Isolation of a yeast artificial chromosome spanning the 8;21 translocation breakpoint t(8;21)(q22;q22.3) in acute myelogenous leukemia
(chromosome 21 Not I library/D21S65/Down syndrome/genome mapping/somatic cell hybrids)

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ABSTRACT The 8;21 translocation is one of the most common specific rearrangements in acute myelogenous leukemia. We have identified markers (D21S65 and a Not I boundary clone, Not-42, referred to as probe B) flanking the chromosome 21 translocation breakpoint (21q22.3) that demonstrate physical linkage in normal genomic DNA, by using at least three restriction endonucleases (Not I, Sac II, and BstHI), and that are located not more than 250–280 kilobases apart. Pulse-field gel analysis of DNA from somatic cell hybrids containing the 8;21 translocation chromosomal demonstrates rearrangement of these markers. A 470-kilobase yeast artificial chromosome, YAC-Not-42, has been isolated that contains both probes. Mapping of λ subclones constructed from YAC-Not-42 suggests that >95% (25/26 probes tested) of the yeast artificial chromosome DNA is located on the proximal (D21S65) side of the breakpoint. In situ hybridization studies using metaphase chromosomes from five acute myelogenous leukemia patients with the 8;21 translocation confirmed these results and demonstrated the translocation of probe B to the derivative chromosome 8. A chromosome walk of ~39 kilobases from probe B has allowed identification of the breakpoint in DNA from a somatic cell hybrid containing the derivative chromosome 8. Since probe B contains conserved DNA sequences and is in close proximity to the translocation breakpoint, it may represent a portion of the involved gene on chromosome 21.

The identification of specific chromosome rearrangements in human malignant diseases has led to a directed search for gene sequences at or adjacent to the breakpoints of these structural alterations. The 8;21 translocation t(8;21)(q22; q22.3) occurs predominantly in the FAB (French-American-British clinical cytological criteria)-M2 subtype of acute myelogenous leukemia (AML) and has been reported to occur in ~18% of patients with AML-M2 and demonstrable karyotypic abnormalities (1). First characterized using banding techniques by Rowley (2), t(8;21) AML is associated with granulocytic maturation (Auer rods and peroxidase-positive granules), a relatively good response to chemotherapy, and an increased incidence of extramedullary tumor masses (3).

Somatic cell hybrids containing the derivative chromosomes 8 and 21, isolated from two patients, have been described (4, 5). Southern blot analyses and in situ hybridization studies on these cell lines have been used to demonstrate the lack of direct involvement of MOS, ETS2, and EGR in the 8;21 translocation (6–8). These and other somatic cell hybrids have facilitated the construction of a detailed pulsed-field gel (PFG) map of human chromosome 21 that confirmed that ETS2 and EGR are physically positioned in segments not immediately adjacent to the chromosome 21 breakpoint (9, 10). Gardiner et al. (10) suggested that the anonymous probe D21S65 might detect the 8;21 rearrangement on PFGs, although confirmatory evidence in the form of physical linkage to a second marker across the breakpoint was lacking.

Our approach to isolate the 8;21 translocation breakpoint was based on earlier work to construct and localize Not I and Eag I clones from chromosome 21 (11). Such probes offer the advantage of identifying CpG islands containing clusters of rare restriction sites. These islands are usually located near the 5’ region of genes (12) and appear to be responsible for many of the gaps in current PFG maps. Of note, regions of chromosome 21 involving translocations often occur near such gaps (10). For the 8;21 translocation, this approach has allowed us to isolate a distal CpG island (probe B) that could be linked by PFG Southern blots to the anonymous probe D21S65 located on the proximal side of the translocation breakpoint. Subsequently, these probes were used to isolate a yeast artificial chromosome (YAC) containing chromosome 21 DNA spanning the translocation site and, by chromosome walking, the breakpoint in one patient has been identified.

MATERIALS AND METHODS

Molecular Probes and Hybridizations. The construction and mapping of 60 individual chromosome 21 Not I and Eag I boundary clones have been described (11). Briefly, these consist of complete Not I–partial Mbo I 15- to 20-kilobase (kb) inserts cloned into Not I/BamHI-digested EMBL6 phage arms. Probe B refers to the boundary clone Not-42 to distinguish it from the corresponding YAC-Not-42-containing this marker. For Southern blot hybridizations, either the entire phage was 32P labeled by random priming and repetitive sequences were blocked using the method of Sealy et al. (13) or a unique sequence subfragment was identified. Phage DNA was prepared using the method of Grossberger (14). The probe D21S65 was kindly provided by Paul Watkins (Life Technologies, Gaithersburg, MD). Hybridizations were carried out at 65°C in 5× standard saline citrate (SSC)/2× Denhardt’s solution/5–10% (wt/vol) dextran sulfate/0.5% SDS/100 μg of salmon sperm DNA as outlined (15). Filters were washed in a final solution of 0.1× SSC/0.1% SDS at 55–60°C and used to expose Kodak XAR film with an intensifying screen for various times.

YAC Library and Screening. We utilized the St. Louis YAC library (16). The screening method was that developed by Gemmill et al. (17). Briefly, microtiter trays were pooled (384 clones per pool) and the DNAs were separated on PFGs.

Abbreviations: AML, acute myelogenous leukemia; PFG, pulsed-field gel; YAC, yeast artificial chromosome.

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After Southern blot hybridization with an individual probe, the specific YAC was identified using colony hybridizations as described (18). We have incorporated the following modifications for colony hybridizations. Autoclaved Whatman 540 paper was used instead of nylon membranes. After the growth of colonies, the filters were sequentially placed on Whatman 3MM paper saturated with the following solutions for the indicated times: (i) 1.0 M sorbitol/0.1 M EDTA/1% 2-mercaptoethanol/zymolyase 100T (30 µg/ml) for 2 hr to overnight at 37°C; (ii) 10% (wt/vol) SDS for 10 min; (iii) 0.5 M NaOH/0.5 M NaCl for 30 min; (iv) 0.5 M Tris-HCl, pH 7.0/0.5 M NaCl for 30 min. Steps ii-iv were performed at room temperature. The filters were baked at 80°C for 2 hr and then hybridized in a solution containing 50% (vol/vol) formamide at 39°C and washed at room temperature.

**PFGE Electrophoresis.** The gel system used was the ED box (19). Restriction endonuclease digests and transfers were carried out as described by Gemmill et al. (20).

**Somatic Cell Hybrids.** Hybrids containing the der(21) chromosome (21pter → 21q22.3::8q22 → 8qter) and the der(8) chromosome (8pter → 8q22::21q22.3 → 21qter) have been described (4, 5). The following hybrids and their chromosome 21 content, indicated in parentheses, have been described: 72532X-6 (q11 → qter), 2FU'1 (q11 → qter), and ACEM (pter → cen::q22 → qter) (refs. 9-10, 21). Hybrid R2-10 contains a complex ring chromosome 21 (22). For the purpose of regional mapping, the 21q22.3 → qter segment is absent in R2-10, and the 21q22.3 breakpoint is distal to that which occurs in the 8;21 translocation. The der(8) and der(21) chromosomes were derived from different patients. In our laboratories, the der(8) and der(21) hybrids have been included among a much larger set of chromosome 21 hybrids for regional mapping studies and tested with >120 chromosome 21 probes. The results obtained with anonymous probes and known genes have been internally consistent and agree with independent data obtained by genetic linkage studies (10). Therefore, there is no evidence that the der(8) and der(21) chromosomes contain additional chromosome 21 material or have deleted chromosome 21 material, other than that expected from the translocations.

**Construction of a λ Library from the Not-42-containing YAC.** DNA was prepared from the Not-42-containing YAC by using a miniprep method (23). Partial MboI digestion and cloning into the BamHI site of EMBL3 were accomplished using standard methods (15). Human recombinants were identified by hybridization to 32P-labeled total human DNA as well as by hybridization to a 3' Kpn-repeat-containing probe (p4A10) kindly provided by Maxine Singer (National Institutes of Health).

**Fluorescence in Situ Chromosomal Hybridization.** Human metaphase cells were prepared from bone marrow samples. The procedure used is a modification of the method described by Lichter et al. (24) and has been described (25).

**RESULTS**

**Identification of Probes That Flank the (8;21) Breakpoint and a YAC Containing Both Probes.** The chromosome 21 probe Not-42, referred to as probe B, is located in the region just distal (telomeric) to the 8;21 translocation (11). This interval is defined by the 8;21 translocation breakpoint (21q22.3) and by the more distal 21q22.3 break in the ring chromosome 21 contained in hybrid R2-10. The probe D21S65 has been mapped to the region just proximal of the 8;21 translocation (10). By analysis of PFGE Southern blots, we were able unequivocally to link these two probes across the 8;21 translocation breakpoint (Fig. 1). By using two cell lines, the hybrid 2FU'1 containing the long arm of chromosome 21 and the human fibrosarcoma line HT1080 (containing chromosome 21 without apparent rearrangement), comigrating fragments were detected with Not I, BssHII, and Sac II. Either partial digestion or methylation differences resulted in additional comigrating bands detected in the HT1080 line, further supporting this linkage. Similar bands were detected with Mlu I and Nru I but these migrated in the compression zone. No common bands were seen with Sal I and Sfi I. These results indicate that probes B and D21S65 are at most 280 kb apart.

The YAC library was screened for a clone that contained both D21S65 and probe B. One clone (YAC-Not-42) containing a single YAC of ~470 kb was identified. Southern blots of the YAC DNA and appropriate genomic DNA controls digested with EcoRI, HindIII, and BamHI confirmed the presence of both D21S65 and probe B in YAC-Not-42 (data not shown).

**Pulsed-Field Analysis of the 8;21 Translocation Somatic Cell Hybrids.** In our initial experiments, we invariably detected an apparently rearranged D21S65 Nru I fragment of ~800 kb in the der(21) hybrid DNA in contrast to control bands migrating in the compression zone when the human kidney cell line

![Fig. 1. (A and B) PFGE Southern blot hybridized sequentially with probes B and D21S65, respectively. Lanes 1 and 2 contain DNA from hybrid 2FU'1 and HT1080, respectively. Molecular size markers correspond to chromosomes from the yeast strain YPH149 (kindly provided by Phil Heiter, Johns Hopkins University, Baltimore). Comigrating bands (with their approximate sizes given in parentheses) were detected with Not I (840 kb), Sac II (280 kb), and BssHII (280 kb). The Not I fragments are weak and have not reproduced well in this photograph. No common bands were detected in the Sal I and Sfi I digests. Between hybridizations, the filter was stripped and no residual bands were detected after autoradiography for 2 days.](image-url)
293 (obtained from the ATCC) and the hybrid 2FU1 (data not shown) were used. However, with Sac II or BssHII and a 60-sec pulse time, only slight and unconvincing differences were noted. To optimize resolution in the 250- to 280-kb range, we carried out a PFG study using a 25-sec pulse time for 40 hr at 6 V/cm. In the experiment, shown in Fig. 2A, the BssHII and Sac II digests indicate a 20- to 30-kb difference with probe D21S65 in the DNA from the der(21) hybrid. With probe B (located distal to the translocation breakpoint), BssHII and Sac II digestions of the der(8) hybrid DNA resulted in bands that migrated in the compression zone, whereas the normal fragment migrated at ~280 kb (Fig. 2B). The similarity of the BssHII and Sac II fragment sizes with D21S65 and the small differences noted in the der(21) DNA suggest that the chromosome 8 material, which is translocated to chromosome 21, contains a CpG island. Probe B contains Sac II and BssHII sites in close proximity to the Not I site (data not shown) and can therefore be considered to contain a CpG island. Thus, the translocation breakpoints on both chromosomes 8 and 21 occur near CpG islands.

Construction of a λ Library from YAC-Not42 and Evidence That the Breakpoint Is in Close Proximity to Probe B. A λ library of several hundred thousand recombinants from YAC-Not42 was constructed using the mcrA-B-, P2 lysogen NM646 (26). Of 26 random human recombinants tested, 24 identified by hybridization to total human DNA (predominantly Alu sequences) mapped to the der(21) chromosome, i.e., proximal to the 8;21 breakpoint. One of two probes identified by hybridization to the Kpn repeat mapped to the distal side of the break and overlapped with a walking clone obtained by screening the λ library with a unique sequence fragment from probe B. This suggests that the breakpoint is quite close to probe B and may be in or at the boundary of a Kpn-rich region.

No evidence for a deletion has been observed with the large number of unique chromosome 21 probes (>120 probes) that have been mapped using these hybrids (10, 11) or with the 26 random probes mapped so far from YAC-Not42. Moreover, no probe has mapped to both hybrids that would likely have been detected if a significant overlap between the two independent translocation breakpoints occurred. These results suggest that the chromosome 21 breakpoints in the two patients [represented by the der(8) and der(21)] hybrids are similar. However, they are not identical. A chromosome walk of ~39 kb from the Not I site in probe B was carried out. This allowed identification by conventional Southern blot analysis of the (t(8;21)) breakpoint in DNA from the der(8) hybrid. These results, shown in Fig. 3, demonstrate that the breakpoint in the der(8) hybrid DNA occurs within the walking clone WC2-1. No hybridization was detected in the der(21) DNA indicating that the breakpoint in this patient is either more centromeric or involves a deletion.

In Situ Hybridization. As an independent verification of these results, fluorescent in situ studies were carried out on metaphase cells from five leukemia patients with an 8;21 translocation (clinical characteristics available upon request). Analysis of four patients using total yeast DNA containing YAC-Not42 and of all five patients using probe B was done. With total yeast DNA, the observed hybridization signal was present on the der(21) chromosome (and the normal chromosome 21) in each of the four patients examined. This was not unexpected since perhaps 96% of the YAC DNA (25/26 random probes tested) was derived from the proximal side of the translocation. In contrast, we found that, by in situ hybridization, probe B DNA sequences were translocated to the reciprocal der(8) chromosome in all of the five patients. To enhance the signal to background ratio, we used a combination of probe B and probe B walking clone, 13', which also maps distal to the translocation breakpoint. An example of these results, shown in Fig. 4, demonstrates translocation of this chromosome 21 DNA segment to the der(8) chromosome. Also shown are the results using the λ probes 2 and 6, which map to the proximal side of the 21q22.3 breakpoint. Thus, the chromosome 21 breakpoint appears to be similar among different patients with the t(8;21).

**DISCUSSION**

Our studies indicate that we have isolated a YAC clone that spans the 8;21 translocation breakpoint. These conclusions are based on the following evidence. (i) Linkage in normal genomic DNA was established by PFG electrophoresis be-
Chromosome 21 may contain a gene that promotes the proliferation of hematopoietic cells. This hypothesis is based on the increased incidence of leukemia in persons with Down syndrome (27) and importantly on the observations that Down syndrome neonates may develop a transient disorder with some features resembling acute leukemia (28–30). Where there has been mosaicism for trisomy 21 detected in bone marrow cells of patients with this "leukemoid reaction," resolution of the "blasts" occurred in parallel with disappearance of the +21 clone (31, 32). It is of interest that the 8;21 translocation breakpoint is located in the segment of chromosome 21 that appears to be responsible for many of the phenotypic features of Down syndrome (10, 33, 34). In addition to Down syndrome, an extra copy of chromosome 21 is one of the most common abnormalities in some forms of lymphoblastic leukemia (31).

Earlier evidence from the cytogenetic analysis of 8;21 variant three-way translocations bears upon the probable position of the involved chromosome 21 gene relative to the breakpoint. In cells from these patients, the distal long arm of chromosome 21 translocates to chromosome 8, whereas the end of chromosome 8 translocates to the third chromosome involved, and the remainder of the third chromosome in turn translocates to chromosome 21 (35, 36). Therefore, the conserved genetic rearrangement in the common two-way and variant three-way translocations is the juxtaposition of sequences from chromosomes 8 and 21 on the der(8) chromosome (36). Thus, if the cause of AML with t(8;21) is...
due to dysregulation of a chromosome 21 gene product by sequences present on chromosome 8, then the gene most likely is telomeric to the breakpoint. The conserved sequences identified in probe B and adjacent clones represent prime candidates for portions of this gene.

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