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Thermostable DNA polymerase chain amplification of t(14;18) chromosome breakpoints and detection of minimal residual disease

(lymphoma/immunoglobulin genes/*BCL2* gene/chromosomal translocation/*Thermus aquaticus* DNA polymerase)

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ABSTRACT **Achieving the capacity to detect minimal numbers of neoplastic cells is a major cancer diagnostic challenge. Chromosomal translocations such as the t(14;18)-(q32;q21) found in follicular and some nonfollicular lymphomas provide a tumor-specific molecular marker. The 14;18 breakpoints are focused at one of six immunoglobulin heavy chain joining (J_H) regions on chromosome 14 and a small major breakpoint region (MBR) of the *BCL2* gene on chromosome 18. We utilized universal oligonucleotide primers of a region 5′ to the *BCL2* MBR and at the 3′ end of J_H segments to initiate a DNA polymerase chain reaction that amplified these *BCL2*–J_H junctures. Use of thermostable DNA polymerase enabled annealing and synthesis steps at temperatures approaching the melting point of the primers, providing a sensitive and specific assay capable of detecting 1 lymphoma cell in 10^6 normal cells. This technique identified the subclinical presence of leukemic cells in all seven patients examined, including two in clinical remission. It also assessed the effectiveness of protocols designed to purge malignant cells from marrow. Moreover, this approach enabled the rapid DNA sequencing of chromosomal breakpoints without their molecular cloning. This assay markedly refines the capacity to detect minimal residual disease and should improve the ability to determine the stage of disease, stratify treatment, and evaluate therapy.**

Specific interchromosomal translocations occur in many types of neoplasms but are not found in their normal cellular counterparts (1–3). The most frequent translocation in human lymphoma is the t(14;18)(q32;q21), which is detected by cytogenetics in approximately 85% of follicular small cleaved cell lymphomas (2, 4). Moreover, a number of cytogenetically indeterminant B-cell lymphomas, approximately 20% of diffuse large cell lymphomas, and 50% of adult undifferentiated lymphomas possess the t(14;18), as determined by gene rearrangement analysis (5).

Despite the mature B-cell phenotype of these lymphomas, the t(14;18) occurs at a very early stage of pre-B-cell development, when rearrangement of diversity (D_H) and joining (J_H) immunoglobulin heavy chain genes occurs (6). This translocation juxtaposes a putative oncogene, *BCL2*, from 18q21 with an immunoglobulin J_H segment on the derivative (der)14 chromosome (7–9). This results in a *BCL2*-immunoglobulin fusion gene, chimeric transcripts, and a marked transcriptional deregulation of the translocated *BCL2* (10–12). The breakpoints are remarkably focused on chromosome segment 18q21, where 70% lie within a 2.8-kilobase (kb) major breakpoint region (MBR) and most cluster within a 150-base-pair (bp) area (6). Thus, a translocation specific rearrangement unique to the neoplastic cells can be identified on Southern blots. This molecular marker can be used to follow the natural history of lymphomas (13) and search for minimal residual disease (MRD).

The rearrangement patterns of immunoglobulin and T-cell receptor genes in tumors have previously been used to identify residual or recurrent disease not detected by routine diagnostic studies (14–16). However, such Southern blot examinations are relatively insensitive, detecting clonal cells only if they make up 1–5% of the total. Approaches to magnify these antigen receptor rearrangements are frustrated by their heterogeneous nature [alternative variable (*V*), *D*, and *J* segments] and their occurrence in normal as well as neoplastic cells. In contrast, the t(14;18) is an attractive tumor-specific marker with focused breakpoints on both chromosomes. We and Lee *et al.* (17) exploited this event to amplify the site of chromosomal juncture by DNA polymerase chain reaction (PCR). We utilized *Thermus aquaticus* (*Taq*) DNA polymerase (18) at temperatures approaching the melting point of chromosome 14- and 18-specific oligonucleotides to amplify the *BCL2*–J_H breakpoint. This approach can amplify a single template copy of the breakpoint, enable its DNA sequence determination without cloning, and detect one neoplastic cell in 10^6 normal cells.

METHODS AND MATERIALS

***BCL2*–J_H *Taq* PCR.** Genomic DNA was obtained from lymph node biopsy samples or the mononuclear cells from peripheral blood or bone marrow aspiration (8). One to 10 μg of genomic DNA was placed in 100 μl of 1× PCR buffer (10 mM Tris·HCl, pH 8.3/50 mM KCl/10 mM $MgCl_2$/0.1% gelatin/dNTPs at 1.5 mM each, with each primer at 1 μM). Oligonucleotide primers were purified by polyacrylamide gel electrophoresis followed by C_{18} Sep-Pak (Waters Associates) purification. Five units of *Taq* DNA polymerase (Cetus, Emeryville, CA; units as defined by the supplier) diluted 1:5 was added and reaction mixtures were overlayed with 100 μl of paraffin oil. Capped tubes were subjected to serial cycles of denaturation (2 min at 95°C, first cycle 5 min), annealing (3 min at 51–61°C), and synthesis and extension (3 min at 72°C). After the final cycle tubes were placed at the annealing temperature for 3 min, followed by 6 min at 72°C. PCR products were extracted twice with $CHCl_3$, precipitated, and size fractionated on nondenaturing 6% polyacrylamide gels or alkaline 4% agarose gels. Products on agarose gels were transferred to GeneScreen*Plus* (New England Nuclear) and

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Abbreviations: MBR, major breakpoint region; MRD, minimal residual disease; *Taq*, *Thermus aquaticus*; PCR, DNA polymerase chain reaction; der, derivative.

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Hinf PBR
DHL-6 10μg
DHL-6 1 μg
DHL-6·1μg+U937·9μg
U937 1μg
DHL-6 10μg
DHL-6 1 μg
DHL-6·1μ+U937·9μg
U937 1μg

298
220/221
154
-238bp

Ig J_H Bcl-2-MBR

U937 .9 .99 ——→μg
DHL-6 $10^{-1}10^{-2}10^{-3}10^{-4}10^{-5}$μg

-238bp

Bcl-2MBR

FIG. 1. Southern blot analysis of Klenow PCR products hybridized with 6.0-kb *Bam*HI–*Hin*dIII immunoglobulin J_H or 2.8-kb *Eco*RI–*Hin*dIII *BCL2* MBR genomic probes. Amounts of genomic DNA from cell lines SU-DHL-6 and U937 are indicated at the tops of the lanes. Hinf PBR indicates size markers of *Hin*fI-digested pBR322. A 72-hr exposure reveals a faint band at 10^{-5} dilution.

hybridized with random primed genomic or T_4 polynucleotide kinase end-labeled oligonucleotide probes. PCR with the Klenow fragment of *Escherichia coli* DNA polymerase ("Klenow PCR") was performed with 37°C annealing and synthesis as previously described (19).

Direct DNA Sequencing of PCR Products. Predicted size fragments from PCR were identified by ethidium bromide staining, eluted from polyacrylamide, purified by C_{18} Sep-Pak, precipitated, and quantitated. Then 120 ng of fragment was subjected to dideoxy chain termination sequence analysis using end-labeled oligonucleotide primers. Samples were subjected to two cycles of denaturation, annealing, and extension at 42°C (20). All dNTPs were at a final concentration of 0.16 mM; 2′,3′-dideoxynucleotides ddGTP, ddATP, and ddTTP were 1 mM; ddGTP was 0.33 mM.

RESULTS

Klenow Amplification of the SU-DHL-6 Breakpoint by Using an Oligonucleotide 5′ to the *BCL2* MBR and 3′ to J_H6. We prepared a 20-residue oligonucleotide of the sequence found immediately 5′ to the 150-bp cluster area within the *BCL2* MBR (PCR-Oligo-1, 5′-TTAGAGAGTTGCTTTACGTG-3′) and a reverse complementary oligonucleotide specific for the 3′ flanking segment of the J_H6 region (PCR-Oligo-2, 5′-TAGAGTGGCCATTCTTACCT-3′). The der(14) breakpoint in the SU-DHL-6 cell line was at J_H6, possessed 11 extra base pairs at the site of juncture (*N* segment), and measured 238 bp between PCR-Oligo-1 and -2. A 25-cycle PCR was performed on genomic DNA that was denatured at 95°C, annealed to oligonucleotides at 37°C, and synthesized with 1 unit of the Klenow fragment of DNA polymerase I per cycle. The presence of SU-DHL-6 DNA resulted in the amplification of a predicted 238-bp fragment hybridizing to both J_H and *BCL2* MBR probes (Fig. 1). No hybridizable product was seen in cell line U937, which lacks the translocation. Serial dilution of SU-DHL-6 DNA to 10^{-5} with U937 DNA indi-

J_H

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CACCGTG   GCTGAATACTTCCAGCACTGGGGCCAGGGCACCCTGGTCACCGTCTCCTCAGGT  IVS J1
GACTGTG  CTACTGGTACTTCGATCTCTGGGGCCGTGGCACCCTGGTCACTGTCTCCTCAGGT  IVS J2
CTGTGTG        ATGCTTTTGATGTCTGGGGCCAAGGGACAATGGTCACCGTCTCTTCAGGT  IVS J3
CAATGTG        ACTACTTTGACTACTGGGGCCAAGGAACCCTGGTCACCGTCTCCTCAGGT  IVS J4
CAATGTG      ACACTGGTTCGACTCCTGGGGCCAAGGAACCCTGGTCACCGTCTCCTCAGGT  IVS J5
CATTGTG AT(TAC)5GGTATGGACGTCTGGGGGCAAGGGACCACGGTCACCGTCTCCTCAGGT  IVS J6

CACTGTG             TCGACTTCTGGGGCCAAGGGACCCTGGTCACCGTCTCCTCAGGT
                                                               CONSENSUS
                                                               SEQUENCE
                                   TGGGACCAGTGGCAGAGGAGTCCA
                                3' <———————————————————————— 5'
                                         PCR-OLIGO-4
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FIG. 2. Sequence comparison of the six J_H regions. Identity with a consensus sequence is indicated by a dot under the letter. A universal J_H primer, PCR-Oligo-4, represents the reverse complement of a 24-nucleotide consensus sequence.

cated that only 10 pg of translocated DNA was needed for amplification (Fig. 1).

A Universal J_H Primer and *Taq* DNA Polymerase. The der(14) juncture of the t(14;18) can involve any of the six J_H regions. A universal J_H primer would make it possible to amplify t(14;18) breakpoints without predetermining the J_H segment utilized. The 3′ portion of each J_H segment is composed of a donor splice site and the highly conserved framework 4 region (21). This enabled the construction of a 24-mer, PCR-Oligo-4, that was identical to three J_H segments and differed by only 1–3 bp from the others (Fig. 2). However, when PCR-Oligo-4 was used in the 37°C Klenow PCR, a nontranslocated product from the germ-line 18q21 segment was also amplified. At 37°C many partially identical sites within the 3×10^9 bp of the human genome also hybridize to such primers (as distant as 10 out of 19 nucleotides, in our experience). Those nonspecific products of J_H or *BCL2* origin also hybridized to the probes. To improve the specificity of primer base pair formation and remove the sink of partially identical sites, we use the DNA polymerase of the thermophilic microorganism *Thermus aquaticus*. This enabled the annealing step to be titrated to the highest optimal temperature for each oligonucleotide (Fig. 3). Moreover, in a 45-cycle amplification, *Taq* polymerase needs to be added only at the beginning. Fig. 4 displays the ethidium bromide-stained polyacrylamide gel of *Taq* PCR products and indicates that an ideal annealing temperature must be determined for each oligonucleotide selection. The number of partially identical sites amplified from the genome is markedly reduced with PCR-Oligo-1 and -4 by changing the annealing temperature from 51°C to 61°C. In addition, the amount of the specific breakpoint fragment synthesized is increased. When the oligomers are shortened to 15-mers, PCR-Oligo-1s, and -4s, virtually all of the nonspecific bands disappear at 61°C.

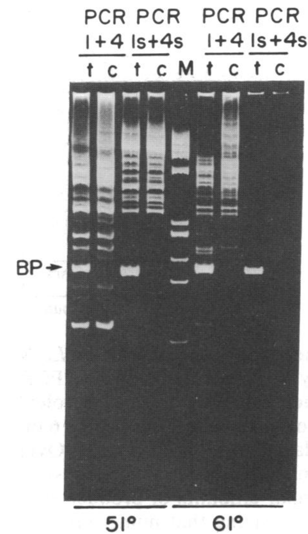

FIG. 4. Ethidium bromide-stained polyacrylamide gel of PCR products of 1 μg of DNA from t(14;18) cell (t) and a control (c) synthesized by using PCR-Oligo-1 (20-mer), -4 (24-mer), -1s (15-mer), or -4s (15-mer) as primers at annealing temperatures of 51°C or 61°C. BP indicates the 222-bp or 217-bp breakpoint fragment. M indicates size markers of *Hae* III-digested phage φX174 DNA.

However, 61°C is very near the functional melting point of these 15-mers and the sensitivity is reduced (Fig. 4).

Direct Sequencing of *Taq* PCR-Amplified Chromosomal Junctures. Hybridization of a genomic *BCL2* MBR probe as well as an oligonucleotide probe representing sequence internal to the primers (Fig. 3) established that the only hybridizable product of the *Taq* PCR was the authentic

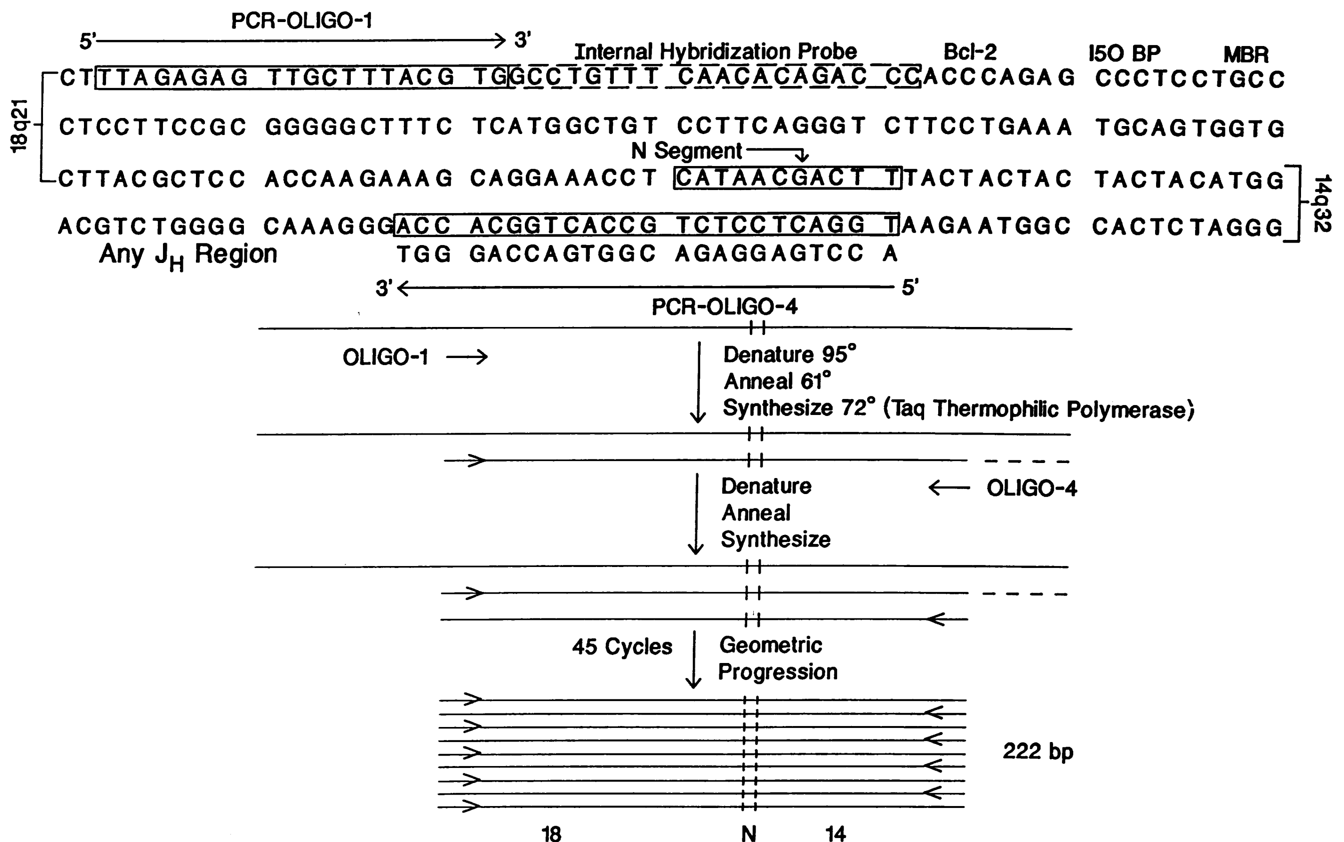

FIG. 3. Schematic presentation of the *Taq* PCR amplification of the der(14) breakpoint from SU-DHL-6. PCR-Oligo-1 is located 5′ to the 150-bp MBR of *BCL2*, while PCR-Oligo-4 is the reverse complement of a universal J_H sequence. An internal oligonucleotide hybridization probe identifies the 222-bp continuous product composed of chromosome 18, *N* segment, and chromosome 14 sequences.

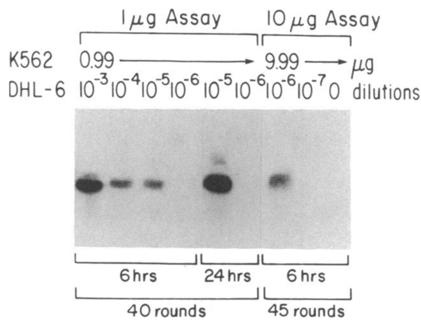

FIG. 5. Southern blot probed with *BCL2* MBR probe, demonstrating sensitivity and specificity of *Taq* PCR in 1-μg and 10-μg genomic DNA reactions. K562 is an erythroleukemia cell lacking a t(14;18). Autoradiograms were exposed for 6 or 24 hr and PCR was allowed to take place for 40 or 45 rounds. Overamplification (10^{-3} dilution) results in faint bands of various sizes upon transfer and hybridization. Similar amounts of product are present at 10^{-4} and 10^{-5} dilution, emphasizing that numerous rounds of amplification can overshadow the effect of initial template copy.

breakpoint (Fig. 5). This assay was capable of detecting 1 lymphoma cell in 10^5 total cells with 1 μg of genomic DNA or 1 cell in 10^6 with 10 μg of DNA (Fig. 5). The universal J_H primer proved capable of amplifying der(14) breakpoints that used different J_H regions (Fig. 6). The capacity of this technique to amplify a previously uncharacterized t(14;18) breakpoint was unequivocally demonstrated by gel purifying PCR-amplified breakpoint products and directly sequencing the fragments by dideoxy termination (Fig. 7). A J_H2 juncture with a 3-bp *N* segment and a classic *BCL2* MBR recombination is shown. Moreover, *Taq* polymerase amplification products produced a valid sequence when breakpoints that had been previously cloned and sequenced were reexamined by this approach (not shown).

Identification of MRD by *Taq* PCR. The sensitivity and specificity of the *Taq* PCR prompted a search for MRD in patients with follicular lymphoma (Fig. 8*A*). All patients examined to date with amplifiable breakpoints in DNA of their diagnostic lymph node cells had PCR evidence of clonal cells in peripheral blood. Several patients had enough clonal cells to be detected by routine genomic blots, whereas patient 3's subclinical leukemia was detectable only by PCR. Patients 5 and 6 were felt to be in complete remission after chemotherapy when examined, but PCR demonstrated clonal cells in their peripheral blood (Fig. 8*A*). Patient 7 was being considered for an ablative chemotherapy regimen with reinfusion of autologous bone marrow. However, the harvested bone marrow demonstrated clonal cells by PCR. The number of lymphoma cells was reduced by treating the marrow with methyl prednisolone or etoposide and most markedly with the drugs in combination (Fig. 8*B*).

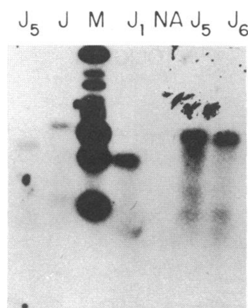

FIG. 6. Southern analysis of PCR products when PCR-Oligo-1 and -4 were used to amplify lymph-node DNAs that involved different J_H segments (J_1, J_5, J_6) at their der(14) breakpoint. The intensity of amplified bands reflects the percentage of lymphoma cells within lymph nodes. NA was a nonamplifiable lymphoma with a breakpoint outside the 150-bp MBR cluster. M is size markers of *Hin*fI/*Eco*RI-digested pBR322.

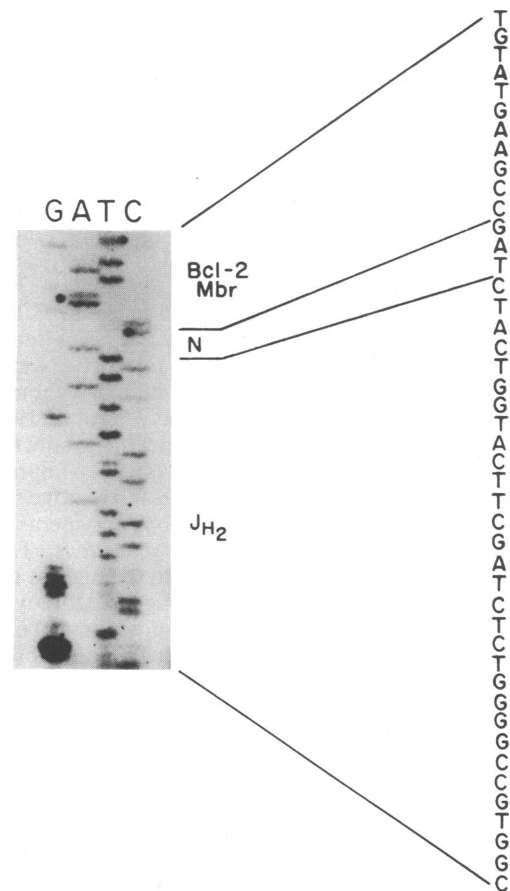

FIG. 7. DNA sequence of a PCR product, determined directly by dideoxy chain termination of the product. The juncture of J_H2, *N* segment, and *BCL2* MBR on the der(14) is shown.

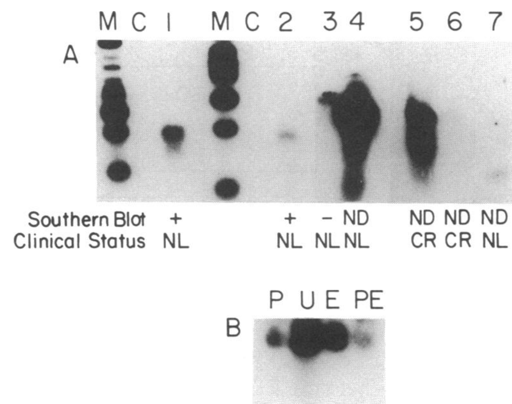

FIG. 8. (*A*) *BCL2*–J_H *Taq* PCR demonstration of amplifiable breakpoints from mononuclear cells from peripheral blood of lymphoma patients. Patients 1 and 2 had over 1–5% clonal cells present, as rearrangements were detected on routine Southern blots of nonamplified genomic DNA. M, marker; C, control (placenta); ND, not done; NL, nonleukemic; CR, clinical remission. (*B*) *BCL2*–J_H *Taq* PCR of bone marrow aspirate of patient 7. There is evidence for t(14;18) cells in U (untreated marrow), but there is improvement after treatment *in vitro* with methylprednisolone (P), etoposide (E), or both drugs (PE).

DISCUSSION

Sensitive techniques able to discern minimal residual tumor might spare therapy for patients without disease and allow others to receive innovative regimens. The malignancy-associated t(14;18) of B-cell lymphoma possesses well-focused breakpoints on both chromosomes such that the majority of translocations are amplifiable with a single procedure. Our first attempt with PCR utilized the Klenow fragment of DNA polymerase I at 37°C. However, the *BCL2* MBR and universal J_H primers selected amplified many partially homologous sites within the genome. These spurious products interfered with specificity and markedly reduced the sensitivity, presumably due to a competition for polymerase. *Taq* thermostable polymerase enabled relatively long, 20- to 24- residue, oligonucleotides to be used at annealing temperatures that approach the functional melting point of the primers. Fig. 4 demonstrates that the sink of nonspecific products can be eliminated and the synthesis of the desired product can be improved. The ideal temperature for each set of oligonucleotides needs to be empirically determined. The *BCL2*–J_H PCR is capable of detecting 1 in 10^5 cells when 1 μg of DNA is used and 1 in 10^6 cells when 10 μg is used; 10 μg is easily obtained from peripheral blood or marrow aspirates. Numerous dilution experiments and theoretical calculations (22) strongly suggest that *Taq* polymerase is capable of amplifying this breakpoint if one genomic copy is present in the reaction. The universal J_H primer allowed all J_H breakpoint sites on chromosome 14 to be amplified. The correct products could be detected with an oligonucleotide probe of sequences found internal to the primers, and even better sensitivity was obtained by using random primed genomic probes. Finally, the fidelity and efficiency of *Taq* polymerase permits direct DNA sequencing of amplified der(14) breakpoints without molecular cloning. This rapid sequencing approach should be applicable to a variety of genes, once an initial sequence allows primers to be constructed.

The t(14;18) is a malignancy-associated event that has not been found in normal B cells. The *BCL2*–J_H PCR unequivocally identified the presence of clonal cells with a t(14;18) in patients felt to be in complete remission and in bone marrow being evaluated for autologous transplantation. Such clones are presumably fully malignant lymphoma cells. However, extensive structural characterization of t(14;18) breakpoints indicated that this translocation occurs at a very early stage of pre-B-cell development (6). Thus, it is theoretically possible that populations of progenitor cells might exist that possess the t(14;18) but lack additional genetic defects that result in a lymphoma clone. This emphasizes the need for prospective studies to assess the natural history of such clones.

PCR amplification should be applicable to other translocations, provided their DNA breakpoints are clustered so that limited numbers of oligonucleotides can be universally applied. Moreover, it should also be possible to amplify hybrid RNA transcripts such as the BCR–Abl products of chronic myelogenous leukemia (23) by converting mRNA to a DNA template with reverse transcriptase and then proceeding with the PCR. These approaches promise to improve our understanding of the biology of neoplasia and our ability to monitor it.

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