Activation of *EVII* gene expression in human acute myelogenous leukemias by translocations spanning 300–400 kilobases on chromosome band 3q26

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ABSTRACT Retroviral activation of *Evi-1* gene expression is one of the most common transforming events in murine myeloid leukemias. To evaluate the role of the *EVII* gene in human acute myelogenous leukemia (AML), leukemic blasts or cell lines from 116 patients were examined. In eight patients the *EVII* gene was expressed and all but one had cytogenetically detectable translocations of chromosome 3q26 where the *EVII* gene has been localized. To identify breakpoints, a restriction map that spans 1700 kilobases (kb) of the *EVII* locus was developed by pulsed-field gel electrophoresis. In one case, t(3;3)(q21;q26), a rearrangement was localized to 170–330 kb 5' of the gene. In a second case, t(3;3)(q21;q26), there was a rearrangement 13 kb 5' of the gene. This rearrangement was cloned and shown to be due to the fusion of sequences from 3q21–22 with the *EVII* locus. In the third case, ins(3)-(q21q25q27), there was a rearrangement that mapped 150 kb downstream from the 5' end of the gene.

Human acute myelogenous leukemia (AML) is associated with a number of recurring chromosomal abnormalities. Only recently have the genes associated with these cytogenetic changes been identified. One region of recurring abnormalities involves chromosome band 3q26 and includes t(3;3)(q21;q26), inv(3)(q21q26), t(2;3)(p21;q26), t(3;21)(q26;q22), and t(3;5)(q25;q34) (1–9). Importantly, the human *EVII* gene maps to this region (10).

Evi-1 was identified as the gene associated with a common site of integration in murine myeloid leukemias. Expression is activated by retroviral insertions in a region that spans 15 kilobases (kb) in the 5' region of the gene (11). Alternatively, *Evi-1* expression is activated by retroviral insertions in the *Cb-1/fim-3* common site of integration, which spans 15 kb and is 90 kb proximal to the gene (12–14). The *Evi-1* gene encodes a zinc finger protein that contains an N-terminal domain of seven fingers and a C-terminal domain of three fingers (11). The 145-kDa protein is localized in the nucleus of transformed cells (15). A portion of the DNA binding sequence has been determined (16). The gene is normally expressed in oocytes and in limited sites in the kidney (17) and may play a role in development. The undifferentiated phenotypes of leukemias expressing the *Evi-1* gene suggest that aberrant expression may interfere with differentiation. Consistent with this, expression in a myeloid cell line (32Dcl) blocks terminal differentiation to granulocyte colony-stimulating factor (18). To determine the potential role of the *EVII* gene in human AML, we screened patients for expression. The results demonstrate activation of *EVII* gene expression in eight patients with AML. In the three patients

examined, rearrangements of the *Evi-1* locus occurred 170–330 and 13 kb 5' of the gene and 150 kb downstream from the 5' end of the gene.

MATERIALS AND METHODS

Cells and Tissue Culture. Patient cells were obtained from St. Jude Children's Research Hospital, from the Southwest Oncology Group, or from The University of New Mexico Cancer Center. The establishment of somatic cell hybrid lines from a t(3;3)(q21;q26) AML and the UCSD/AML1 cell line has been described (19, 20).

Detection of *EVII* Transcripts. RNA was prepared as described (11) and poly(A)⁺ was isolated on oligo(dT)-cellulose. Five micrograms of poly(A)⁺ or 20 µg of total RNA was fractionated on 1% agarose gels in 2.2 M formaldehyde (10). The filters were hybridized with the H1-1 cDNA probe containing the entire coding region (10). For detection of *EVII* transcripts by PCR, 1 µg of total RNA was hybridized to oligo(dT) primers and extended with 20 units of avian myeloblastosis virus reverse transcriptase. After 1 hr, the samples were diluted and denatured by heating; 100 pmol of 5' and 3' oligonucleotide primers was added with 2.5 units of *Taq* polymerase, 200 µM each dATP, dGTP, dTTP, and 100 µM dCTP and [³²P]dCTP. The reactions were cycled for 30 cycles of 1 min at 92°C, 1 min at 62°C, and 2 min at 72°C followed by one cycle of 1 min at 92°C, 1 min at 62°C, and 15 min at 72°C. After amplification, the products were subjected to electrophoresis and the fragments were detected by autoradiography. The 30-mer 5' primer (AGCAACGTCGAATCAAGACCTGCTTCAGAT) initiates at base 2110 and the 30-mer 3' primer (TCAGACTGTAAGAGCTCACTGGCCTCAGGT) terminates at base 2390 of the human *EVII* cDNA (10).

Pulsed-Field Gel Electrophoresis (PFGE). Agarose plugs from cryopreserved patient cells or from cultured cells were prepared by adding 1% low melting point agarose in phosphate-buffered saline (PBS) to an equal volume of cells suspended in PBS (1.2–2.4 × 10⁷ cells per ml). The plugs were incubated in 0.5 M EDTA/1% *N*-laurylsarcosine/1 mg of proteinase K per ml overnight at 50°C, washed twice at room temperature for 2 hr in 10 mM Tris-HCl, pH 7.5/1 mM EDTA/1 mM phenylmethylsulfonyl fluoride, and were digested with the indicated enzymes for 6–8 hr. The plugs were loaded onto a 1% agarose gel. PFGE was carried out on a Bio-Rad CHEF-DR II PFGE apparatus. Gels were run at 11°C at ramped pulses from 120 to 180 sec over 36 hr at a constant 150 V, the DNA was transferred, and the filters were hybridized as described (10).

FIG. 1. Detection of *EVII* transcripts in AML. (A) Expression was examined by Northern analysis. The samples are human endometrial carcinoma cell line HEC1B (lane 1) and leukemia cell lines UCSD/AML1 (lane 2) (19), TF-1 (lane 3) (25), and Mo-7 (lane 4) (26). (B) Detection of *EVII* transcripts by PCR. The samples included HEC1B (lane 1), individual cases of AML (lanes 2–5) including patients 3 (lane 2) and 1 (lane 4) (Table 1), somatic cell hybrids [H3-4/6(3q–) (lane 6), H10-c(3q+) (lane 7), 2A3CT (normal 3) (lane 8)], human myeloid cell line HL-60 (lane 9), and a human ovarian carcinoma cell line (lane 10). The products with human DNA are shown in lane 11 as a control.

Fluorescence *in Situ* Hybridization. The 32A clone was hybridized to metaphase spreads of phytohemagglutinin-stimulated human peripheral blood lymphocytes (PBLs). Phage DNA was nick-translated with digoxigenin-11 UTP and was hybridized in a buffer consisting of 50% formamide/2× standard saline citrate (SSC)/75 μg of C₀t 1 DNA per ml/10% Dextran sulfate at 37°C overnight. Hybridization was followed by one 20-min wash in 50% formamide/2× SSC at 39°C and was followed by two 4-min washes in 2× SSC at 39°C. Hybridization was detected with fluorescein-conjugated sheep anti-digoxigenin antibodies.

RESULTS

To determine whether the *EVII* gene was involved in human AML, RNAs from 96 patients with AML and from 20 AML cell lines were screened for *EVII* gene expression by Northern blot analysis and PCR. Typical results are illustrated (Fig. 1). Eight patients were found to contain *EVII* transcripts (Table 1); all but one had cytogenetically detectable chromosomal rearrangements involving 3q26. The biological and clinical properties will be considered in detail elsewhere (C.L.W., K.M., and J.N.I., unpublished data).

Table 1. Diagnosis and chromosome 3 rearrangements of AML patients 1–8 in which *EVII* gene expression was detected

	FAB	Cytogenetics
1	AML-M1	45,XY,–7,del(3)(q27);del(5)(q23q31)
2	AML-M1	46,XY,del(7)(p15),t(2;3)(q21;q27)
3	AML-M0	45,XY,–7,ins(3)(q21q25q27)
4	AML-M1	45,XX,–7,t(3;3)(q21;q26)
5	AML-M1	45,XX,–7,t(3;3)(q21;q26)
6	AML-M2	46,XY,del(7)(q22),t(3;3)(q21;q26)
7	AML-M1	45,XX,–7
8	AML-M1	45,XX,–3,–5,–7,der(3),t(3;5)(q11.2;p11)der(5)t(3;5)(qter→3q29::3q11.2→3q29::5p11→5qter)

RNA was obtained from blast cells of patients 1,2,3,4,7, and 8. RNA for patient 5 was obtained from a myeloid leukemia cell line established from the patient. RNA for patient 6 was obtained from somatic cell hybrids with CHO cells that contained the derivative 3q+ chromosome seen in the patient's blast cells. *EVII* transcripts were detected in these samples by Northern analysis and by a PCR approach as described in the legend to Fig. 1.

To determine whether aberrant expression of the *EVII* gene was due to chromosome rearrangements, we focused on three patients for which sufficient cells were available. One patient (patient 6, Table 1) was an AML FAB-M2 containing a t(3;3)(q21;q26) for which somatic cell hybrids were available containing the 3q+ and 3q– chromosomes (20). As shown in Fig. 1B (lane 7), *EVII* transcripts were found in the hybrid containing the 3q+ but not in hybrids containing the 3q– or a normal 3. As shown in Fig. 1B, *EVII* transcripts were not detected in HL-60 cells (lane 9) and in blast cells from two AML patients, an AML-M5, inv(3)(q21q26) and an AML-M4, t(3;7)(q27;q32). Transcripts were detected in an endometrial carcinoma cell line (HEC-1B) (lane 1) as anticipated from previous studies (10) and in blast cells from two patients with AML (patients 1 and 3, Table 1; Fig. 1B, lanes 2 and 4, respectively).

The second AML patient (patient 5, Table 1), a FAB-M1 leukemia patient with a t(3;3)(q21;q26), was particularly useful because a cell line (UCSD/AML1) had been established (19). By Northern analysis, the UCSD/AML1 cells expressed *EVII* transcripts (Fig. 1A, lane 2). The third case (patient 3, Table 1) involved a patient with mixed lineage AML and an ins(3)(q21q25q27). By PCR analysis, *EVII* transcripts were detectable (Fig. 1B, lane 4). Although all three patients expressed the *EVII* gene, rearrangements were not detectable by Southern blot analysis with probes to *EVII* or *CB-1/FIM3*.

To detect breakpoints distant from the gene, the human *EVII* locus was further characterized. Initially, we isolated cosmid and phage clones by chromosomal walking and conserved murine probes. These clones spanned ≈150 kb of the human *EVII* locus (Fig. 2B) and contained the indicated *Sal*I and *Not*I restriction sites. Within this region, there were no *Sfi*I sites. The organization of the murine locus is shown for comparison.

The 5' region of genes often contains CpG-rich islands with restriction sites for *Bss*HII, *Nae*I, *Sac*II, and *Eag*I that are useful for PFGE mapping (27). Sequencing the 5' region of the *EVII* gene identified a CpG island that contained single potential restriction sites for *Bss*HII, *Mlu*I, *Nar*I, and *Nru*I (Fig. 2C). This fragment contained exon 1 (E1A) (11, 14) and an alternatively utilized first exon (E1B).

A PFGE restriction map of the human *EVII* locus was developed with several probes (Fig. 2B and C). The P1 probe is from the *CB-1/FIM3* site of integration. The P2 probe is from immediately 5' of the CpG island, while the P3 probe is immediately 3' of the CpG island. The P4 probe is from a region after exon 2 and the P5 probe contains the fifth exon. Probes P1 and P2 detected common restriction fragments of 700 kb (*Mlu*I), 610 kb (*Bss*HII), and 650 kb (*Nru*I). Similarly, probes P3, P4, and P5 detected common restriction fragments of 950 kb (*Bss*HII), 950 kb (*Nru*I), and 980 kb (*Mlu*I) and allowed the mapping of the 3' sites. Restriction sites for *Sfi*I and *Sal*I were also identified. Together these restriction sites covered ≈1700 kb of the *EVII* locus.

We next examined cells for rearrangements. Examples with the somatic cell hybrids and the P1 probe are shown (Fig. 3A). No hybridization was detected in the 3q– chromosome-containing hybrid derivative indicating that the *EVII* locus had moved to the 3q+ chromosome. The hybrids containing the normal 3 and the 3q+ derivative contained the expected normal 650-kb *Nru*I and 610-kb *Bss*HII fragments. In addition, new fragments were seen in the hybrid containing the 3q+ of 400 kb (*Bss*HII) and 330 kb (*Nru*I). No rearrangements were detected with *Sal*I or *Sfi*I or with the P5 probe, which detects the 3' region of the gene (data not shown). Therefore, a rearrangement had occurred between 170 and 330 kb 5' of the *EVII* gene.

The results with the UCSD/AML1 cells (patient 5, Table 1) are also shown (Fig. 3B) and compared with PBLs. Using

FIG. 2. Restriction map of the human *EVI-1* locus. (A) A PFGE map was developed by digesting normal lymphocyte DNA. (B) Clones containing the 5' region of the human *EVI-1* gene were used to develop a restriction map. Arrows indicate the individual cosmid and phage clones. Top line shows the restriction map for the comparable region in murine DNA. Sites of integration of retroviruses are shown (DA3, DA34, etc.) and arrows indicate the orientation. Boxes 1–6 indicate the exons of the gene. The third exon contains the first ATG. (C) Restriction map and organization of the immediate 5' end of the gene. A detailed restriction map of the 5' region of the human *EVI-1* locus is shown. This region contains the two alternative first exons of the gene (boxed areas E1A and E1B). The position of CpG dinucleotides within the region is shown on the bottom. This region contains 63% G+C. The bar with an asterisk indicates the *Bam*HI fragment, which is rearranged in the UCSD/AML1 cell line. The restriction enzymes indicated include, in A, *Mlu* I (MI), *Nru* I (Nr), *Bss*HI (B), *Sfi* I (Sf), and *Sal* I (Sa); in B, *Sal* I (S) and *Not* I (N); in C, *Bam*HI (B), *Hind*III (H), *Eco*RV (RV), *Xba* I (X), *Sma* I (Sm), *Sac* I (Sc), *Bss*HI (Bs), *Mlu* I (MI), *Nru* I (Nr), and *Eco*RI (RI). The probes used included the P1 probe, which is a 2-kb *Eco*RI/*Sac* I fragment from a region that corresponds to the murine *Cb-1/fim-3*. The P2 probe is a 350-bp *Hind*III/*Bss*HI fragment from the 5' end of the 2-kb *Hind*III fragment containing the CpG island. The P3 probe is a 400-bp *Nru* I/*Hind*III fragment from the 3' region of the 2-kb *Hind*III fragment. The P4 probe is a 500-bp *Bam*HI/*Eco*RI fragment from a region after exon 2. The P5 probe is a 4.5-kb *Eco*RI/*Bam*HI fragment derived from a region that contains the fifth exon of the gene.

the P1 probe, new fragments were found for *Bss*HI (500 kb), *Sfi* I (290 kb), and *Nru* I (>850 kb), indicating a rearrangement in the 5' region of the gene. It should be noted that polymorphic variations exist with *Nru* I and give rise to normal fragments of 720 and 650 kb. Of particular importance was a 630-kb *Sal* I fragment, which indicated that the rearrangement had occurred within the 5' *Sal* I site and the CpG island. When the blots were probed with the P4 probe, a unique *Sal* I fragment of 240 kb was detected (data not shown). This allowed the mapping of the rearrangement to within 30 kb 5' of the CpG island.

In contrast to the above, no rearrangements were detected with the 5' probes (data not shown) in the third case (patient 3, Table 1). However, when a 3' probe was used (P5), a rearrangement was evident (Fig. 3C). In particular, fragments of 120 (*Bss*HI), 290 (*Sal* I), 390 (*Sfi* I), and 270 (*Nru* I) kb were identified. The size of the unique *Bss*HI fragment indicated that the rearrangement was <120 kb downstream from the CpG island.

Since the translocation in UCSD/AML1 cells mapped to a cloned region, we could more precisely localize it. Screening of Southern blots with probes identified a 2-kb *Bam*HI fragment (indicated in Fig. 2C) that contained the rearrangement (data not shown). To localize the rearrangement, *Eco*RI/*Bam*HI subfragments of 700 (E2) and 1300 base pairs (bp) (E1) were used to probe Southern blots. Specific fragments were evident with most of the restriction enzymes and were generally distinct for the two probes (data not shown). Based on these data, the rearrangement could be further localized to within a 700-bp *Eco*RI/*Bam*HI fragment.

The E1 and E2 probes were then used to clone the rearranged alleles. As illustrated in Fig. 4A, clone 32A contained ≈1 kb from the *EVI-1* locus and the remainder contained unique restriction sites. Similarly, clone 27A contained ≈1.5 kb from the *EVI-1* locus and unique restriction sites. The region of breakpoint in clone 27A was sequenced and, as illustrated in Fig. 4B, the point of divergence of the *EVI-1* locus sequence was identified. To determine whether this region was derived

FIG. 3. Identification of rearrangements by PFGE and Southern blotting. (A) Hybridization of the P1 *EVII* probe to DNA from somatic cell hybrids containing a normal 3q (3q control), the derivative 3q+ chromosome (H10; 3q), and the derivative 3q- chromosome (H3-4; 3q-). (B) Hybridization of the P1 *EVII* probe to DNA from normal PBLs [PBL(N)] or to DNA from the UCSD/AML1 cell line. (C) Hybridization of the P5 *EVII* probe to DNA from normal PBLs [PBL(N)] or of DNA from blast cells of patient 1 with AML-M4 containing a complex *ins(3)(q21q25q27)*. Restriction enzymes are indicated at the top. DNA markers are shown on the sides.

FIG. 4. Rearrangement in UCSD/AML cells is due to the *t(3;3)(q21;q26)*. (A) Restriction maps of the normal *EVII* locus (lane 2) and rearranged alleles (lanes 1 and 3). Restriction maps were generated from phage clones containing the normal allele (8B, 17-1) or the rearranged alleles (32A, 27A) containing the right and left breakpoints. Restriction sites indicated are *Bam*HI (B), *Hind*III (H), *Eco*RI (RI), *Eco*RV (RV), *Sma*I (Sm), *Xba*I (Xb), *Sac*I (Sc), and *Sal*I (Sa). The position of the first noncoding exon (E1A) of the *EVII* gene is shown. (B) Sequence of the translocation breakpoint. Regions containing the normal allele (17-1, I) and the rearranged allele (27A, II) were sequenced. Sequences surrounding the breakpoint of the normal allele (I) and the rearranged allele (II) are shown. E1 and E2 indicate the probes used for cloning. Restriction sites indicated include *Bam*HI (B), *Hind*III (H), *Eco*RI (RI), *Sac*I (Sc), and *Kpn*I (K). The region of the breakpoint was subcloned and sequenced. (C) Fluorescence *in situ* hybridization localization of the breakpoint sequences to chromosome 3q21-22.

from 3q21, the 32A clone was used in fluorescence *in situ* hybridization. As shown in Fig. 4C, hybridization was localized to the q21–22 region of chromosome 3, demonstrating that the rearrangement was due to the translocation.

DISCUSSION

The aberrant expression of the *EVII* gene was observed in 8/116 (7%) patients with AML. This frequency is comparable to the occurrence of many of the recurring translocations in AML (5). A potential role for the *EVII* gene in these cases is supported by its common occurrence in murine myeloid leukemias in which activation of *Evi-1* expression occurs in ≈20% of the cases. In studies of a large spectrum of tumors (21), retroviral insertions in the *Evi-1* locus were found predominantly in myeloid leukemias, indicating that the *Evi-1* gene shows lineage specificity for transformation.

All but one of the AML patients expressing the *EVII* gene contained cytogenetically detectable changes in chromosome 3q, suggesting a role in the aberrant expression. It is possible that in the patient without a cytogenetically detectable alteration of 3q26 it is also rearranged. For example, activation of the *TAL1* gene in acute lymphocytic leukemias often occurs as a consequence of a cytogenetically undetectable 90-kb deletion (22). Unfortunately, sufficient cells were not available from this patient for PFGE analysis. It is also interesting to note that all the patients that expressed the *EVII* gene had either monosomy 7 or deletions of chromosome 7. Conversely, a number of the patients had monosomy 7 but were negative for *EVII* expression (data not shown). Thus, it can be hypothesized that alterations in chromosome 7 may contribute to the evolution of AMLs that involve the *EVII* gene.

Interestingly, 7/7 patients with typical *inv(3)(q21;q26)* did not express the *EVII* gene (data not shown) while 3/3 patients of *t(3;3)(q21;q26)* did. In the case of *t(3;5)*, one patient with a complex rearrangement expressed the *EVII* gene while an AML-M2 with a typical *t(3;5)(q25;q34)* was negative (data not shown). In the latter case, no rearrangements were detected by PFGE analysis of 1700 kb surrounding the locus. Therefore, the breakpoint in the cases that do not involve the *EVII* gene may be quite distant from the *EVII* locus.

In UCSD/AML1 cells, transcripts for the *EVII* gene initiate at the normal transcriptional start sites (data not shown) and the translocation breakpoint is 13 kb 5' of these sites. The rearrangements in the other two patients were detected by PFGE. The detection of the rearrangements by multiple enzymes rules out the possibility that they are due to polymorphisms and the presence of rearrangements of 3q26 strongly suggests that the rearrangements are due to *t(3;3)* or *ins(3)*. The possibility that these rearrangements affect *EVII* gene expression is supported by several observations. First, long-range activation occurs in murine myeloid leukemias (14). Second, the ability of translocations to affect gene expression over long distances has been demonstrated for the *MYC* gene (23). More recently, translocations that are quite distant from *PRAD1* have been implicated in overexpression of the gene (24).

The phenotypic properties that aberrant expression of the *EVII* gene contributes to the transformed AML blasts are not known. However, all the cases were FAB M0, M1, or M2 leukemias and, among the cases examined, were CD34 positive (data not shown), suggesting they were immature myeloid cells. It is also important to note that the UCSD/AML1 cells are dependent on hematopoietic growth factors for proliferation and maintenance of viability (19), indicating that transformation has not abrogated growth factor requirements. The immature phenotype, inability to terminally differentiate, and growth factor dependence are characteristic of murine myeloid leukemia cell lines expressing the *Evi-1*

gene. Therefore, aberrant *Evi-1* gene expression may specifically affect the ability of the cells to differentiate. Consistent with this, *Evi-1* retroviral expression vectors block the granulocyte colony-stimulating factor-induced differentiation of murine myeloid cells (18).

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