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t(8;21) breakpoints on chromosome 21 in acute myeloid leukemia are clustered within a limited region of a single gene, *AML1*

(chromosomal translocation/cancer genetics)

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ABSTRACT **The t(8;21)(q22;q22) translocation is a nonrandom chromosomal abnormality frequently found in patients with acute myeloid leukemia (AML) with maturation (M2 subtype). We report here the cloning of a gene, named *AML1*, on chromosome 21 that was found to be rearranged in the leukemic cell DNAs from t(8;21) AML patients. The breakpoints in 16 out of 21 patients were clustered within a limited region of *AML1*, and detailed analysis in 3 patients revealed that the breakpoints occurred in the same intron of the gene. Sequencing of cDNA clones identified a long open reading frame encoding a 250-amino acid protein. Northern blot analysis detected four constant mRNA species in t(8;21) leukemic and normal cells; the largest species was more abundant in the leukemic cells than in normal cells. In addition, two mRNA species limited to the leukemic cells were found. These findings indicate that the *AML1* gene may be involved in neoplastic transformation of AML with the t(8;21) translocation.**

Specific chromosomal translocations are closely associated with a variety of human hematopoietic malignancies. Molecular analyses of translocation breakpoints in lymphoid malignancies have resulted in the isolation of many genes that were located at the breakpoint regions and were found to play important roles in control of cellular growth and differentiation. Recently, genes involved in the t(15;17) acute promyelocytic leukemia and the t(6;9) acute myeloid leukemia (AML) have been isolated (1–4). However, for other translocations in AML, molecular analyses of breakpoint regions have been less successful.

The t(8;21)(q22;q22) translocation is one of the most frequent karyotypic abnormalities in AML, especially in the M2 subtype according to the French–American–British (FAB) classification (5, 6). The incidence of t(8;21) was 18% of the AML-M2 cases (5) and displayed a remarkable geographic variation (7). Leukemic cells with this translocation can differentiate into mature neutrophils and eosinophils (8) and occasionally form tumors called myeloblastomas (9). Several genes were located near the t(8;21) breakpoints, but none of these genes has been shown to be rearranged and involved in AML with the t(8;21) translocation (10–12).

We previously reported (13) that the rearrangement in leukemic cell DNAs from t(8;21) AML patients was detected with clone LL263 (one of the *Not* I linking clones specific for chromosome 21) on pulsed-field gel electrophoresis. With the use of clone LL263, we have now isolated and sequenced cDNA clones for a gene we have named *AML1*, on chromosome 21, that was rearranged by the t(8;21) translocation. The t(8;21) breakpoints are clustered within a limited region of this gene, probably within the same intron.¶

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MATERIALS AND METHODS

Genomic and cDNA Cloning. Genomic clones λE3 and λE4 were isolated from a human leukocyte genomic library (Clontech, EMBL-3) with LL263L as a probe, which is one of two fragments of the linking clone LL263 digested with *Not* I (13). λE12 was isolated from the same library with λE4 (Fig. 1). The cDNA clones C6 and B1-3 were isolated from a human bone marrow cDNA library (Clontech, λgt10) with λE4 and C6E6H2, a *Hin*dIII–*Eco*RI fragment of the C6 clone, as probes, respectively. Other cDNA clones shown in Fig. 1 were from a human peripheral blood leukocyte cDNA library (Clontech, λgt10). Genomic clones λD11 and λD13 were isolated from a human lymphocyte genomic library (Stratagene, λDASH) by using C6E6H2 as a probe. Library screening and plaque purification were performed by standard procedures (14).

DNA Sequencing. The cDNA inserts of phage clones were recloned into the *Eco*RI site of pBluescript II KS(+) (Stratagene). Both strands of the cDNA clones were sequenced by the dideoxy chain-termination method, using Sequenase version 2.0 (United States Biochemical).

Patient Samples and Somatic Cell Hybrids. Peripheral blood or bone marrow samples were obtained from t(8;21) AML patients at diagnosis and in remission. The diagnosis was established in each patient according to the FAB classification. The somatic cell hybrids established by fusion of leukemic cells from patient 2 with mouse myeloma X63 cells have been described (13).

Southern Blot Analysis. Genomic DNA (10 μg) was digested with appropriate restriction enzymes, separated by electrophoresis in agarose gels, and transferred to Hybond-N (Amersham). Hybridization with a random-primer-labeled probe was performed at 42°C in 6× standard saline citrate (SSC)/10% dextran sulfate/1% SDS/1× Denhardt's solution/50% formamide containing denatured salmon sperm DNA (100 μg/ml). The final washing was in 0.1× SSC/0.1% SDS at 65°C. Autoradiography was performed using a bioimage analyzer, Fujix BAS 2000.

Northern Blot Analysis. Total RNA was isolated by the acid guanidinium thiocyanate/phenol/chloroform method (15). The poly(A)$^+$ RNA was purified on Oligotex-dT30 (Roche). Poly(A)$^+$ RNA samples (1 μg of each) were electrophoresed in formaldehyde/1% agarose gel. Hybridization, washing, and autoradiography were performed as for Southern blot analysis.

RESULTS

Isolation of the cDNA Clone Located at the t(8;21) Breakpoint on Chromosome 21. We have reported (13) the rear-

Abbreviation: AML, acute myeloid leukemia.

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¶The sequence reported in this paper has been deposited in the GenBank data base (accession no. D90525).

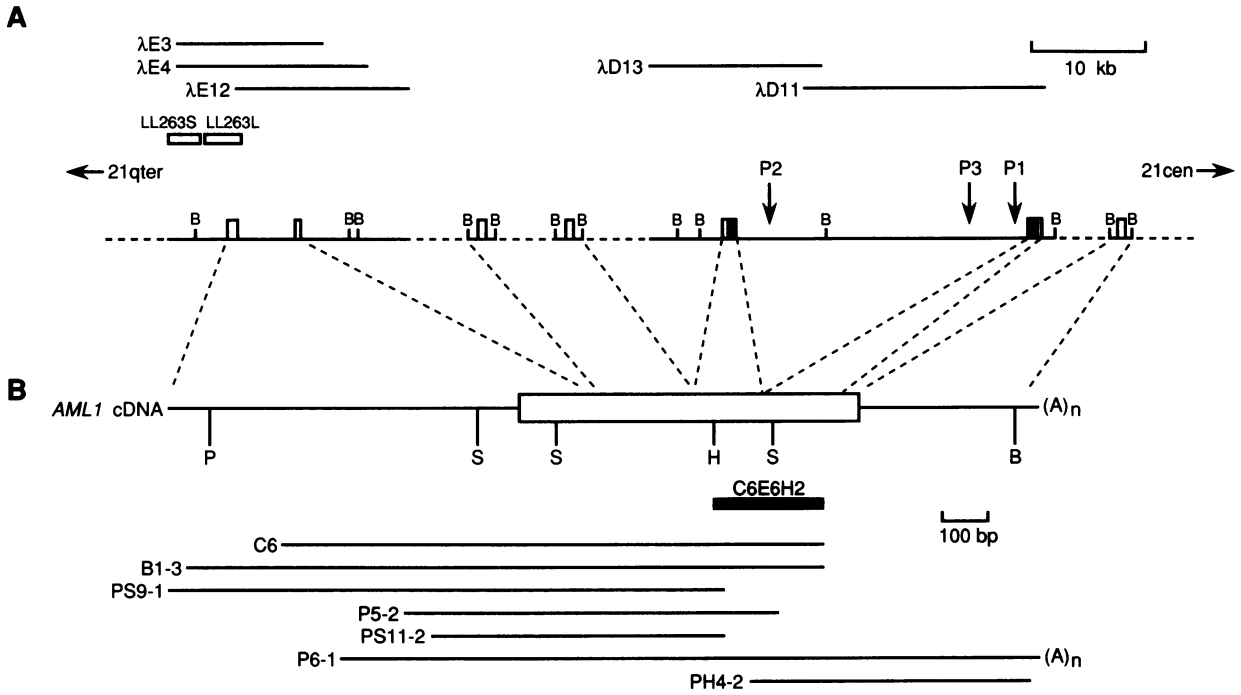

FIG. 1. Restriction maps of the t(8;21) breakpoint region on chromosome 21 and the *AML1* cDNA. (*A*) Genomic clones λE3, λE4, λE12, λD11, and λD13 are represented by horizontal bars above the map. LL263L and LL263S are fragments of the linking clone LL263 digested with *Not* I. The partial restriction map of *Bam*HI was derived from Southern blot analysis of genomic DNA and genomic clones using several restriction fragments of the cDNA clones as probes. Exons of *AML1* (not shown to scale) are represented by boxes; solid boxes correspond to the C6E6H2 fragment. The breakpoints in patients 1, 2, and 3 (P1, P2, and P3) are indicated by vertical arrows. (*B*) Open box represents the open reading frame deduced from the sequence of cDNA clones indicated below. The cDNA clone P6-1 contains a poly(A) tract of >90 nucleotides, and several other overlapping cDNA clones with distinct sequences in the 3′ region were also isolated (not shown). Dashed lines show the approximate locations of exons in genomic DNA. C6E6H2, a *Hin*dIII–*Eco*RI fragment of C6 clone, is indicated by a solid bar. B, *Bam*HI; H, *Hin*dIII; P, *Pst* I; S, *Sma* I; bp, base pairs; kb, kilobases.

rangement in leukemic cell DNAs from t(8;21) AML patients detected with *Not* I linking clone LL263, and the t(8;21) breakpoints were located about 13–100 kb proximal to the LL263 *Not* I site. LL263 contains a CpG island that is present in the 5′ regions of most "housekeeping" genes (16), so we screened a human bone marrow cDNA library with the LL263 clone, but we failed to isolate any cDNA clones. We then performed "chromosome walking" from LL263L and isolated three genomic clones, λE3, λE4, and λE12 (Fig. 1). Genomic clone λE4 insert was used to rescreen the cDNA library, and cDNA clone C6 was identified (Fig. 1). Leukemic cell DNAs from t(8;21) AML patients digested with *Bam*HI were examined by Southern blot analysis with a 0.24-kb *Hin*dIII–*Eco*RI fragment (C6E6H2) of clone C6 as a probe. Rearranged *Bam*HI fragments were detected in leukemic DNAs of four patients but not in their DNAs in remission (Fig. 2*A*). This finding showed that the translocation breakpoints in the four patients occurred within the *Bam*HI fragments that were recognized by the cDNA probe (C6E6H2). Comparison of the intensities of the germ-line bands in DNAs of leukemic cells and of cells in remission suggested that the rearranged DNA fragment of patient 2 was derived from a

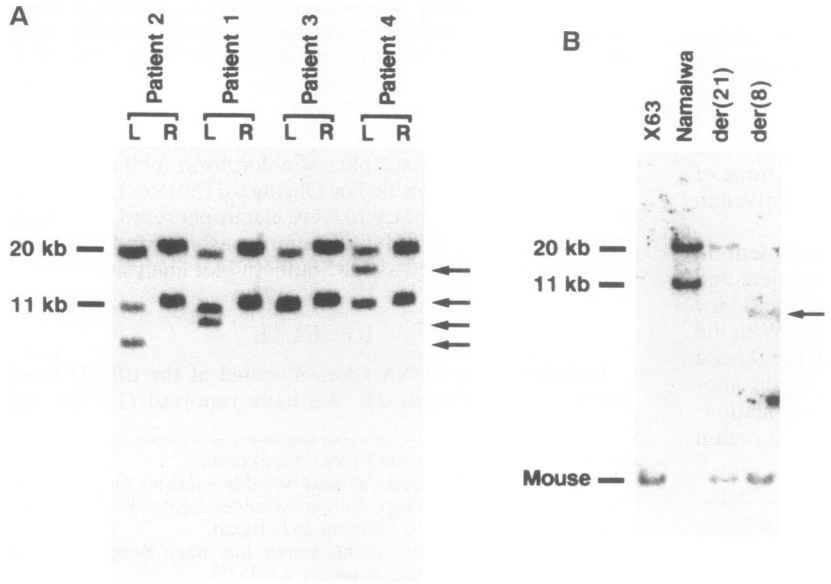

FIG. 2. Detection of the t(8;21) breakpoints by Southern blot analysis of *Bam*HI-digested DNAs with cDNA probe C6E6H2. (*A*) DNAs from leukemic cells (L) and cells in remission (R) from four patients are compared. Rearranged bands are indicated by arrows. In DNAs obtained in remission, only germ-line fragments of 11 and 20 kb were detected. The rearranged band in patient 3 overlapped the 11-kb germ-line band. (*B*) Mouse–human somatic cell hybrid DNA containing the der(8) or der(21) chromosome of patient 2. X63 (mouse myeloma parental cell line) and Namalwa (Burkitt lymphoma cell line) were used as controls for mouse and human DNAs, respectively.

germ-line *Bam*HI fragment of 11 kb, while those of patients 1, 3, and 4 were from a fragment of 20 kb.

The chromosomal breakpoint of patient 2 was subsequently confirmed by Southern blotting of mouse–human somatic cell hybrid DNA containing only the der(8) or der(21) chromosome from leukemic cells of patient 2 (Fig. 2*B*). Both the hybrid containing the der(8) and leukemic cell DNAs of patient 2 shared the same rearranged band, whereas the hybrids containing either der(8) or der(21) lacked the 11-kb band, indicating that the breakpoint in patient 2 occurred in the 11-kb *Bam*HI fragment. Furthermore, detection of the 20-kb *Bam*HI fragment in the hybrid containing the der(21) chromosome indicated that this 20-kb fragment was located centromeric to the 11-kb fragment. The localizations of breakpoints in patients 1 and 3 were confirmed by use of genomic clone λD11, as described later.

Nucleotide Sequence and Predicted Amino Acid Sequence of *AML1*. We used the C6E6H2 probe to screen human bone marrow and peripheral blood leukocyte cDNA libraries and isolated several overlapping cDNA clones (Fig. 1), including clone P6-1, which contained the poly(A) sequence. Sequencing of these cDNA clones revealed a long open reading frame encoding a protein of 250 amino acids (Fig. 3). The nucleotide sequence surrounding the predicted initiation codon agreed well with the Kozak consensus sequence (17). The predicted amino acid sequence showed no significant homology with any known protein sequences in the Swiss-Prot data base. Therefore, we named this novel gene *AML1*. The predicted *AML1* gene product is rich in proline (9.6%) and arginine (9.2%). An ATP- or GTP-binding-site motif A (18) was found at amino acid residues 138–145 by using the PCGENE PROSITE program. With several fragments of cDNA clones as probes, Southern blot analysis of the hybrids demonstrated the orientation of *AML1* on chromosome 21 as 5′ telomeric and 3′ centromeric (see Fig. 1).

The t(8;21) Breakpoints on Chromosome 21 Are Clustered Within a Limited Region of *AML1*. By screening a human lymphocyte genomic library with the C6E6H2 probe, we obtained two overlapping clones, λD11 and λD13. Southern blot analysis using various subfragments of these genomic clones as probes identified the locations of breakpoints in patients 1, 2, and 3 on chromosome 21 (Fig. 1). Since the *AML1* cDNA hybridized to six genomic *Bam*HI fragments, and one of them covered by λE4 contained at least two exons, the *AML1* cDNA contained at least seven exons. Exon mapping on λD11 and λD13 with the C6E6H2 probe and subsequent sequencing of intron/exon boundaries of the cloned DNA revealed that each of the two clones contained one exon (Fig. 1). Consequently, the breakpoints of patients 1, 2, and 3 occurred within the same intron between these two exons (Fig. 3). In addition, to determine whether the breakpoints clustered in this region, leukemic cell DNAs from other t(8;21) AML patients were digested with *Bam*HI and hybridized with the C6E6H2 probe. As a result, 16 (including patients 1–4) out of 21 patients examined showed the rearranged *Bam*HI fragments (data not shown). The findings described so far indicated that the breakpoints on chromosome 21 of t(8;21) AML patients were clustered within a limited region, possibly the same intron, of *AML1*.

Expression of *AML1*. We used Northern blot analysis with the C6E6H2 probe to analyze RNA from normal individuals and patient 1 (Fig. 4). Furthermore, we examined another t(8;21) AML patient and a human AML cell line (Kasumi-1) with t(8;21) (19). Four species of mRNA were commonly observed in normal individuals, the two patients, and the cell line, probably due to alternative splicing. This possibility was supported by the isolation of some overlapping cDNA clones with distinct sequences in the 3′ region (data not shown). The level of the smallest mRNA detected (about 2.1 kb, corresponding to the size of the cloned cDNA) was very low; this may have been due to its rapid degradation, because its 3′ untranslated region contained an A+U-rich sequence involving the AUUUA motif, which has been shown to mediate selective degradation of mRNA (20).

The largest mRNA (about 8.2 kb) was more abundant in the two patients and the cell line than in normal individuals. Two species of mRNA (about 5.4 kb and 4.3 kb) were observed only in the patients and the cell line but not in normal individuals, although those expression levels were low. These findings suggest that the t(8;21) translocation caused some deregulation of *AML1* expression or production of a fusion mRNA between *AML1* and a counterpart gene on chromosome 8.

DISCUSSION

We have isolated and sequenced a gene, *AML1*, that was rearranged in the leukemic cell DNAs from t(8;21) AML patients. Southern blot analysis with cDNA probe C6E6H2

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ATTGAGAGAACAGAGAACATGACAAGTACTTCTAGCTCAGCACTGCTCCAACTACTGAAG    60
CTGATTTTCAAGGCTACTTAAAAAAATCTGCAGCGTACATTAATGGATTTCTGTTGTGTT   120
TAAATTCTCCACAGATTGTATTGTAAATATTTTATGAAGTAGAGCATATGTATATATTTA   180
TATATACGTGCACATACATTAGTAGCACTACCTTTGGAAGTCTCAGCTCTTGCTTTTCGG   240
GACTGAAGCCAGTTTTGCATGATAAAAGTGGCCTTGTTACGGGAGATAATTGTGTTCTGT   300
TGGGACTTTAGACAAAACTCACCTGCAAAAAACTGACAGGCATTAACTACTGGAACTTCC   360
AAATAATGTGTTTGCTGATCGTTTTACTCTTCGCATAAATATTTTAGGAAGTGTATGAGA   420
ATTTTGCCTTCAGGAACTTTTCTAACAGCCAAAGACAGAACTTAACCTCTGCAAGCAAGA   480
TTCGTGGAAGATAGTCTCCACTTTTTAATGCACTAAGCAATCGGTTGCTAGGAGCCCATC   540
CTGGGTCAGAGGCCGATCCGCAGAACCAGAACGTTTTCCCCTCCTGGACTGTTAGTAACT   600
TAGTCTCCCTCCTCCCCTAACCACCCCCGCCCCCCCCCACCCCCCGCAGTAATAAAGGCC   660
CCTGAACGTGTATGTTGGTCTCCCGGGAGCTGCTTGCTGAAGATCCGCGCCCCTGTCGCC   720
GTCTGGTAGGAGCTGTTTGCAGGGTCCTAACTCAATCGGCTTGTTGTGATGCGTATCCCC   780
                                                M  R  I  P     4

GTAGATGCCAGCACGAGCCGCCGCTTCACGCCGCCTTCCACCGCGCTGAGCCCAGGCAAG   840
 V  D  A  S  T  S  R  R  F  T  P  P  S  T  A  L  S  P  G  K    24

ATGAGCGAGGCGTTGCCGCTGGGCGCCCCGGACGCCGGCGCTGCCCTGGCCGGCAAGCTG   900
 M  S  E  A  L  P  L  G  A  P  D  A  G  A  A  L  A  G  K  L    44

AGGAGCGGCGACCGCAGCATGGTGGAGGTGCTGGCCGACCACCCGGGCGAGCTGGTGCGC   960
 R  S  G  D  R  S  M  V  E  V  L  A  D  H  P  G  E  L  V  R    64

ACCGACAGCCCCAACTTCCTCTGCTCCGTGCTGCCTACGCACTGGCGCTGCAACAAGACC  1020
 T  D  S  P  N  F  L  C  S  V  L  P  T  H  W  R  C  N  K  T    84

CTGCCCATCGCTTTCAAGGTGGTGGCCCTAGGGGATGTTCCAGATGGCACTCTGGTCACT  1080
 L  P  I  A  F  K  V  V  A  L  G  D  V  P  D  G  T  L  V  T   104

GTGATGGCTGGCAATGATGAAAACTACTCGGCTGAGCTGAGAAATGCTACCGCAGCCATG  1140
 V  M  A  G  N  D  E  N  Y  S  A  E  L  R  N  A  T  A  A  M   124

AAGAACCAGGTTGCAAGATTTAATGACCTCAGGTTTGTCGGTCGAAGTGGAAGAGGGAAA  1200
 K  N  Q  V  A  R  F  N  D  L  R  F  V  G  R  S  G  R  G  K   144
                                        ATP- or GTP-binding site
AGCTTCACTCTGACCATCACTGTCTTCACAAACCCACCGCAAGTCGCCACCTACCACAGA  1260
 S  F  T  L  T  I  T  V  F  T  N  P  P  Q  V  A  T  Y  H  R   164
motif A                                  ↓
GCCATCAAAATCACAGTGGATGGGCCCCGAGAACCTCGAAGACATCGGCAGAAACTAGAT  1320
 A  I  K  I  T  V  D  G  P  R  E  P  R  R  H  R  Q  K  L  D   184

GATCAGACCAAGCCCGGGAGCTTGTCCTTTTCCGAGCGGCTCAGTGAACTGGAGCAGCTG  1380
 D  Q  T  K  P  G  S  L  S  F  S  E  R  L  S  E  L  E  Q  L   204

CGGCGCACAGCCATGAGGGTCAGCCCACACCACCCAGCCCCCACGCCCAACCCTCGTGCC  1440
 R  R  T  A  M  R  V  S  P  H  H  P  A  P  T  P  N  P  R  A   224

TCCCTGAACCACTCCACTGCCTTTAACCCTCAGCCTCAGAGTCAGATGCAGGAGGAAGAC  1500
 S  L  N  H  S  T  A  F  N  P  Q  P  Q  S  Q  M  Q  E  E  D   244

ACAGCACCCTGGAGATGTTAAGGCAGAAGTCAGTTCTTCTGTCCATCCCTCTCCCCAGCC  1560
 T  A  P  W  R  C  -                                           250

AGGATAGAGCTATCTTTTCCATCTCATCCTCAGAAGAGACTCAGAAGAAAGATGACAGCC  1620
CTCAGAATGCACGTTATGAGGAAGGCAGAATGTGGGTCTGTAATTCCTCCGTGTCCCTTC  1680
TCCCCCTCTGCAAACCGTCGTAACAATAATAGTTCCTAACACATGGGACAATTGTGAGGA  1740
TTAAATGAGTTAGCCTGCAGAAATCACTTGATGCACAGCACATGGGAAGCATTGTGTGTA  1800
TTTATTAATCCTTCACAAAGTCTTTGAGATATATTTTTATCAAATATTTAGCATGGATCC  1860
CGGTACACTTTCAATACTTAATAAATGGTCAATGTTATTCTTTTTCACTATT(A)n       1912
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FIG. 3. Nucleotide sequence of *AML1* cDNA and deduced amino acid sequence (single-letter code). Numberings of nucleotides and amino acids are shown at right. The underlined amino acid sequence represents an ATP- or GTP-binding-site motif A. The 3′ untranslated region contains the AUUUA motif (underlined) implicated in selective destabilization of mRNA. The polyadenylylation signal, AATAAA, is boxed. An arrow indicates the exon boundary that was interrupted by the t(8;21) translocation.

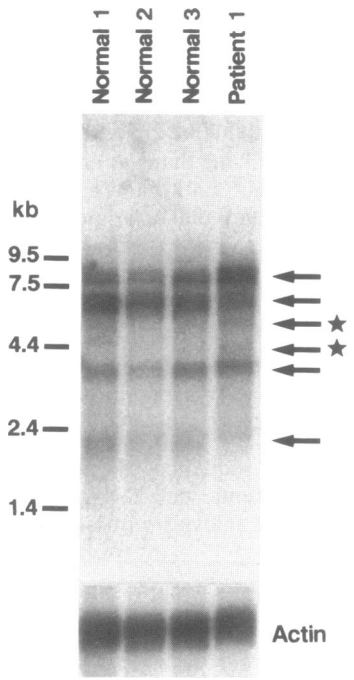

FIG. 4. Northern blot analysis of poly(A)$^+$ RNAs from patient 1 and normal individuals. Northern blots were hybridized with the C6E6H2 probe and rehybridized with a β-actin probe. RNA size markers (BRL) are shown at left. Arrows mark four species of mRNA commonly observed in patient 1 and normal individuals, and starred arrows mark two species in patient 1 that were not detected in normal samples.

revealed that the breakpoints in 16 of 21 patients occurred in a limited region of *AML1*. Detailed analysis in three patients showed that their breakpoints occurred within the same intron between the two exons identified by C6E6H2 probe. Thus the t(8;21) breakpoints probably are clustered within the same intron of *AML1*. The clustering of breakpoints implies that *AML1* may play an important role in leukemogenesis of t(8;21) AML.

The function of the *AML1* gene product is not known at present. However, several features in primary structure suggest that *AML1* may be involved in the control of cellular proliferation and/or differentiation. First, the coding sequence of the gene is conserved in both humans and mouse as indicated by strong hybridization of the cDNA fragment to mouse DNA (Fig. 2*B*). This strong conservation of *AML1* will be useful for characterization of its functions and production of a mouse model for the study of AML. Second, the *AML1* gene seems to have a housekeeping function, since our preliminary Northern blot analysis indicated continuous expression of *AML1* in various human tissues and cell lines. This is consistent with the existence of the CpG island in the 5′ region of *AML1*. Third, the predicted *AML1* gene product has an ATP- or GTP-binding-site motif, though its biological functions are unknown. Further, the *AML1* gene has some similarity in sequence to protooncogenes. The 5′ untranslated region of *AML1* is very long (>700 nucleotides) and similar in length to that of most protooncogenes, in contrast with the 5′ untranslated region of most vertebrate mRNAs, which fall in the size range of 20 to 100 nucleotides (17). In addition, the 3′ untranslated region contains an AUUUA motif, a selective mRNA degradation signal that has been found in mRNAs of certain protooncogenes, lymphokines, and cytokines (20).

Northern blot analysis in two patients and an AML cell line with t(8;21) showed higher expression of the largest *AML1* mRNA than in normal individuals and the presence of two mRNA species not detected in normal individuals. This finding may possibly be ascribed to the t(8;21) translocation in AML, because chromosomal translocations are implicated in the pathogenesis of many hematopoietic malignancies through genetic alteration of protooncogenes. Two general mechanisms are well known for the roles of translocation in activation of protooncogenes: transcriptional deregulation of a normal gene or production of a chimeric protein (21–25). Although the interruption of *AML1* in a single specific intron between two coding exons may suggest production of a chimeric protein, we cannot distinguish between the two possibilities on the basis of the present results. Further study is needed to identify the genetic alteration resulting from the t(8;21) translocation.

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