Detection of myc Translocations in Lymphoma Cells by Fluorescence In Situ Hybridization With Yeast Artificial Chromosomes

By Maria Luisa Veronese, Masataka Ohta, Janet Finan, Peter C. Nowell, and Carlo M. Croce

Translocations involving chromosome 8 at band q24 and one of the Ig loci on chromosomes 14q32, 22q11, and 2p11 are the hallmark of Burkitt’s lymphoma (BL). It has been previously observed that the exact localization of the breakpoints at chromosome 8q24 can vary significantly from patient to patient, scattering over a distance of more than 300 kb upstream of c-myc and about 300 kb downstream of c-myc. To generate probes for fluorescence in situ hybridization (FISH) that detect most c-myc translocations, we screened a yeast artificial chromosome (YAC) library from normal human lymphocytes by colony hybridization, using three markers surrounding the c-myc gene as probes. We obtained 10 YAC clones ranging in size between 500 and 200 kb. Two nonchimeric clones were used for FISH on several BL cell lines and patient samples with different breakpoints at 8q24. Our results show that the YAC clones detected translocations scattered along approximately 200 kb in both metaphase chromosomes and interphase nuclei. The sensitivity, rapidity, and feasibility in nondividing cells render FISH an important diagnostic tool. Furthermore, the use of large DNA fragments such as YACs greatly simplifies the detection of translocations with widely scattered breakpoints such as these seen in BL.

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MATERIALS AND METHODS

Cell lines and patients. The BL cell lines Daudi, P3HR1, AG876, and EW36 (kindly provided by I. Magrath, National Institutes of Health, Bethesda, MD); Manca and BL2 were used in this study. All cell lines carry translocations involving chromosome 8 at band q24. Each of the five patient samples studied had a t(8;14) translocation. Four of the patients were diagnosed with sporadic BL (sBL) and one with large-cell immunoblastic lymphoma (LCIL; see Table 2).

Isolation and characterization of YAC clones. A YAC library established from normal human lymphocytes was screened by colony hybridization, as previously described, with probes pRyc7.4, pPA1,35B, and pD4AH1.6. The size of isolated YAC clones was determined by pulse-field gel electrophoresis (PFGE) and probes 8q267, pEW36-9H2.0, p380-8A1.8, p380H9 0.8s, pEW36-7D.
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G4

E2

P72

I2

Fig 1. Map of the region surrounding the c-myc locus on 8q24 showing the position of overlapping YAC clones P72, E2, G4, and I2 as well as the breakpoints (arrows) in BL cell lines BL2, Daudi, P3HR1, EW36, and Manca. Probes H4.1 (A), pEW36-7D (B), pD4AH1.6 (C), p380j9 (D), p380-8A1.8 (E), pEW36-9H2.0 (F), pPA1.3SB (G), and 8q267 (H) are also shown.

RESULTS

Ten YAC clones were isolated from the YAC library after screening with probes pRyc7.4, pPA1.3SB, and pD4AH1.6 (Fig 1). PFGE indicated sizes ranging between 200 and 500 kb. All clones were analyzed by Southern blotting using the probes 8q267, pEW36-9H2.0, p380-8A1.8, p380j9 0.8ss, pEW36-7D, and H4.1, in addition to the probes used for the screening of the library to define overlaps and to map the clones with respect to known breakpoints around the c-myc locus. The clones were further screened for chimerism by FISH on normal human lymphocyte chromosomes. Two nonchimeric clones, P72 and I2 (which gave a strong signal on both chromatids of chromosome 8 only), were used to detect the chromosomal breakpoints in the Burkitt’s cell lines and patient samples.

Four YAC clones forming a contig spanning the c-myc locus are depicted in Fig 1, which shows their relative sizes and position with respect to c-myc. Clones E2 and G4 were not used for FISH on BL cell lines or fresh tumor samples, because E2 showed cross-hybridization with the centromere of D group chromosomes and G4 was shown to be chimeric. I2 contains an insert of approximately 200 kb that includes the c-myc gene and the pPA1.3SB marker. The 8q267 marker, located approximately 60 kb downstream of c-myc, is not present in the insert that we were then able to position with respect to c-myc as shown in Fig 1. P72 is approximately 500 kb in size and contains the p380-8A1.8, p380j9 0.8ss, pEW36-7D, and H4.1 markers.

To determine the effectiveness of our YAC clones in detecting different breakpoints on chromosome 8 both in metaphase chromosomes and interphase nuclei, we performed two-color FISH analysis on six BL cell lines, five of them carrying the t(8;14) translocation and one the t(8;22) translocation (Table 1). We also examined tumor samples obtained from five patients whose karyotype had shown a t(8;14)

Table 1. Location of Chromosomal Breakpoint in BL Cell Lines and FISH Results With YAC Clones P72 and I2

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Translocation</th>
<th>Breakpoint on Chromosome 8*</th>
<th>FISH Signals With I2 and P72</th>
</tr>
</thead>
<tbody>
<tr>
<td>BL2</td>
<td>t(8;22)</td>
<td>20 kb 3'</td>
<td>N8, der (8), der (22)</td>
</tr>
<tr>
<td>Daudi</td>
<td>t(8;14)</td>
<td>170-190 kb 5'</td>
<td>N8, der (8), der (14)</td>
</tr>
<tr>
<td>AG876</td>
<td>t(8;14)</td>
<td>Unknown</td>
<td>N8, der (8), der (14)</td>
</tr>
<tr>
<td>P3HR1</td>
<td>t(8;14)</td>
<td>170-190 kb 5'</td>
<td>N8, der (8), der (14)</td>
</tr>
<tr>
<td>EW36</td>
<td>t(8;14)</td>
<td>170-190 kb 5'</td>
<td>N8, der (8), der (14)</td>
</tr>
</tbody>
</table>

* Breakpoint positions are given with respect to c-myc.

and H4.1 were used by Southern blot analysis to map their location within the 8q24 breakpoint region.

FISH. Metaphase chromosomes from cell lines were prepared according to standard techniques. Patient samples were cultured in situ Medium (Irvine Scientific, Santa Ana, CA) at 37°C with 5% CO₂ for 24 hours and metaphase spreads were then prepared in the same manner as cell lines. Slides were pretreated with RNase A (100 µg/mL; Boehringer Mannheim, Mannheim, Germany) in 2× SSC (pH 7.0) for 60 minutes at 37°C, followed by two washes in 2× SSC and sequential dehydration in 70%, 85% and 100% ethanol. Slides were then denatured in 70% formamide, 2× SSC at 70°C for 2 minutes and dehydrated. YAC clone DNA was labeled by nick translation with biotin-14dATP (BRL, Gaithersburg, MD), coprecipitated with 10 to 15 µg of unlabeled human Cot-1 DNA (BRL), and resuspended in 30 µL of hybridization solution (50% formamide, 10% dextran sulphate). The probe was denaturated, allowed to preanneal at 37°C for 1 hour, and combined with the denaturated digoxigenin-labeled α satellite probe D14Z1/D22Z1 (Oncor, Gaithersburg, MD) or with the Tel14q(14q32.3-pter) probe (Oncor) immediately before adding the mixture to the slides. After overnight hybridization and washes in 50% formamide 2× SSC at 45°C and 2× SSC at 37°C, the digoxigenin- and biotin-labeled probes were detected immunologically using fluorescein isothiocyanate conjugated to avidin (FITC-avidin DCS; Vector Laboratories, Burlingame, CA) and rhodamine-conjugated antidigoxigenin antibodies (Oncor). The chromosomes were counterstained with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) and visualized with a triple band pass filter from Chromatech (Brattleboro, VT).
translocation (Table 2). Biotin-(green) labeled clones P72 and I2 were combined with digoxigenin-(red) labeled centromeric probe D14Z1/D22Z1 or Tel114q probe. The D14Z1/D22Z1 probe hybridizes with the centromeres of chromosomes 14 and 22, whereas the Tel114q probe detects only the telomere of chromosome 14. Using this approach, we were able to identify unequivocally the partner chromosome in metaphase spreads, whereas, in interphase nuclei, the presence of the translocation was demonstrated by the presence of three green signals (the biotin-labeled YAC), one of which colocalizes with one red signal (the digoxigenin-labeled D14Z1/D22Z1 or Tel114q probes; Fig 2). Figure 3 shows representative results of FISH analysis on three cell lines: BL2, Daudi, and AG876. The breakpoints on chromosome 8 of the BL2 and Daudi cell lines have been well characterized. The BL2 breakpoint is located 10 kb downstream of c-myc and the Daudi breakpoint is 170 to 190 kb upstream of c-myc. The exact location of the breakpoint in the AG876 cell line is not yet defined. When applied on the BL2 cell line (Fig 3A and B) carrying the t(8;22) translocation, I2 and P72 hybridized to normal chromosome 8 (green dots) and split signals on both the der(8) and der(22). On the Daudi and AG876 cell lines (Fig 3C and D) carrying the t(8;14) translocation, both clones hybridized to the normal chromosome 8 and to both the der(8) and der(14) chromosomes. The breakpoint in the AG876 cell line is shown here to be located within the region covered by our YACs. I2 and P72 also detect the t(8;14) translocation in the P3HRI and EW36 cell lines with a breakpoint at 170 to 190 kb upstream of c-myc. Analysis of the Manca cell line, in which the breakpoint occurs within the c-myc gene, showed that both YAC clones effectively detect the t(8;14) translocation.

FISH analysis on patients samples was performed using the two YAC clones together with the Tel114q probe. Breakpoints in tumor samples have not been characterized. Figure 4A, B, and D shows representative metaphases, whereas an interphase is depicted in Fig 4C. Fifty cells, including both metaphases and interphases, were examined for each patient. In four patients (no. 1 through 4), the majority of cells, from 30 to 35 (60% to 70%), showed three signals and colocalization of the YAC clones with the Tel114q probe, indicating that the YAC clones effectively detect the breakpoint on chromosome 8. In 5 to 10 cells (10% to 20%), only two green signals were visible, one of which colocalized with the Tel114q probe, indicating the presence of the der(8) chromosome. The number of normal cells, with only two green and two red signals without colocalization, ranged from 5 to 15 cells (10% to 30%). In patient no. 5, only interphases were analyzed and two green signals were visible, one of which colocalized with the Tel114q probe in 75% of cells, again demonstrating the presence of the der(8) chromosome.

DISCUSSION

Rearrangements involving the c-myc oncogene are the hallmark of BL. Moreover BL, with the characteristic translocations and c-myc activation, represents the most frequent lymphoma developing in AIDS patients. In all translocations, overexpression of c-myc results from its juxtaposition to Ig or T-cell receptor enhancers. c-myc is overexpressed even when the breakpoints occur more than 150 kb from the gene, indicating that the enhancers are active over large
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Fig 3. Representative results of FISH analysis with YACs 12 and P72 on BL cell lines. (A) BL2 metaphase. (B) DAPI image of same metaphase as in (A). (C) Daudi metaphase. (D) AG876 metaphase. Long arrows mark the position of normal chromosome 8, arrowheads indicate the der(8), and short open arrows indicate the der(22) (A and B) or the der(14) (C and D). The red signal marks the centromeres of chromosomes 22 and 14 (A and C) or the telomere of chromosome 14 (D). In (D), the der(8) is labeled by both the YAC clones (green) and the Tel14q probe (red).

distances and allowing great flexibility in the location of breakpoints. Indeed, this phenomenon is well documented\textsuperscript{9,12} and has so far generally limited the use of molecular diagnostic methods in BL.

Using nonchimeric YAC clones containing large DNA fragments surrounding the c-myc locus, we examined the efficacy of FISH analysis on metaphase spreads and interphase nuclei in the detection of the t(8;14) translocation characteristic of BL. Six BL cell lines with known breakpoints at 8q24 and five patient samples with unknown breakpoint were used as a model in this study.

Cytogenetic analysis, Southern blotting, and PCR have been widely used for the detection of rearrangements and translocations. However, the need of good quality metaphases and a good mitotic index constitute major limitations in cytogenetic analysis. Southern blot analysis can detect a clone of cells representing as little as 1% of the total cell population and it is suitable for observing patients serially. However, only one probe can be used at a time and only some rearrangements can be detected with one probe, making it cumbersome for routine diagnosis and follow-up. Because of its great sensitivity, PCR could provide a valuable means of detecting chromosome translocations and localizing breakpoints to specific genetic regions. PCR has been used in the detection of the ber-abl transcripts in chronic myeloid leukemia and adult acute lymphoblastic leukemia carrying the t(9;22) translocation.\textsuperscript{16} It has also been successfully used in the detection of bcl-2 rearrangements in follicular lymphomas with the t(14;18) translocation.\textsuperscript{21} However, it is limited to rearrangements of which the sequence is known and that are clustered in a well-defined region, again making this method impractical in the case of BL.
Since its development, FISH has had great impact on diagnosis and basic research. FISH is a quick and simple method that is highly specific and independent on the cycling status of the cells. In addition, the feasibility of direct correlation between cytogenetic and cytologic/morphologic features allow accurate diagnosis even in equivocal cases.\(^{32}\) The possibility of unequivocally identifying chromosomes with chromosome-specific probes simplifies the recognition of numerical and other cytogenetic abnormalities. In addition, the use of combinations of differently labeled probes allows high resolution ordering and mapping of DNA sequences. Because FISH is not limited by probe size or by the presence of repetitive sequences, large genomic fragments such as YACs can be used in the analysis of chromosomal abnormalities. The advantage of YAC clones, covering several hundred kilobases within a chromosome region of interest, has been shown in the case of acute myelogenous leukemia for the detection of the t(8;21) translocation, \(bcr-abl\) fusion in chronic myeloid leukemia,\(^{33}\) and translocations involving chromosome 11 at q23 band.\(^{34}\) YAC clones were also used to identify the partner sites of 14q32 translocations in B-cell malignancies, even in cases not identified by cytogenetic analysis.\(^{35}\)

These characteristics make FISH ideally suited for the analysis of the translocations in BL. Indeed, a pool of bacteriophage clones has been used to detect the breakpoint of Burkitt’s cell lines.\(^{36}\) However, more than 30 phage clones measuring 20 kb each would be necessary to cover the approximately 600-kb region involved in the rearrangements and their combined use is too time consuming to be performed routinely. For this reason, larger probes such as YACs covering a large region surrounding the breakpoints represent a great advantage.

In this study, we have used two YAC clones spanning approximately 700 kb surrounding the \(c-myc\) locus to detect the different breakpoints of six Burkitt’s cell lines. Two-color FISH analysis shows that the clones effectively detect the breakpoints. We also presented data from tumor samples.

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**Fig 4.** Representative results of FISH analysis with YACs 12 and 72 on patient samples. (A) Metaphase from patient no. 1. (B) DAPI image of same metaphase as in (A). (C) Interphase from patient no. 1. (D) Metaphase from patient no. 4. Long arrows mark the position of normal chromosome 8, arrowheads indicate the der(8), and short open arrows indicate the der(14). The red signal marks the telomere of chromosome 14.
In four of them, the hybridization experiments showed both the det(8) and det(14) of the t(8;14) translocation. In patient no. 5, the presence of only two green signals suggests that the breakpoint is in the 3' region of c-myc, outside the region covered by our clones. This problem can be easily overcome by the isolation of a YAC clone extending further 3' than the contig presented in this work. Although studies including a larger number of patients are necessary, our ability to detect the presence of the translocations using the approach described here indicated that it represents a sensitive and specific tool in confirming a histopathologic diagnosis of BL in cases in which classical cytogenetic analysis is not possible. In addition, three-color FISH could be used in the same manner described in this study to differentiate between the t(8;14) and variant t(8;22) and t(2;8) translocations. The ability to detect a tumor-specific genetic marker may have significant impact not only in diagnosis but also in the therapy and in follow-up of disease. The same approach could also prove particularly relevant in other types of non-Hodgkin's lymphoma that show the same variability in breakpoint locations. 3

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