901\(^5\) was from Dr T. Miki (Tokyo Medical and Dental University School of Medicine, Tokyo, Japan). The cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum and antibiotics at 37°C in 5% CO₂. Cyto- genetic analysis was performed for cell lines and selected tumor materials.

DNA probes and genomic Southern blot hybridization. DNA probes for oncogenes involved in translocations were as follows: poc1.4P probe for the BCL3 gene\(^6\); F372 probe representing the major translocation cluster (MTC) region of the BCL6 gene\(^7\); and pMYc6514-1 (exon 1) and pMYc6514-2 (exon 2) probes for the c-MYC gene, which were provided by the Japanese Cancer Research Resources Bank (JCRB; Tokyo, Japan). DNA probes for the IG genes were as described previously. Genomic DNA extracted from tumor materials and cell lines was analyzed with regard to rearrangements of oncogenes by Southern blot analysis. DNA was digested with restriction enzymes, electrophoresed through 0.8% agarose gels, and transferred onto nylon membrane filters (GeneScreen Plus; NEN Research Products, Boston, MA). Each reaction mixture (10 mL) contained 100 ng of genomic DNA and/or appropriate amount of cloned DNA, reaction buffer, 8 mL of dNTP mixture, 20 pmol of each primer, and 2.5 U Taq DNA polymerase (TaKaRa LA Taq polymerase); included in TaKaRa LA PCR Kit, Takara Shuzo), which was im- proved for longer PCR product synthesis. The PCR protocol was as follows: denaturation at 94°C for 1 minute; 14 cycles of denaturation at 98°C for 20 seconds, and primer annealing and DNA extension at 68°C for 20 minutes; 16 cycles of denaturation at 98°C for 20 seconds, and annealing and extension at 68°C for 20 minutes with 15-second increments per cycle (auto-segment extension); further extension at 72°C for 10 minutes; and rapid cooling to 4°C. Aliquots of the PCR mixtures were analyzed by agarose gel electrophoresis (0.7% agarose, Sigma, St Louis, MO) and visualized under ultraviolet (UV) illumination after ethidium bromide (EtBr) staining. Southern transfer onto membranes and hybridization with DNA probes for oncogenes were performed as described above. Oligonucleotide probes were end-labeled with [γ-32P]ATP (Amersham) by T4 poly- nucleotide kinase (MEGALABEL Kit, Takara Shuzo).

RESULTS

Reliability of LD-PCR amplification. To establish reaction conditions and to evaluate the reliability of LD-PCR amplification, we first performed reactions using isolated clones as templates, and the most suitable primer pair and optimal conditions for efficient PCR amplification of the junctional sequences were defined. These reaction conditions were then used for LD-PCR on genomic DNA.

pIM8.4G plasmid clone (Fig 1A) was isolated from a patient with chronic lymphocytic leukemia characterized by rearrangement of the BCL3 gene.\(^8\) The clone included the BCL3 sequences on chromosome 19, which were joined to the 5' side of the switch region of the Am(2) allototype of the Cα constant gene.\(^9\) The breakpoint on chromosome 19 was 0.8 kb upstream of the first exon of BCL3. Thus, the clone represented junctional sequences of the t(14;19) transloca- tion, although cytogenetic data were not available. AMH-1 phage clone (Fig 1B) was isolated from a patient with high-grade small noncleaved cell lymphoma (no. 744; this patient was reported as case 2 in Ohno\(^8\)) and case 8 in Ohno\(^10\)). The lymphoma cells had a t(3;22)(q27;q11) translocation and the BCL6 gene on chromosome band 3q27 was rearranged with the IGA light chain gene. Restriction mapping of the clone showed that chromosome 3 sequences were joined to the 5' side of the third constant region gene of the IGA gene (Cα3).\(^10\) The breakpoint on chromosome 3 was within the MTC region of the BCL6 gene\(^11\) and that on chromosome 22 presumably occurred at the J segment associated with the Cα3 constant gene.

The t(14;19) translocation involved the Sa switch region composed of regular pentamer repeat sequences, hindering the design of primers capable of specific hybridization to this particular region. Thus, we prepared a primer for the chromosome 14 sequences to be hybridized with the CH1 exon of the Cα constant gene,\(^2\) which is located downstream of the Sa switch region. Oligonucleotide primers for the chromosome 19 sequences were designed to complemen-
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Table 1. Sequences and Specificities of Oligonucleotide Primers for Long-Distance PCR

<table>
<thead>
<tr>
<th>Primer Designation</th>
<th>5' to 3' Sequences</th>
<th>Specificity</th>
<th>Strand/Orientation</th>
<th>Reference No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCL3/02</td>
<td>GGCCCTATCCTCTACATTCTCAGTCTCTGAGTGCTG</td>
<td>BCL3, 5' flanking</td>
<td>A/R</td>
<td>26</td>
</tr>
<tr>
<td>MYC/O4</td>
<td>ACAGTCCTGGATCATGTCAGTCTCTTGTAGAAGGTCG</td>
<td>c-MYC, exon 2</td>
<td>A/R</td>
<td>28</td>
</tr>
<tr>
<td>MYC/O6</td>
<td>TGCTGCTGCTGCTGCTGCGG</td>
<td>c-MYC, exon 2</td>
<td>A/F</td>
<td>28</td>
</tr>
<tr>
<td>MYC/O7</td>
<td>ACCTCAGGGAATGGTGATGCTCTGGTCGCTGCTGCTG</td>
<td>c-MYC, exon 1</td>
<td>S/F</td>
<td>28</td>
</tr>
<tr>
<td>MYC/O8</td>
<td>AGTCTGATGATATGGTGGTGCTGCTGCTGCTGCTG</td>
<td>c-MYC, exon 2</td>
<td>A/R</td>
<td>28</td>
</tr>
<tr>
<td>BCL6/02</td>
<td>CAATCCCCGGCTCAGTATGTTGATGCTGCTGCTG</td>
<td>BCL6, exon 2</td>
<td>A/R</td>
<td>15</td>
</tr>
<tr>
<td>BCL6/04 (5'-BCL6)</td>
<td>TTATCACGACCCACAGATGATCACCTTCTTAGA</td>
<td>BCL6, approximately 4 kb</td>
<td>S/F</td>
<td></td>
</tr>
<tr>
<td>C5/o1</td>
<td>GGCGGCTGGGTGCTGAGGCTGATGCTGTGCTGCTG</td>
<td>C5 constant region</td>
<td>A/R</td>
<td>34</td>
</tr>
<tr>
<td>C5/o2</td>
<td>TGCGGCTGGCTGCTGCTGCTGCTGCTGCTGCTGCTG</td>
<td>C5 constant region</td>
<td>A/R</td>
<td>24</td>
</tr>
<tr>
<td>C5/o3</td>
<td>TCCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTG</td>
<td>C5 constant region, CH1</td>
<td>A/R</td>
<td>29</td>
</tr>
<tr>
<td>C5/o1</td>
<td>AAGATGGTGATGGTGGAGGGATGGAGGGATGGAGG</td>
<td>C5 constant region, membrane exon 1</td>
<td>A/R</td>
<td>31</td>
</tr>
<tr>
<td>C5/o2</td>
<td>AGGGCGGCTGGCTGAGGAGGAGGAGGAGGAGG</td>
<td>C5 constant region, CH1</td>
<td>A/R</td>
<td>30</td>
</tr>
<tr>
<td>C5/o1</td>
<td>TCCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTG</td>
<td>C5 constant region, CH1</td>
<td>A/R</td>
<td>25</td>
</tr>
</tbody>
</table>

Abbreviations: S, sense strand; A, antisense strand; F, forward direction; R, reverse direction.

MYC/O6 was used as an internal oligonucleotide probe for c-MYC. MYC/O7 and MYC/O8 primer pair was used for amplification of germline c-MYC sequence. The other primers were for LD-PCR of junctional sequences.

PCR amplification was performed using a newly available Taq polymerase, which was improved to allow amplification of longer fragments. Figure 2A shows the results of LD-PCR amplification using the cloned and genomic DNA as templates. LD-PCR analysis detected a 1.8-kb fragment representing the 5'-BCL3/Ca junction in t(14;19) and a 10-kb discrete single band representing the BCL6/Ca junction in t(3;22). The sizes of the PCR product from the genomic DNA were identical to those from the cloned DNA, as expected from the restriction maps of the clones. To confirm amplification, the amplified fragments were transferred onto nylon membranes and hybridized with the appropriate radiolabeled probes; strong hybridization with the probes was observed (Fig 2B). These pilot studies clearly demonstrated...
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Fig 2. (A) Ethidium bromide-stained gel following electrophoresis of PCR products. PCR amplification was performed using the primer pairs indicated with genomic DNA (100 ng), plasmid DNA (10 ng), or bacteriophage DNA (10 ng) as a template. Aliquots of the PCR amplification mixtures were electrophoresed through a 0.7% agarose gel. HindIII-digested λ phage DNA was used as a molecular size marker. (B) Southern blots prepared from the same gel hybridized with the 32P-labeled Cr2 genomic probe5 and the F372 probe from the BCL6 gene, respectively.

Representative data for optimization of the conditions of LD-PCR are shown in Fig 3. To maximize the polymerase activity and to increase the yield of amplification products, annealing/extension time after the fifteenth cycle was extended for each cycle. Using the LD-PCR conditions described in Materials and Methods, as little as 0.01 pg of AMH-1 phage DNA in unrelated human genome DNA could be amplified to be visualized on EtBr-stained gels.

Application to t(8;14) translocation in BL. We applied the LD-PCR approach to the t(8;14) translocation, which results in recombination of the c-MYC gene on chromosome 8 with the IGH gene on chromosome 14 in divergent orientation. Molecular cloning of the translocation has shown a remarkable heterogeneity in genetic architecture surrounding the breakpoints.6 Sites of breakage on chromosome 14 are distributed throughout the IGH locus; translocations have been shown to occur near Dµ segments, JH segments, and within Sµ, Sγ, and Sε switch regions. On chromosome 8, translocations involve far 5' of the c-MYC gene, upstream of the gene or within the first intron, leaving the coding regions of c-MYC, composed of the second and third exons, intact. We thus designed primers for the second exon of the c-MYC gene (MYC/04)3 and constant region genes of the IGH.35,29,32 For the Cγ gene, two independent primers were prepared: one for the membrane exon (Cγ/01) and the other for the CH1 exon (Cγ/02). The former was located approximately 3.5 kb downstream of the latter. Orientation of the primer pairs was the same as that described for t(14;19) and t(3;22).

Figure 4A shows the results of LD-PCR for BL cell lines, in which the genomic structures of t(8;14) have been well

Fig 3. Representative results for optimization of LD-PCR conditions. (A) AMH-1 phage DNA was 105-fold diluted in unrelated genomic DNA and LD-PCR for BCLL6/Cα junction was performed with various annealing/extension times. An aliquot of 2 μL was loaded in each lane. The yield of PCR products declined at 20 minutes extension. However, auto-segment extension (AE) after the fifteenth cycle, as described in Materials and Methods, resulted in maximal amounts of the PCR products. (B) LD-PCR was performed with various concentration of dNTP. The amounts of dNTP solution included in the LA PCR kit are indicated. (C) Comparison of standard Taq polymerase and LA Taq. (D) AMH-1 DNA was serially diluted into unrelated genomic DNA and LD-PCR for BCL6/Cα junction was performed. As little as 0.01 pg of phage DNA could be detected on ethidium bromide-stained gels following amplification.
characterized.\textsuperscript{9-11} Genomic linkage on the 14q+ chromosome in Ramos cells was reported to occur between a point 5' of c-MYC exon 1 and within the S\textsubscript{\mu} switch region. In the Manca cell line, breakpoints were found at c-MYC intron 1 and a point close to the IG enhancer element between the J\textsubscript{H} and S\textsubscript{\mu} regions. The 5' sequences of c-MYC in Raji cell line linked the switch region associated with the Cy constant gene. As shown in Fig 3A, LD-PCR using appropriate primer pairs successfully detected c-MYC/IGH fusions in these cell lines. The sizes of the amplified fragments were compatible with those expected from the reported genomic structures.

We next applied the LD-PCR approach to large numbers of established cell lines and fresh tumor materials from cases of BL. All materials were subjected to LD-PCR using the primers for the c-MYC exon 2 (MYC/04) and four IGH constant gene loci. Table 2 summarizes the results in BL cells with some of their properties. Figure 4B shows a representative EtBr-stained gel; specific amplification were confirmed by hybridization with a DNA probe (pMyc6514-2) representing the c-MYC exon 2 and an oligonucleotide probe (MYC/06) for the c-MYC sequence internal to the PCR primer. Figure 5 shows a negative amplification study of LD-PCR for c-MYC/IGH junction in BL and other hematological tumor cell lines in which no c-MYC rearrangement with IGH gene was apparent on genomic Southern blot hybridization. Daudi and P3HR-1, which carried a t(8;14) translocation
involving a region far 5' of c-MYC, gave negative results on amplification.

Of the 22 materials from BL, LD-PCR detected c-MYC/IGH linkage in 17; five with the Cμ, nine with the Cγ, and three with the Ca constant genes. The sizes of the amplified products ranged from 2.3 to 12 kb, and they were unique to each tumor material. In the HBL-5 cell line, LD-PCR with the Cy/02 primer failed to amplify the junctional sequences, suggesting that the Cy constant gene corresponding to the primer may have been deleted. In contrast, only the Cy/02 primer detected a 12-kb fragment in case no. 839. Thus, yields of amplified fragments longer than approximately 15 kb may be lower than those detectable by gel electrophoresis. With respect to the c-MYC rearrangement, all LD-PCR-positive materials had a c-MYC rearrangement detected by conventional Southern blot analysis, suggesting that breakpoints of these materials were located adjacent to the c-MYC exon 1.

**Table 2. Long-Distance PCR Detection of (t(8;14) in BL**

<table>
<thead>
<tr>
<th>Cell Lines and Tumor Materials</th>
<th>Origin</th>
<th>EBv</th>
<th>Cytogenetics</th>
<th>c-MYC Rearrangement</th>
<th>LD-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ramos</td>
<td>American</td>
<td>–</td>
<td>t(8;14)</td>
<td>+</td>
<td>Cμ</td>
</tr>
<tr>
<td>Manca</td>
<td>American</td>
<td>–</td>
<td>t(8;14)</td>
<td>+</td>
<td>Cμ</td>
</tr>
<tr>
<td>Raji</td>
<td>African</td>
<td>+</td>
<td>t(8;14)</td>
<td>−</td>
<td>Cγ</td>
</tr>
<tr>
<td>Daun</td>
<td>African</td>
<td>–</td>
<td>t(8;14)</td>
<td>–</td>
<td>Ca</td>
</tr>
<tr>
<td>P3HR-1</td>
<td>African</td>
<td>–</td>
<td>t(8;14)</td>
<td>–</td>
<td>Cα</td>
</tr>
<tr>
<td>KS-Bu3</td>
<td>Japanese</td>
<td>–</td>
<td>t(8;14)</td>
<td>+</td>
<td>Cγ</td>
</tr>
<tr>
<td>L-1091</td>
<td>Japanese</td>
<td>–</td>
<td>14q+</td>
<td>+</td>
<td>Ca</td>
</tr>
<tr>
<td>DL-4</td>
<td>Japanese</td>
<td>–</td>
<td>t(8;14)</td>
<td>+</td>
<td>Cμ</td>
</tr>
<tr>
<td>Kobayashi</td>
<td>Japanese</td>
<td>–</td>
<td>t(8;14)</td>
<td>+</td>
<td>Cγ</td>
</tr>
<tr>
<td>Kimura</td>
<td>Japanese</td>
<td>–</td>
<td>t(8;14)</td>
<td>+</td>
<td>Ca</td>
</tr>
<tr>
<td>HBL-4</td>
<td>Japanese</td>
<td>–</td>
<td>t(8;14)</td>
<td>+</td>
<td>Cγ</td>
</tr>
<tr>
<td>HBL-5</td>
<td>Japanese</td>
<td>+</td>
<td>t(8;14)</td>
<td>+</td>
<td>Cμ</td>
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<td>ITO</td>
<td>Japanese</td>
<td>–</td>
<td>14q+</td>
<td>–, amplification</td>
<td>Cγ</td>
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<tr>
<td>KPB-L2</td>
<td>Japanese</td>
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<td>t(8;14)</td>
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</tr>
<tr>
<td>Middle91</td>
<td>Japanese</td>
<td>+</td>
<td>t(8;14)</td>
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<td>Ca</td>
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<td>Tree92</td>
<td>Japanese</td>
<td>–</td>
<td>t(8;14)</td>
<td>–</td>
<td>Cμ</td>
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<td>Cμ</td>
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<td>BW-1</td>
<td>Japanese</td>
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<td>Cμ</td>
</tr>
<tr>
<td>No. 836</td>
<td>Japanese</td>
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<td>NA</td>
<td>+</td>
<td>Cg</td>
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<tr>
<td>No. 837</td>
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<td>+</td>
<td>Cμ</td>
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<tr>
<td>No. 838</td>
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<td>NA</td>
<td>–</td>
<td>Ca</td>
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<tr>
<td>No. 839</td>
<td>Japanese</td>
<td>–</td>
<td>t(8;14)</td>
<td>–</td>
<td>Cγ</td>
</tr>
</tbody>
</table>

Abbreviation: NA, not available.  
* Cy/01 primer.  
† Cy/02 primer.

coding regions linked the Jα segment of the IGH. Thus, to detect recombination of the 5' region of BCL6 with the IGH gene, we designed another BCL6 primer, 5'-BCL6 primer, representing a sequence approximately 4 kb upstream of BCL6 (Fig 1B).

We performed LD-PCR amplification on genomic DNAs, in which rearrangement of the BCL6 gene was determined by Southern blot hybridization with the MTC probe. Of 34 materials examined, eight fresh materials and one cell line were positive for amplification (Fig 6, Table 3). The BCL6/Ca junctional products from two patients, including one (no. 744) described above, and MD901 cell line were similar in size, suggesting that breakage and joining in these three samples occurred at similar positions on the two genes. The amplified product representing (2;3) was detected in a patient who carried both BCL2 and BCL6 gene rearrangements. Primer pairs for the 5'-BCL6 and IGH constant genes successfully amplified fragments including a junction of t(3;14) from five cases; two had a 5'-BCL6/Cμ junction and three had a 5'-BCL6/Cγ junction, respectively.

**Sensitivity of LD-PCR.** To test the sensitivity of LD-PCR amplification, we performed a serial dilution mixing experiment with genomic DNA from Kobayashi cells carrying a c-MYC/Cγ junction and unrelated human DNA. The experiment consistently demonstrated a single band on agarose gel electrophoresis stained with ExBr at the level of sensitivity of 10−2. Hybridization with a radiolabeled probe improved the level of sensitivity by one order of magnitude (10−3; Fig 7A). To mirror the clinical conditions, Kimura cells, which also carried a c-MYC/Cγ junction, were diluted
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Fig 5. (A) Negative amplification of LD-PCR for c-MYC/Cp junction using MYC/04 and Cp/03 primer pair. HindIII-digested λ phage DNA was used as a molecular size marker. FL-18, FL-218, FL-318, FL-418, and FL-518, follicular lymphoma cell lines carrying t(14;18); HBL-1, HBL-2, and KIS-1, B-cell lymphoma cell lines; HBL-3 and TA-1, acute lymphocytic/biphenotypic leukemia cell lines; HL-60, an acute promyelocytic leukemia cell line; and LCL, a lymphoblastoid cell line from a normal volunteer. Except for the Ramos cell line shown in Fig 4, no amplification products were detected in other cell lines. (B) Integrity of the DNA was confirmed by LD-PCR amplification of germline c-MYC using primer pair for exon 1 (MYC/07) and exon 2 (MYC/08), under the same amplification protocol. Daudi cells showed slightly smaller products than those from other lines; this may have reflected a small deletion within the intron 1 of c-MYC, which has been described in endemic BL cells.

Fig 6. Ethidium bromide-stained gel showing LD-PCR amplification of junctional sequences of t(3;22)q27;q11), t(2;3)(p11;q27), and t(3;14)(q27;q32). Primer pairs used for each PCR amplification are indicated. HindIII-digested λ phage DNA was used as a molecular size marker.

DISCUSSION

Because malignant lymphoma is a disease curable by appropriate treatment, the ability to detect lymphomatous infiltration is important not only for accurate staging of the disease at presentation, but also for the evaluation of therapy. Considerable effort has been made to develop newer, more sensitive techniques capable of detecting minimal residual disease (MRD), which is almost certainly the source of recurring disease. The PCR technique, which specifically amplifies DNA sequences of interest, has greatly increased the sensitivity of MRD detection. Because the vast majority of B-cell neoplasms are characterized by clonal rearrangement of the IGH gene, PCR amplification of unique sequences of the complementarity-determining region III (CDRIII) of the IGH, which was originally developed for detection of B-cell lineage acute lymphoblastic leukemia, has been applied to mature B-cell neoplasms. However, one of the potential problems of the application of CDRIII amplification to malignant lymphoma is that the PCR product may not represent a neoplastic clone, as lymphoma tissues are apparently composed of heterogeneous cell populations in addition to neoplastic cells. Another disadvantage of this PCR approach is the occurrence of clonal changes within this particular region during the course of the disease, potentially leading to false negative amplification in tumor tissue obtained at relapse.

In contrast to the CDRIII sequences, junctional sequences created by chromosomal translocations in B-cell neoplasms, which involve IG loci and cellular oncogenes on the reciprocal partner chromosome, are unique to lymphoma cells characterized with particular histological and immunological phenotypes. Moreover, the junctional sequences would be stable during the course of the disease, as the fusion of two genes is most likely critical for the malignant phenotype. Thus, detection of these sequences provides valuable information for diagnosis and subsequent management of B-cell neoplasms. Here, we have described a sensitive method to detect lymphoma cells characterized by chromosomal translocation using a novel strategy based on long distance PCR amplification, as well as specific primer pairs for two genes involved in the translocation. The PCR strategy apparently
Table 3. Long-Distance PCR Detection of 3q27 Translocations Involving Immunoglobulin Gene Loci

| Materials and Cell Line | Diagnosis (WF) | Cytogenetics | LD-PCR  
<table>
<thead>
<tr>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td>1. No. 744</td>
<td>NHL (IBL)</td>
<td>t(3;22)(q27;q11)</td>
<td>BCL6 exon 2/Cx</td>
</tr>
<tr>
<td>2. No. 850</td>
<td>NHL (DL)</td>
<td>t(3;22)(q27;q11)</td>
<td>BCL6 exon 2/Cx</td>
</tr>
<tr>
<td>3. MD901 cell line</td>
<td></td>
<td>t(3;22)(q27;q11)</td>
<td>BCL6 exon 2/Cx</td>
</tr>
<tr>
<td>4. No. 514</td>
<td>NHL (FMIX)</td>
<td>t(2;3)(p11;q32)</td>
<td>BCL6 exon 2/Cx</td>
</tr>
<tr>
<td>5. No. 737</td>
<td>NHL (DL)</td>
<td>t(3;14)(q27;q32)</td>
<td>5'-BCL6/Cy</td>
</tr>
<tr>
<td>6. No. 119</td>
<td>NHL (FMIX)</td>
<td>t(7;14)(q27;q32)</td>
<td>5'-BCL6/Cy</td>
</tr>
<tr>
<td>7. No. 210</td>
<td>NHL (FMIX)</td>
<td>ND</td>
<td>5'-BCL6/Cy</td>
</tr>
<tr>
<td>8. No. 200</td>
<td>NHL (DL)</td>
<td>ND</td>
<td>5'-BCL6/Cy</td>
</tr>
<tr>
<td>9. No. 418</td>
<td>NHL (FL)</td>
<td>14q+</td>
<td>5'-BCL6/Cy</td>
</tr>
</tbody>
</table>

Abbreviations: NHL, non-Hodgkin’s lymphoma; WF, International Working Formulation for Clinical Usage; IBL, immunoblastic lymphoma; DL, diffuse large cell lymphoma; FMIX, follicular mixed small and large cell lymphoma; FL, follicular large cell lymphoma; ND, not done.

circumvents problems in standard PCR techniques, i.e., difficulty in design of unique primers adjacent to breakpoints on IG genes and distribution of breakpoints over a large region on the reciprocal partner chromosome. Other advantages of this LD-PCR approach are that it can be applied to translocations in which the DNA sequences of the regions immediately adjacent to the breakpoint have not yet been defined, and that amplified fragments have sequences unique to individual patients. Thus, this PCR approach could be applied to analysis of translocations in leukemias; PCR using DNA is considerably easier than RT-PCR, which requires RNA isolation and cDNA synthesis.

During the past decade, an increasing number of centers have treated patients with malignant lymphoma on first relapse with high-dose myeloablative therapy and autologous hematopoietic stem cell support. It is generally accepted that patient selection is a major factor in determining the disease-free survival in this trial. Diffuse large cell lymphoma (DLCL) of B-cell phenotype constitutes 30% to 40% of adult non-Hodgkin’s lymphoma and is a good candidate for this aggressive treatment. Thus, sensitive genetic markers for assessment of MRD of this particular lymphoma subtype are urgently required. Association of 3q27 translocations with DLCL have been described, and t(8;14) has been observed not only in BL, but also in DLCL; Offit et al showed that t(8;14) is the second and t(3;22) is the third most common translocation in this lymphoma subtype. Thus, the present PCR strategy should be useful for determining the efficiency of experimental approaches such as in vitro purging of grafts for transplantation obtained from autologous bone marrow or peripheral blood stem cell collections, which have been performed for lymphoma carrying the t(14;18) translocation. It would be of interest to determine whether patients with PCR-detectable disease ultimately relapse. The present study provided a potentially useful tool for further understanding of the contribution of MRD to subsequent relapse of malignant lymphoma.

The translocation t(8;14) differs at the molecular level in endemic and sporadic cases of BL. Breakpoints in endemic tumors lie far 5’ of the c-MYC exon 1 and

![Fig 7. Determination of the sensitivity of LD-PCR amplification.](image)
within switch regions of the IGH. The LD-PCR approach is apparently capable of detecting translocations of the sporadic type. In the present study, all cell lines and tumor materials in which c-MYC rearrangements were detectable by conventional Southern blot analysis were positive for amplification. Thus, it is evident that the current LD-PCR approach is an important adjunct to time-consuming Southern hybridization. It has been suggested that t(8;14) probably occurs in sporadic cases at a later stage of B-cell differentiation where the machinery for isotype class switching is active.6 6 Our present study showed an unexpected higher frequency of involvement of the y and e gene loci than the of the p gene, confirming further the correlation between t(8;14) and the class switch mechanism.

The 3q27 translocation affecting the BCL6 gene is unique in that it can involve not only the IG loci, but also other partner chromosomes.1 1,2 2,4 4,6 In this study, nine of 34 (26.5%) materials with a BCL6 gene rearrangement were positive for amplification, indicative of recombination with the IG loci. However, two materials with a cytogenetically identified translocation with chromosomal loci for the IGH and the IGs genes lacked fragments amplifiable by the present LD-PCR method (data not shown). Thus, the proportion of the involvement of IG loci in 3q27 translocations could be underestimated. It is of interest to determine whether breakpoints on the IG genes in the negative cases were at uncharacterized loci, which could be unrelated to known constant genes, or translocations generated sequences, which could prevent polymerase activity. Further improvement of primer design and reaction conditions will be necessary to elucidate this point.

Deweindt et al,1 7 cloned a complicated junctional sequence of t(3;14) from a patient with non-Hodgkin's lymphoma. They showed that chromosome 3 materials were translocated to an inverted 14q32 VNt-containing fragment, which was itself translocated to the Jc segment; the BCL6 coding regions joined the IGH gene in divergent orientation. In the present study, however, we observed no head-to-head fusion of BCL6 exon 2 with the IGH, as expected from the observations reported by Deweindt et al even in cases with t(3;14) on cytogenetic analysis. Because the BCL6 gene lies in telomere-to-centromere orientation on chromosome 3, t(3;14) would result in juxtaposition of 5'-BCL6 with the IGH on the 14q+ chromosome and junction of the VDJ complex-containing IGH segment with the coding exons of the BCL6 on the 3q- chromosome.4 7 Our LD-PCR study using primer pairs for the 5'-BCL6 and IGH constant genes provided persuasive evidence of this recombination on 14q+. This molecular consequence is in contrast with that of other translocations involving the IGH gene; coding regions are invariably juxtaposed with the downstream sequences of IGH, including a second enhancer located 3' of the Cae2 gene.4 8 Thus, it is possible that the critical molecular consequence of the 3q27 translocations may be the deletion of the first exon and 5' regulatory element, leading to enhanced expression. Of course, this does not exclude the possibility of transcriptional activity in the VDJ-containing segment of the IGH gene.

ACKNOWLEDGMENT

The authors thank Dr C. Bastard for providing the F372 probe.

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Application of long-distance polymerase chain reaction to detection of junctional sequences created by chromosomal translocation in mature B-cell neoplasms

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