Identification of Breakpoints in t(8;21) Acute Myelogenous Leukemia and Isolation of a Fusion Transcript, AML1/ETO, With Similarity to Drosophila Segmentation Gene, runt

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We have developed a restriction map of the chromosome 21 breakpoint region involved in t(8;21)(q22;q22.3) acute myelogenous leukemia (AML) and have isolated a genomic junction clone containing chromosome 8 and 21 material. Using probes from these regions, rearrangements have been identified in each of nine cases of t(8;21) AML examined. In addition, we have isolated cDNA clones from a t(8;21) AML cell library that contain fused sequences from chromosome 8 and 21. The chromosome 8 component, referred to as ETO (for eight twenty-one), is encoded over a large genomic region, as suggested by the analysis of corresponding yeast artificial chromosomes (YACs). The DNA sequence of the chromosome 21 portion of the fusion transcript is derived from the normal AML1 gene. A striking similarity (67% identity over 387 bp, with a corresponding 69% amino acid identity) was detected between AML1 and the Drosophila segmentation gene, runt. The critical consequence of the translocation is the juxtaposition of 3' sequences of AML1 to 3' sequences of ETO, oriented telomere to centromere on the der(8) chromosome.

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MATERIALS AND METHODS

Somatic cell hybrids. The somatic cell hybrids 2Fu1 and 706B6CL17 contain, respectively, the long arm of human chromosome 8 and an intact chromosome 8 as their only identifiable human material.9,10 Somatic cell hybrids containing the der(8) and 550 kb, isolated using the genomic chromosome 8 junction fragment probe, contain a portion of these same fragments, confirming that the chromosome 8 gene is indeed derived from the region involved in the 8;21 translocation and suggesting that the ETO gene may be encoded over a large stretch of DNA. Partial DNA sequence analysis of the fusion transcript demonstrates that the chromosome 21 gene corresponds to its normal counterpart, AML1, recently isolated by Miyoshi et al.9 Our analysis of AML1 demonstrates a striking, and previously unrecognized, similarity to the Drosophila segmentation gene runt. Of interest, the sequence of the fusion transcript diverges from AML1 at the end of the runt homology, thereby interrupting a possible DNA binding basic region and removing a potential transactivating domain.

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der(21) chromosomes, isolated from separate patients, have been previously described. Before DNA preparation, cytogenetic analysis was used to verify the chromosome content of each hybrid.

Restriction mapping. The inserts of the lambda clones represent partial MboI-digested DNAs cloned into the BamHI sites of EMBL3, which are flanked by SalI sites. End-fragments were identified by single and double digests using EcoRI and SalI. Complete SalI/partial EcoRI digest products from each clone were hybridized with end-probes that had been gel isolated and 32P-labeled. The localization of BamHI sites within the cloned DNA was determined by double and triple digests with EcoRI-SalI and EcoRI-SalI-BamHI, respectively. The positioning of these BamHI sites is in agreement with the results observed with BamHI-digested genomic DNA hybridized with corresponding probes.

YAC isolation. Screening of the CEPH YAC library was performed using a variation of the hybridization-based approach described by Mendez et al. Briefly, DNA was prepared from each of 500 96-well microtiter plates containing individual YACs using a miniprep method. Approximately 0.5 μg of EcoRI-digested DNA from each plate was separated by standard agarose electrophoresis (100 wells per 15 × 25-cm gel), transferred to charged nylon membranes (Oncor, Gaithersburg, MD), and hybridized to a 32P-labeled probe in a 7% sodium dodecyl sulfate (SDS)/10% polyethylene glycol (PEG) containing buffer as described by Amasino. Individual wells were identified using DNA prepared from rows and columns. The YACs from several individual colonies from each positive well were examined using pulsed-field gel electrophoresis to determine the size of the insert and to look for any instability arising in culture.

cDNA library construction. Total RNA was isolated using the method of Chirgwin et al from leukemic blasts of AML-M2 morphology containing the 8:21 translocation. PolyA-RNA was selected as described by Aviv and Leder and double-stranded cDNA was prepared as described by Gubler and Hoffman. cDNA libraries were prepared in lambda gt11 and lambda ZAP II (Stratagene, La Jolla, CA) according to the procedure of Huynh et al.

Genomic library construction. A genomic library was constructed from the der(8) chromosome containing somatic cell hybrid 8:21 using standard methods. Approximately 1 million plaques were screened using probes derived from walking clone 2 (WC2-1) previously used to detect a rearrangement in this cell line. Positive clones were plaque-purified and SalI-EcoRI end-probes developed as described above. These probes were mapped to either chromosome 8 or 21 using DNA from the somatic cell hybrids 706B6C17 or 2FUr1, respectively.

DNA sequencing. Double-stranded templates were obtained by either directly rescuing plasmids from lambda ZAP II using helper virus and protocols provided by the manufacturer (Stratagene) or by subcloning cDNA inserts from lambda gt11 clones into pBlue-Script (Stratagene). Plasmid DNA was prepared by standard methods24 and the DNA was further purified on QIAGEN columns (Studio City, CA). Dideoxy chain-termination sequencing24 was performed using standard protocols.

RESULTS

Analysis of t(8;21) AML breakpoints. An EcoRI and BamHI restriction map from 60 kb of overlapping chromosome 21 lambda clones is shown in Fig 1. The position of the two translocation breakpoints contained in somatic cell hybrids is indicated by arrows, and probes used in the analysis of t(8;21) AML DNAs are indicated by darkened boxes. A genomic library was constructed from the der(8) hybrid DNA. From approximately 1 million plaques screened with the 1.1- and 2.5-kb chromosome 21 probes contained in WC2-1, one recombinant containing both chromosome 8 and 21 material was found (indicated as junction clone in Fig 1).

Using the restriction map for appropriate choices of probe and restriction enzyme combinations, we have identified rearrangements in each of the nine cases of t(8;21) AML examined. Five of the nine chromosome 21 breakpoints occur within a 20-kb BamHI fragment recognized by the 2.6-kb probe from WC3-1 (as indicated by a second hybridizing fragment in Fig 2A, lanes 6, 7, 8, 10, and 11-12). The breakpoint in the der(21) hybrid also occurs within this fragment (data not shown), although we do not have the corresponding der(8) chromosome from the same patient to allow detection with the 2.6-kb probe. Thus, six of the nine chromosome 21 breakpoints occur in the 20-kb BamHI fragment. We have found no BamHI polymorphisms in 10 normal DNA samples or cell lines containing two copies of apparently normal chromosome 21s with this probe (data not shown). One additional rearrangement was detected in the adjacent telomeric 11-kb BamHI fragment using the 1.6-kb probe (Fig 2B, lane 5). A chromosome 8 genomic probe from the junction clone identified germine BamHI fragments of 3 and 15 kb (Fig 2C). Rearrangements were detected in two samples with this probe (Fig 2C, lanes 1 and 2). Both rearrangements occur within the 3-kb fragment, which is absent in the somatic cell hybrid containing only the der(8) chromosome (lane 1) and is reduced in intensity in DNA from patient 3 (lane 2). Also shown in Fig 1 are additional chromosome 8 lambda clones that extend in both directions and the restriction map of one of these, JG3.

Isolation of a fusion transcript. cDNA libraries constructed from t(8;21) AML cells were screened with the 0.8-kb EcoRI fragment from Not42 (Fig 1). We identified 12 clones from approximately 100,000 plaques, suggesting the transcript was moderately abundant in these cells. Two of these clones, PS400-1 and 9b, are shown schematically in Fig 3A. Clone PS400-1, containing a 1.23-kb insert with internal EcoRI sites in the transcript was moderately abundant in these cells. Two of these clones, PS400-1 and 9b, are shown schematically in Fig 3A. Clone PS400-1, containing a 1.23-kb insert with internal EcoRI fragments of 0.6 and 0.63 kb, mapped to the 21q22.3-qter segment that is translocated to chromosome 8 in t(8;21) AML (data not shown). No hybridization could be detected to DNA from the normal chromosome 8 contained in 706B6C17. Using this probe and DNA from the overlapping lambda clones indicated in Fig 1, we detected hybridization to the 0.8- and 2.8-kb EcoRI fragments in clone Not42 (data not shown). Clone 9b contains an insert of approximately 3 kb with an internal EcoRI site that results in subfragments of 0.6 and 2.4 kb. Partial DNA sequence analysis of the 0.6-kb fragment and of one end of the 2.4-kb fragment shows complete identity to the 0.6- and 0.63-kb fragments from PS400-1, respectively. Therefore, this portion of the cDNA clone is derived from chromosome 21. Importantly, the 2.4-kb fragment also contains DNA sequences derived from chromosome band 8q22. This is shown in Fig 3B with the 2.4-kb cDNA probe detecting chromosome 8-specific genomic bands of 6.0, 4.0, 3.7, 2.2, 0.87, and 0.63 kb. DNA from three chromosome 8 YACs, isolated using an 8q22 probe from the junction clone, are

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Fig 1. Restriction map of the chromosome 21 breakpoint region in t(8;21) AML. EcoRI (E), BamHI (B), and NotI (N) sites are indicated. The breakpoints identified in two somatic cell hybrids containing the der(8) and der(21) chromosomes from separate patients are indicated by arrows. A junction clone, isolated from the der(8) hybrid, is also indicated. Darkened boxes indicate probes used in the analysis of rearrangements. These are [1] a 2.6-kb EcoRI fragment from walking clone (WC) 3-1; [2] a 1.6-kb EcoRI-SalI fragment from walking clone 2-1 (the SalI site is vector-derived); and [3] a 5.9-kb FcoRI fragment from the junction clone consisting primarily of chromosome 8 material. The lambda clones represent partial Mbol digests of DNA cloned into the BamHI site of EMBL3. Each insert is therefore flanked by SalI sites. End-fragments were identified by single and double digests using EcoRI and SalI. Complete SalI partial EcoRI digest products from each clone were hybridized with 32P-labeled end-probes. The localization of BamHI sites within the cloned DNA was determined by double and triple digests with EcoRI-SalI and EcoRI-SalI-BamHI, respectively. The positioning of these BamHI sites is in agreement with the results observed with BamHI-digested genomic DNA hybridized with corresponding probes. To obtain the junction clone, a genomic library was constructed from the der(8) chromosome containing somatic cell hybrid using standard methods and screened using probes derived from walking clone 2 (WC2-1) previously used to detect a rearrangement in this cell line. End-probes were mapped to either chromosome 8 or 21 using DNA from the somatic cell hybrids 70686CL17 or 2FUrl, which contain, respectively, an intact chromosome 8 and the long arm of human chromosomes 21 (21q11-qter) as their only identifiable human material. Additional chromosome 8 clones (JG 1, JG 2, and JG 3), obtained by chromosome walking, are also indicated.

also included. The 360- and 550-kb YACs contain the 6.0-, 4.0-, 3.7-, and 2.2-kb bands, but do not contain those of 0.87 and 0.63 kb, while the 320-kb YAC contains only the 6.0- and 4.0-kb fragments. Therefore, the chromosome 8 gene, referred to as ETO (for eight twenty-one), maps to the region of the 8;21 translocation at 8q22 and may extend over a large distance, possibly greater than 0.5 Mb. The DNA sequence, from the opposite side of clone 9b, although not yet complete, shows no relationship to the AML1 gene, in agreement with its chromosome 8 origin. The DNA sequence across the breakpoint is given in Fig 4A and was found to be identical in two independent clones.

**DISCUSSION**

Two major goals of molecular and cytogenetic research of leukemia have been to develop reagents for the sensitive diagnosis and monitoring of patients, and to gain a better understanding of the disease pathogenesis. Combined, the chromosome 8 and 21 genomic clones should provide excellent diagnostic reagents for the detection of the 8;21 translocation by either Southern blot or fluorescence in situ approaches. Using the restriction map and appropriate choices of probe and enzyme combinations, we have been able to identify each of the nine cases of t(8;21) AML examined. The nucleic acid sequence provided from the chromosome 8 portion of our fusion transcript (Fig 4A), along with the AML1 sequence, allows for the development of polymerase chain reaction (PCR) diagnostic reagents. The results of our investigations by reverse transcription PCR of t(8;21) AML will be presented elsewhere.

The chromosome 8 genomic probe from the junction clone identified germline bands of 3 and 15 kb. Two
Fig 2. Southern blot analysis of breakpoints using coded patient samples obtained from four children and five adults with t(8;21) AML. (A) BamHI digest of DNA separated on 0.6% agarose gel hybridized with 2.6-kb probe from WC3-1. The germline fragment is 20 kb. Arrowheads indicate rearranged fragments. Lanes 1, 2FUrI (normal 21q hybrid); 2, der(21) containing hybrid (21pter-21q22.3::8q22-8qter) from patient 1. This breakpoint occurs within the 6.6-kb EcoRI fragment in clone WC3-1 (not shown) and DNA sequences corresponding to the 2.6-kb probe are not present in the hybrid. Lane 3, der(8) containing hybrid (8pter-8q22::21q22.3-21qter) from patient 2. In this case, the breakpoint occurs in the 7.2-kb EcoRI fragment (not shown) and only chromosome 21 sequences distal to the probe are contained in the hybrid (see lanes 11 and 12 for corresponding der(21) hybrid and leukemic DNA from this patient. Lane 4, patient 3 (leukemic DNA); 5, patient 4; 6, patient 5; 7, patient 6; 8, patient 7; 9, patient 8; 10, patient 9; 11, leukemic DNA from patient 2; 12, hybrid containing normal 21 and der(21) from patient 2. (B) BamHI-digested DNA hybridized with the 1.6-kb SalI-EcoRI fragment from WC2-1. The germline fragment is 11 kb. Arrow indicates rearrangement. Lane 1, 2FUrI; 2, der(21) hybrid from patient 1; 3, der(8) hybrid from patient 2; 4-8, patients 3-7, respectively. (C) BamHI-digested DNA hybridized with a 5.9-kb chromosome 8 probe from junction clone. Germline fragments are 15 and 3 kb. Lane 1, der(8) hybrid (patient 2); 2-8, patients 3-9, respectively; 9, total human DNA from patient 2. Note that the rearranged fragment is identical to that in the somatic cell hybrid containing the der(8) chromosome.

rearrangements were detected with this probe, both of which occurred within the 3-kb fragment. This suggests that if there is any clustering in the chromosome 8 breakpoints, it will not be in the direction of the 15-kb fragment. We have isolated additional chromosome 8 clones in both directions for future examination of this question.

Miyoshi et al. recently described the identification of a chromosome 21 gene, AML1, isolated from normal cDNA libraries, that was rearranged in t(8;21) AML. The 5' to 3' orientation of the AML1 gene is telomere to centromere. DNA sequence information from the chromosome 21 portion of our fusion transcript shows complete identity with the AML1 gene up to nucleotide 1300 (Fig 4A), but then diverges from that point onward. Identity to the AML1 sequence begins approximately 50 bp 3' to the internal EcoRI site in PS400-1, and continues throughout the remaining 580 bp. Therefore, the first 600 bp of PS400-1 likely represents additional 5' untranslated material. Since
the orientation of the \textit{AML1} gene is telomere to centromere, the orientation of the \textit{ETO} gene should also be telomere to centromere.

Other than a putative adenosine triphosphate (ATP)-binding site, no similarities to known genes were previously identified in \textit{AML1}.\textsuperscript{9} We performed a BLAST search\textsuperscript{25} of GenBank DNA sequences and detected a 67\% identity between \textit{AML1} (from nucleotides 915 to 1301) with nucleotides 564 to 950 from the \textit{Drosophila} segmentation gene, \textit{runt}.\textsuperscript{26} A comparison of the amino acid sequence homology, which extends over 118 residues, is given in Fig 4B. Sixty-nine percent of the amino acids are identical and most of the differences are conservative changes. After submission of this manuscript, we became aware that the homology between \textit{AML} and \textit{runt} has independently been reported by others.\textsuperscript{27}

There is evidence for regulatory interactions between \textit{runt} and the pair-rule genes \textit{hairy}, \textit{even-skipped}, and \textit{fushi tarazu} during the establishment of a segmentation pattern in \textit{Drosophila}.\textsuperscript{28} The \textit{runt} gene is also necessary for the uniform expression of the sex-determining gene, \textit{sex-lethal}.\textsuperscript{29} Although localized to the nucleus, \textit{runt} does not have similarity to any described transcription factors.\textsuperscript{26} We have identified aspects of the normal \textit{AML1} sequence, not present in \textit{runt}, that suggest it might be a transcription factor. First, it has a cluster of basic amino acids, commonly found in transcription factors,\textsuperscript{26,30} adjacent to the carboxy end of the homology region (Fig 4B). Second, in its carboxy terminal region, \textit{AML1} has a stretch containing five prolines and four glutamines.\textsuperscript{9} Such residues are present in the activating domains of many eukaryotic transcriptional factors.\textsuperscript{31} This glutamine/proline region in \textit{AML1} is part of a block of homology with both the rat transcription factor, \textit{C/EBP}, and the \textit{Drosophila} homeotic caudal protein (Fig 4C). The portion of the \textit{AML1/ETO} transcript represented by clone 9b contains the entire region of \textit{AML1} that is homologous to \textit{runt}. However, the DNA sequence diverges just at the end of the \textit{runt} homology and therefore the translocation disrupts not only the cluster of basic amino acids, but also eliminates the glutamine/proline-rich region.

Based on the analysis of complex three-way translocations, the junction on the der(8) chromosome, namely the 5’ portion of the \textit{AML1} gene and the 3’ portion of the \textit{ETO}...
gene, is the conserved and therefore critical genetic rear-

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