A Novel Chromosomal Inversion at 11q23 in Infant Acute Myeloid Leukemia Fuses *MLL* to *CALM*, a Gene That Encodes a Clathrin Assembly Protein

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Rearrangements involving the *MLL* gene at chromosome band 11q23 are common in infant acute myeloid leukemias (AMLs). We recently encountered an infant patient with rapidly progressive AML whose leukemic cells harbored a previously undescribed *MLL* rearrangement involving an inversion of 11q [inv(11)(q14q23)]. We used panhandle PCR to determine that this rearrangement juxtaposed the *MLL* (*M*ixed-*L*ineage *L*eukemia) gene to the *CALM* (*C*lathrin *A*ssembly *L*ymphoid *M*yeloid leukemia) gene at 11q14–q21. The CALM protein participates in recruitment of clathrin to internal membrane surfaces, thereby regulating vesicle formation in both endocytosis and intracellular protein transport. Intriguingly, *CALM* has been identified in other cases of AML as a translocation partner for the *AF10* gene, which has independently been found to be an *MLL* partner in AML. We identified the *MLL-CALM* fusion transcript (but not the reciprocal *CALM-MLL* transcript) in leukemia cell RNA by RT-PCR. The predicted 1803 amino acid MLL-CALM fusion protein includes amino-terminal MLL domains involved in transcriptional repression, and carboxy-terminal CALM-derived clathrin-binding domains. The genomic breakpoint in *MLL* is in the 7th intron (within the breakpoint cluster region); the corresponding *CALM* breakpoint is in the 7th *CALM* intron. In contrast, breakpoints in *CALM-AF10* translocations lie in the 17th–19th *CALM* introns (30 kb downstream); also, in these translocations, *CALM* provides the 5′ end of the fusion transcript. Together with its previously recognized association with *AF10* in AML, the identification of *CALM* as an *MLL* fusion partner suggests that interference with clathrin-mediated trafficking pathways may be an underappreciated mechanism in leukemogenesis.

INTRODUCTION

Acute myeloid leukemia (AML) accounts for approximately 15 to 20% of all cases of childhood leukemia (Golub et al., 1997). In particular, AML constitutes the most frequent type of congenital leukemia. In spite of improvements in understanding some of the molecular mechanisms involved in AML pathogenesis, fewer than 40–50% of newly diagnosed children with AML are cured.

Although the specific factors that lead to myeloid leukemogenesis remain unknown, a number of chromosomal abnormalities have been described in association with AML. Translocations involving band q23 of the long arm of chromosome 11 (11q23) are seen in 5–6% of all AML cases, as well as in 5–10% of all acute lymphoblastic leukemias (ALLs). Strikingly, the frequency of 11q23 translocations is significantly higher in AML in infants (50%), and also in infant ALL (80%) (Felix and Lange, 1999). The presence of 11q23 translocations correlates with a very poor prognosis (Dreyling et al., 1998). In 1993, it was determined that the *MLL* (*M*ixed-*L*ineage *L*eukemia) gene at this locus is involved in nearly all 11q23 translocations (Thirman et al., 1993). The *MLL* gene encodes for a large, complex transcription factor, and translocations involving *MLL* result in the formation of novel hybrid or chimeric genes, whose protein products are thought to play a key role in leukemogenesis.

To date, there have been more than 30 gene fusion partners documented for *MLL* (Ayton and Cleary, 2001a,b). Many of these genes encode for nuclear transcription factors; for example, the *AF10* gene at 10p13, which is involved in t(10;11)(p13;q23) translocations in AML-M4 and AML-M5, encodes for a zinc finger transcription factor (Beverloo et al., 1995; Chaplin et al., 1995). Of note, *AF10* is one of the few *MLL* fusion partners that have been found to be involved in a translocation with another gene in both myeloid and lymphoid malignancies

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(Lillington et al., 1998). Specifically, *AF10* is fused to the *C*lathrin *A*ssembly *L*ymphoid *M*yeloid leukemia (*CALM*) gene at 11q14–q21 in t(10;11)(p13; q14) translocations (Dreyling et al., 1996, 1998). The *CALM* gene encodes a 1956-bp cDNA and a predicted 652 amino acid (aa) protein product that binds to clathrin, which mediates vesicle formation in both endocytosis and intracellular protein transport through the Golgi network (Tebar et al., 1999; Kim et al., 2000). The CALM protein is thought to participate in recruitment of clathrin to internal membrane surfaces, thereby regulating vesicle formation (Tebar et al., 1999). Leukemias with *CALM– AF10* translocations are extremely aggressive and are associated with a very poor prognosis (Bohlander et al., 2000). The disruption of normal CALM function by the CALM–AF10 fusion protein may significantly alter the dynamics of clathrin-mediated growth factor receptor turnover, and potentially alter the sensitivity of cells to external growth factors (Bohlander et al., 2000). To date, although the precise roles of the reciprocal gene fusion products *AF10-CALM* and *CALM-AF10* in leukemogenesis have not been firmly established (Silliman et al., 1998; Kumon et al., 1999; Bohlander et al., 2000; Carlson et al., 2000), the involvement of the CALM protein implicates perturbed vesicle trafficking as having a previously underappreciated role in leukemogenesis (Bohlander et al., 2000).

We recently encountered a pediatric patient with rapidly progressive AML whose leukemic cells demonstrated a previously unidentified *MLL* rearrangement involving an inversion within 11q. This rearrangement juxtaposed the *MLL* gene to the *CALM* gene at 11q14–q21. This is the first instance of a fusion gene involving *MLL* and *CALM*, and the implications of this novel rearrangement in myeloid leukemogenesis are discussed.

MATERIALS AND METHODS

Patient

A 12-week-old Caucasian female infant with fever, congestion, and rhinorrhea developed significant respiratory distress. On physical examination, hepatosplenomegaly was present and multiple petechiae were noted. The initial white blood cell (WBC) count was 272,000/μL, hematocrit was 13%, and the platelet count was 10,000/μL. The peripheral blood smear showed extreme hypercellularity with mature and immature monocytes and granulocytes; increased eosinophils; nucleated red blood cells; and two populations of blasts, one larger with increased cytoplasm and one smaller. The morphologic pattern was suggestive of either juvenile myelomonocytic leukemia (JMML) or AML. Immunophenotyping of the peripheral blood revealed a majority of cells with phenotypic features of mature monocytes [CD11C (98.9%), CD13 (88.1%), CD14 (88.6%), CD33 (97.1%), and CD56 (51.1%)]. Evaluation of the bone marrow indicated 100% cellularity, with a monotony of the neoplastic cell population, and periosteal involvement by neoplastic cells. A 300-cell differential count of the bone marrow aspirate smear revealed a high percentage of blasts (19.6%) and promonocytes (10%), with evidence of monocytic differentiation, as demonstrated by alpha naphthyl butyrate esterase and myeloperoxidase. These findings fulfilled the FAB criteria for acute monocytic leukemia (M5b), with a significant component of monocytic differentiation.

Leukapheresis was performed on a single occasion to decrease the tumor cell burden, and immediately reduced the WBC to 163,200/μL. Over the next 4 days, without additional therapy, the WBC fell to 70,000/μL (where it remained until the 10th hospital day). At the same time, the differential WBC count showed a dramatically higher percentage of monocytes (42–73%) and monocytoid blasts (16–36%). The patient developed respiratory failure (requiring intubation) and renal failure (necessitating continuous veno-venous hemodialysis). Once a diagnosis of AML was confirmed, hemodynamic and respiratory instability precluded chemotherapy initiation with high-dose cytosine arabinoside. On the 10th hospital day, the peripheral WBC began to increase, and chemotherapy was initiated, despite ongoing multiorgan system failure. On the following day, the patient sustained prolonged hypotensive episodes with bradycardia and brainstem herniation. Support was withdrawn, and the patient expired.

Cytogenetic Analysis and Fluorescence In Situ Hybridization (FISH)

Direct and 24-h cultures of leukemic cells without mitogens were prepared by use of standard techniques from bone marrow, peripheral blood, and leukapheresis samples obtained before chemotherapy. Slides with metaphase cells were banded by a trypsin–Giemsa technique; a total of 20 cells from all cultures were analyzed.

FISH with the *MLL* gene probe (Vysis, Downers Grove, IL) was performed following the procedures recommended by the manufacturer. The *MLL* "break-apart" probe consists of a 350-kb por-

tion centromeric of the *MLL* gene breakpoint cluster region labeled with SpectrumGreen and a 190-kb portion telomeric of the breakpoint region labeled with SpectrumOrange. A yellow fusion signal is produced by the juxtaposition of the probes on a normal *MLL* gene; distinct orange and green signals are observed when a rearrangement involving *MLL* is present. A DAPI (4′-6-diamidino-2-phenylindole) counterstain is used to visualize interphase nuclei and to identify metaphase chromosomes. Slides were examined using a Leica DMRA fluorescence microscope (Leica, Deerfield, IL) equipped with a filter set, including SpectrumRed/Green dual-band pass and DAPI filters and imaged with a CCD camera using the CytoVysion software system (Applied Imaging, Santa Clara, CA). A total of more than 300 interphase cells and 10 metaphase cells were analyzed from two hybridizations, which included a negative control.

cDNA Panhandle PCR

The published technique of Megonigal et al. (2000a,b) was used with 1 μg of leukemia cell-derived total RNA. After first-strand cDNA synthesis by use of *MLL*–random hexamer oligonucleotides (MLLRH: 5′-CCTGAATCCAAACAGGCCA CCACTCCAGCTTCNNNNNN-3′), second-strand stem-loop generation was performed. After nested PCR amplification (first with primers MLP1: 5′-TCCTCCACGAAAGCCCGTCGAG-3′ and MLP2: 5′-TCAAGCAGGTCTCCCAGCCAGCAC-3′, then with primers MLP3: 5′-GGAAAAGAGTGAAGA-AGGGAATGTCTCGG-3′ and MLP4: 5′-GTG-GTCATCCCGCCTCAGCCAC-3′), panhandle PCR products were subcloned by recombination PCR into the pUC19 vector in MAX efficiency DH5α cells (Gibco BRL, Gaithersburg, MD). Subclones containing suitable products were identified by PCR (using primers MLP3 and MLP4), and DNA sequencing was performed using the fluorescent dideoxy terminator method on a PE/Abd 373a automated DNA sequencer at The University of Michigan DNA Sequencing Core Facility.

Reverse Transcriptase–Polymerase Chain Reaction (RT-PCR)

A 1 μg sample of total leukemic cell RNA was reverse-transcribed with SuperScript II (Invitrogen Life Sciences, Carlsbad, CA). The resulting first-strand cDNA was amplified by use of a Perkin–Elmer GeneAmp 2400 Thermocycler (Applied Biosystems/Perkin–Elmer, Foster City, CA) with combinations of *MLL*- and *CALM*-specific primers (*MLL*Ex5F1: 5′-AGTCAGAAACCTACCCCATC-AGC-3′; *MLL*Ex5F2: 5′-ACTCTAGTCAGAA-ACCTACCCCATC-3′; *MLL*Ex7F: 5′-TCCTCA-GCACTCTCTCCAATGG-3′; *MLL*Ex8R: 5′-CTC-CCACACATTTTCTGCTTCAC-3′; *MLL*Ex9R: 5′-AGGGCTCACAACAGACTTGGC-3′; *MLL*Ex10R: 5′-CACTCAGGGTGATAGCTGTTTCG-3′; *CALM*Ex6F: 5′-CAAATGGGGTAATAAATGCT-GCC-3′; *CALM*Ex7F: 5′-AGAACCAATGCAAA-GAAGGTCTTG-3′; *CALM*Ex8R1: 5′-GGTCTG-GTATATCACCTCTGTCAATTC-3′; *CALM*Ex-8R2: 5′-TGTGAAAGGTCTGGTATATCACCTC-TG-3′; *CALM*Ex9R: 5′-GTTCCAAAGCATCAA-GAAGACTGC-3′; *CALM*Ex10R: 5′-AGAGATA-GACCAGTGCTTGCCAGG-3′), HF *Taq* polymerase (Roche, Indianapolis, IN), and nucleotide mixtures. Initial denaturation was at 94°C for 1 min, followed by 30 cycles of 94°C for 10 sec, annealing at 56°C for 30 sec, and extension at 68°C for 2 min, with 5 sec added per cycle, and a final extension step at 68°C for 7 min.

Long-Range PCR for Detection of Genomic Breakpoint

The Expand 20 kb^{PLUS} PCR System (Roche) was used with 5′-*MLL* and 3′-*CALM* specific primers (*MLL*Ex5F1, from *MLL* exon 7; and *CALM*Ex9R, from *CALM* exon 9) to amplify genomic DNA from leukemic cells. Amplifications were performed in the absence or presence of DMSO (5 or 10% final concentration), and products were cloned into the pCRII-TOPO TA cloning vector (Invitrogen, Carlsbad, CA), then sequenced. Intron-specific *MLL* and *CALM* primers (*MLL*INT7-450: 5′-CAATCCCAGTGTAT-TTTCGC-3′; *CALM*INT7-700: 5′-AGCCGTGAT-GTCATTTTTC-3′; *CALM*INT7BK+562: 5′-CG-GGTTCAAACGATTCTC-3′; *CALM*INT7R-695: 5′-CCCTTCCTTTTGTGACCTC-3′) were then used to confirm the breakpoint under the PCR amplification conditions defined above.

Guthrie Card PCR Analysis

The neonatal blood spot procured on the patient's second day of life was obtained from the State of Michigan Department of Community Health. Genomic DNA was amplified from this Guthrie card using a modification of a procedure developed by Makowski et al. (1995). Briefly, an office supply hole puncher was used to produce a $\frac{1}{2}$-in.-diameter punch, which was further cut into four equivalent segments. Each segment was incubated in 1 mL of ddH_2O twice for 30 min, then added directly to PCR amplification reactions as described above. PCR amplification cycles included an initial 94°C 10-min incubation, followed

by 37 cycles of denaturation at 94°C (15 sec), annealing at 56°C (30 sec), and extension at 72°C (150 sec), followed by a 7-min extension at 72°C. Amplifications were repeated four times with punches from different Guthrie card sections.

Data Analysis

Sequence data were analyzed using MacVector 6.5.3 and Assemblylign (Accelrys, San Diego, CA). BLAST and BLASTX searches were performed at http://www.ncbi.nlm.nih.gov.

RESULTS

Cytogenetics and FISH Identify a Novel 11q Rearrangement

A paracentric inversion of the long arm of chromosome 11, inv(11)(q14q23), was observed in all 20 metaphase cells examined by standard cytogenetic analysis (Fig. 1A). No other abnormal chromosomes were observed. Metaphase FISH confirmed that the inversion split the *MLL* gene, moving the centromeric 5′ region to band 11q14, whereas the 3′ region remained in band 11q23 (Fig. 1B). Interphase nuclei with normal *MLL* fusion signals were observed in 41.3% (124/300) of cells examined from a peripheral blood sample, indicating that the rearrangement is an acquired cytogenetic abnormality.

Identification of an *MLL-CALM* Fusion Transcript by cDNA Panhandle PCR

We used the technique of cDNA panhandle PCR (Megonigal et al., 2000a,b) to identify hybrid transcripts containing the *MLL* gene. cDNA generated from total RNA resulted in panhandle products of various sizes, and recombination PCR yielded several hundred subclones, of which 65 were selected for further PCR analysis. Eight representative clones containing inserts, ranging from 250 to 2500 bp, were sequenced. Clones with smaller inserts included a sequence from *MLL* exons 5 and 6, followed by 60–110 bp of *MLL* exon 5, then 60–70 bp of *MLL* exon 5 in reverse orientation, consistent with previously described exon scrambling (Megonigal et al., 2000a). These were not studied in any further detail. Five remaining clones contained inserts that ranged in size from 1500 to 2500 bp, and all included a sequence that stopped abruptly after *MLL* exon 7, sharing an identical sequence thereafter (Fig. 2A). BLAST analysis of the sequence immediately following *MLL* exon 7 indicated 100% identity with the human *CALM* gene, also known as *CLTH* (GenBank accession no. U45976/XM_006305). The human *CALM* cDNA coding sequence is 1956 bp, and the predicted 652-aa CALM protein plays a role in regulation of vesicle formation by recruiting clathrin to internal membrane surfaces (Tebar et al., 1999). The full-length *MLL-CALM* transcript is 5409 bp, including 4218 bp of *MLL* sequence (*MLL* exons 1–7) and 1191 bp of *CALM* sequence, corresponding to *CALM* exons 8–20 (GenBank accession no. AF477006). The predicted 1803-aa MLL-CALM fusion protein (Fig. 2B) includes MLL-derived A-T hooks and a repression domain, and a CALM-derived clathrin-binding domain.

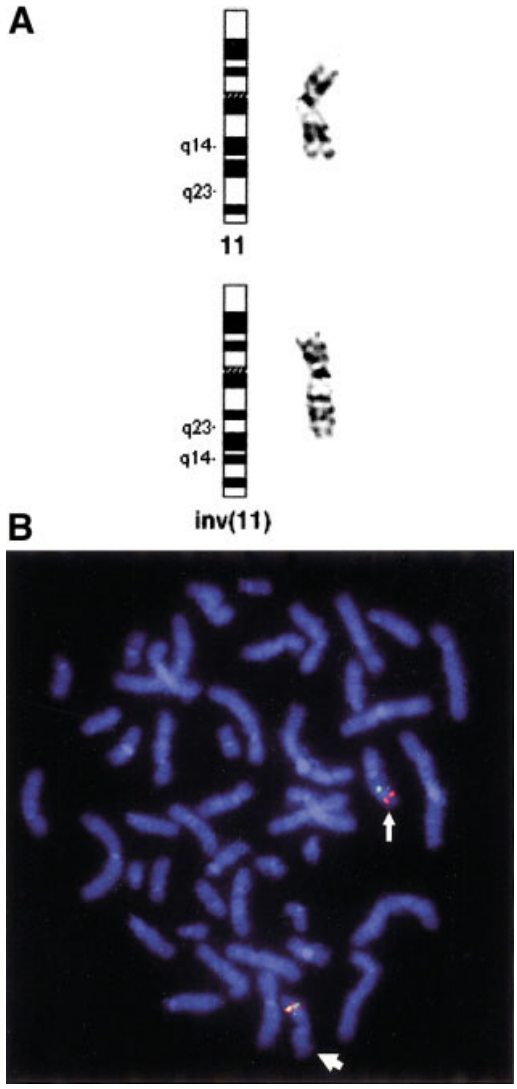

Figure 1. Cytogenetics and FISH from representative leukemic cells. **A:** GTG-banded chromosomes and ideograms depicting the rearrangement of chromosome 11. **B:** Metaphase cell from the patient hybridized with the *MLL* FISH probe. The inverted chromosome (long arrow) has a split in *MLL*, which separates into green (centromeric) and red (telomeric) signals, whereas the normal chromosome 11 homolog (short arrow) has a yellow fusion signal.

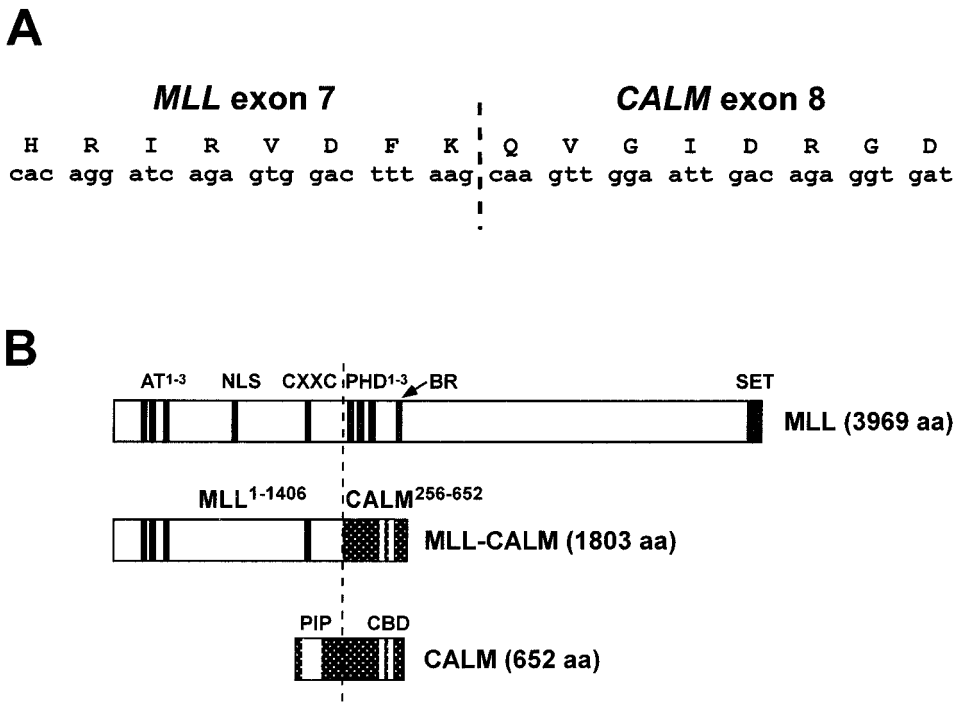

Figure 2. Panhandle PCR identifies *CALM* as an *MLL* fusion partner. **A:** Subclone sequence analysis identified *CALM* as an *MLL* partner in five separate recombination PCR-generated subclones (GenBank accession no. AF477006). **B:** Predicted full-length MLL-CALM fusion protein. Schematic of native MLL (*top*) and CALM (*bottom*) proteins, and predicted 1803-aa MLL-CALM fusion protein (*middle*). The fusion protein retains the three AT-hooks (AT^{1-3}), nuclear localizing sequence (NLS), and CXXC domains of MLL, but lacks PHD, Basic (BR), and SET domains. The fusion protein also includes the putative CALM clathrin-binding domains (CBD), but not the phosphatidylinositol phosphate (PIP)–binding domain.

Confirmation of *MLL-CALM* Transcript in Leukemic Cells by RT-PCR

The presence of the *MLL-CALM* fusion transcript in leukemia cell RNA was confirmed by RT-PCR using 5′-*MLL*- and 3′-*CALM*-specific primers (Fig. 3, lanes b and c). We also used primers from the 5′ end of *CALM* and the 3′ end of *MLL* to demonstrate that the reciprocal *CALM-MLL* fusion transcript is *not* present in the patient's leukemia-derived RNA (Fig. 3, lane f). In contrast, mRNAs from both native *MLL* and *CALM*, presumably derived from the unaffected chromosome 11, are detectable (Fig. 3, lane a and lanes d and e).

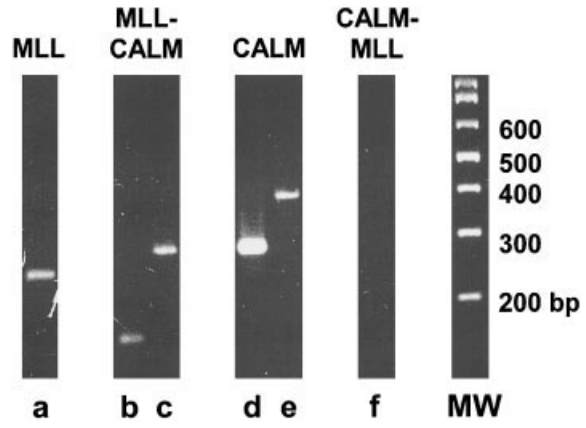

Figure 3. Identification of *MLL-CALM* transcripts in leukemic cells by RT-PCR. RT-PCR analysis of leukemia cell RNA by use of *MLL*- and *CALM*-specific primers. Amplification with primers for native *MLL* (*MLL*4159 and *MLL*Ex9R1055, lane a) and *CALM* (*CALM*F713 and *CALM*R985, lane d; *CALM*F713 and *CALM*R1094, lane e) transcripts yields products of the expected size, as do primers for the hybrid *MLL-CALM* transcript (*MLL*4159 and *CALM*R985, lane b; and *MLL*4159 and *CALM*R1094, lane c). No product was detected using a forward *CALM* primer together with a reverse *MLL* primer (*CALM*F713 and *MLL*Ex9R1055, lane f), indicating the absence of the *CALM-MLL* transcript.

Determination of Genomic Breakpoint

To determine the genomic breakpoint that results in the *MLL-CALM* fusion transcript, we used long-range PCR with 5′-*MLL*- and 3′-*CALM*-specific oligonucleotide primers and leukemia-derived genomic DNA, and amplified a 6.2-kb product (data not shown). DNA sequencing analysis (Fig. 4A) revealed that the breakpoint in *MLL* lies in the 7th intron (444–446 bp from the end of *MLL* exon 7) within the breakpoint cluster region, a well-characterized region of chromosome instability (Felix et al., 1998; Felix and Lange, 1999). The breakpoint within *CALM* lies in the 7th *CALM*

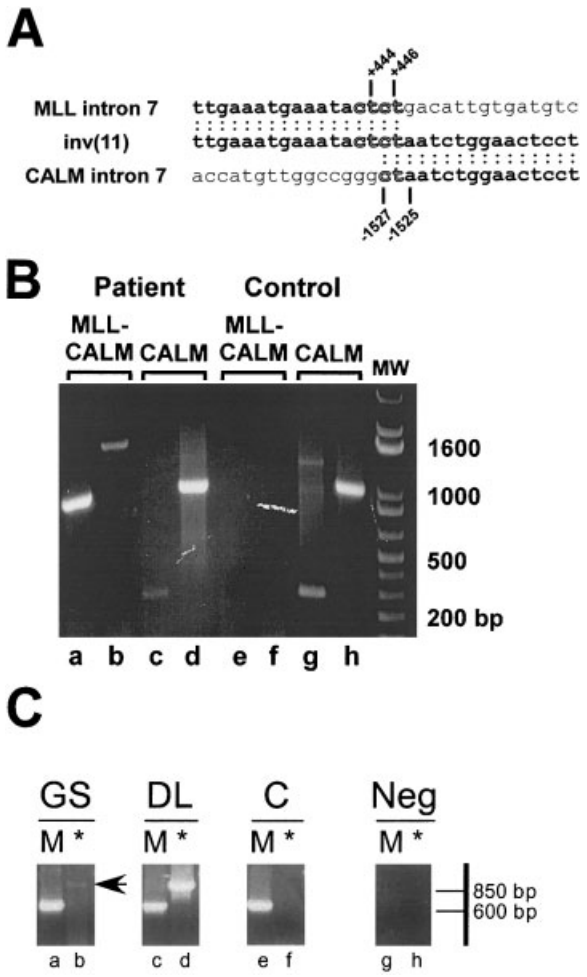

Figure 4. Definition of genomic breakpoints within *MLL* and *CALM* introns. **A:** Genomic sequences within the 7th introns of *MLL* (*upper*) and *CALM* (*lower*) are indicated, with homologies to the derivative sequence (*middle*) indicated by colons. The genomic breakpoint within the *MLL* gene is 444, 445, or 446 bp from the beginning of intron 7, and the *CALM* breakpoint is 1527, 1526, or 1525 bp upstream of the end of intron 7. Identical 5′-CT-3′ sequences in *MLL* and *CALM* (outlined) preclude a more precise assignment of breakpoint positions (GenBank accession no. AF477007). **B:** PCR amplification by use of genomic DNA as a template with forward and reverse intron 7 *MLL* and *CALM* primers yields a band from leukemia cell–derived DNA (lanes a–d), but not control DNA (lanes e–h). Primers in lanes a and e: *MLL*INT7-450 and *CALM*INTR-695; lanes b and f: *MLL*INT7-450 and *CALM*R946; lanes c and g: *CALM*INT7BK+562 and *CALM*INTR-695; lanes d and h: *CALM*INT7BK+562 and *CALM*R946. **C:** PCR amplification by use of genomic DNA derived from neonatal Guthrie spot (GS), in comparison with diagnostic leukemia cells (DL), control non-leukemic cells (C), and no DNA control (Neg). Primers used were specific for *MLL* (lanes a, c, e, and g indicated by M: *MLL*4159 and *MLL*Ex8R942) or *MLL-CALM* (lanes b, d, f, and h indicated by *: *MLL*INT7-450 and *CALM*INTR-695). Gel shown is representative of four amplifications performed with four different GS punches. Arrow indicates *MLL-CALM* band in GS DNA.

intron (1525–1527 bp upstream of *CALM* exon 8), but precise assignment of breakpoint positions was not possible because of the presence of identical 5′-CT-3′ sequences in *MLL* and *CALM* (GenBank accession no. AF477007). This rearrangement results in an *MLL–CALM* fusion transcript that positions *MLL* exon 7 immediately upstream of *CALM* exon 8. By using oligonucleotide primers flanking the putative breakpoints in *MLL* intron 7 and *CALM* intron 7, we were able to confirm the genomic breakpoint in DNA derived from the diagnostic leukemia specimen (Fig. 4B). Furthermore, genomic PCR performed with several sets of 5′-*CALM* and 3′-*MLL* primers failed to yield a product (data not shown), consistent with the absence of a *CALM-MLL* mRNA transcript (Fig. 3, lane f). The *MLL-CALM* fusion gene was also present at least 10 weeks earlier, as evidenced by the presence of an identical PCR amplification product in the Guthrie spot blood obtained on the second day of life (Fig. 4C, lane b). Although the PCR reaction is not quantitative, the intensity of the *MLL-CALM* PCR product in Guthrie spot DNA (lane b) relative to that in the diagnostic leukemia specimen (lane d) is clearly diminished, in comparison with *MLL* products amplified from the same specimens (lanes a and c).

Genomic Structure of Human *CALM*: A Novel Exon and Alternatively Spliced Transcripts

Based on the draft genomic sequence of human chromosome 11 (GenBank accession no. NT_001984), the human *CALM* gene spans approximately 110 kb and is composed of 20 exons (Fig. 5A). Sequencing analysis of the clones derived from our patient indicates the presence of a novel exon near the 3′ end of *CALM*. Two of the initially isolated *MLL-CALM* clones, with 1600- and 1800-bp inserts, included an identical 24-bp insert that was not present in the human *CALM* sequence, or in rat *CALM* (GenBank accession nos. AF041373/AF041374). This sequence was, however, present in the murine *CALM* cDNA (GenBank accession no. BC011470) (Fig. 5B). RT-PCR by use of *CALM*-specific primers with both leukemia cell RNA and control, non-leukemia RNA indicated the presence of transcripts both with and without the 24-bp insert (data not shown). Because a 24-bp insert may be spliced out while still maintaining the reading frame of the remainder of the protein, this sequence represents a previously undescribed human *CALM* exon (17a; GenBank accession no. AF477006). Indeed, a survey of the available human chromosome 11 genomic sequence (GenBank accession no. NT_009184) indicated that the 24-bp sequence was present within the *CALM* gene between exons 17 and 18 (nucleotides 1059895–1059918) (Fig. 5A), flanked by AG and GT splice acceptor and donor sites (Fig. 5C). The functional significance of the CALM protein that includes the 8-aa NGMHFPQY sequence re-

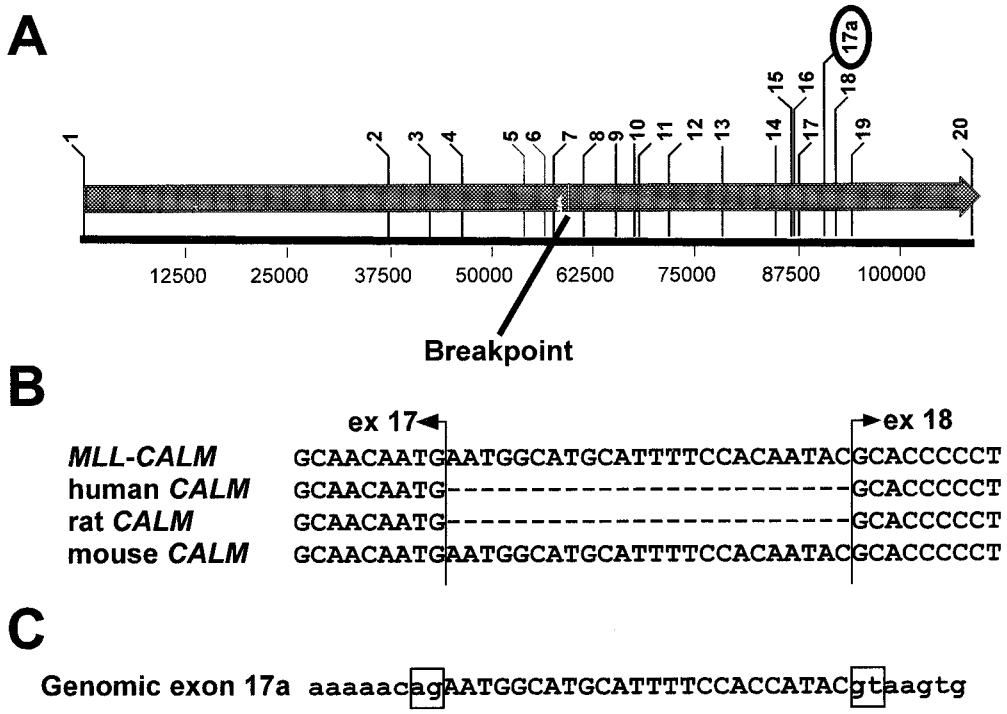

Figure 5. Genomic organization of human *CALM*. **A:** Schematic representation of exon structure of the human *CALM* gene, derived from available chromosome 11 sequence. The locations of the breakpoint within *CALM* as well as the novel 24-bp exon (exon 17a) are indicated. Numbers below the bar indicate distance (in bp) from *CALM* ATG. **B:** Alignment of *MLL-CALM* sequence with human, rat, and mouse *CALM* cDNA sequences indicates the presence of a 24-bp insert in both *MLL-CALM* and mouse *CALM*, but not human or rat cDNAs. **C:** Genomic sequence flanking putative human *CALM* exon 17a (*uppercase*), with flanking intron sequence (*lowercase*) indicating presence of consensus splice donor and acceptor sites (*boxed*).

mains to be determined. Of note, this region is within the putative CALM carboxy-terminal clathrin-binding domain.

Finally, RNA derived from our patient includes transcripts that lack 21 bp of sequence at the beginning of *CALM* exon 13 (data not shown). This region is missing from rat *CALM* but is present in murine *CALM*. It is also present in the human genomic chromosome 11 sequence, and does not constitute a distinct exon because it is contiguous with the remainder of exon 13. However, because the last 2 bp of this region correspond to an AG 3′ splice acceptor site, it is possible that they constitute a cryptic splice site that is used to generate some *CALM* transcripts. Indeed, this region bears a high degree of similarity to the consensus sequence of 3′ splice acceptor sites (Shapiro and Senapathy, 1987). A similar cryptic splice site at the beginning of exon 15 could account for the alternative splice product detected in the original description of *CALM* (Dreyling et al., 1996).

DISCUSSION

Translocations involving the *MLL* gene play a role in the pathogenesis of both lymphoid and myeloid leukemias, particularly those that develop in infants. Although more than 30 *MLL* partner genes have been identified (Ayton and Cleary, 2001b), the precise mechanisms by which most MLL fusion proteins contribute to leukemic transformation remain unknown. In this report, we describe the use of panhandle PCR to identify *CALM* as a novel translocation partner for the *MLL* gene in an infant with AML. The patient's leukemia was extremely aggressive, and was characterized by an unusual JMML-like phenotype. The *MLL-CALM* translocation was present in DNA derived from the neonatal Guthrie spot, indicating a prenatal origin of the leukemic clone. We have documented the genomic structure of human *CALM* for the first time, and identified a novel human *CALM* exon, as well as several alternatively spliced *CALM* isoforms. Because *CALM* has been identified as a translocation partner for *AF10* in leukemias with t(10;11)(p13;q14), the involvement of *CALM* in separate translocations with both *MLL* and *AF10* suggests that CALM-dependent endocytosis pathways may be important in leukemogenesis.

The *CALM* gene is ubiquitously expressed (Dreyling et al., 1996), and encodes for a 652-aa

protein with homology to AP180, a synaptic protein involved in clathrin assembly and endocytic vesicle formation. The full-length CALM protein is primarily associated with cellular membrane fractions, and co-localizes with clathrin in endocytic pits (Tebar et al., 1999). Binding to clathrin is mediated primarily by carboxy-terminal CALM domains (aas 414–652) (Tebar et al., 1999), whereas a lysine-rich amino-terminal ENTH homology domain is responsible for binding to membrane inositol polyphosphates (Ford et al., 2001; Itoh et al., 2001). CALM also contains amino-terminal proline-rich sequences that direct binding to the SH3 domain of PLCγ1 (Kim et al., 2000; Kim and Kim, 2001). Together with carboxy-terminal DPF and NPF motifs that may mediate binding to AP-2 and Eps15 homology (EH) domains, respectively (Santolini et al., 1999; Ford et al., 2001), these features suggest a role for CALM in tethering clathrin and its adaptor proteins to membranes during the early stages of endocytic vesicle formation. That CALM plays a definitive role in endocytic vesicle formation is demonstrated by the ability of overexpressed CALM to interfere with endocytosis of receptors for both transferrin and epidermal growth factor (Tebar et al., 1999). CALM overexpression also results in loss of clathrin accumulation in the trans-Golgi network (Tebar et al., 1999). Thus, perturbation of normal CALM activity might interfere with endocytosis.

Intriguingly, a transcriptional regulatory domain has been described in the carboxy-terminal region of CALM (Bohlander and Bartels, 1997), and CALM has recently been shown to act as a transcriptional activator in GAL4-based transcriptional assays (Vecchi et al., 2001). The same report indicated that the CALM protein also undergoes nucleocytoplasmic shuttling (Vecchi et al., 2001), suggesting that, in addition to its role in endocytosis, CALM might also participate in transcriptional regulation.

The *CALM* gene was first described as a translocation partner for *AF10* in the U937 monocytic cell line (Dreyling et al., 1996, 1998). Notably, *AF10* was initially identified as an *MLL* translocation partner in AML (Chaplin et al., 1995), and it is one of only two *MLL* partner genes [the other being *CBP* (Borrow et al., 1996)] that are known to participate in translocations with genes other than *MLL* (Ayton and Cleary, 2001b). Subsequent investigations have confirmed the presence of *CALM-AF10* transcripts in a total of 25 patients, including 12 myeloid leukemias, 6 T-cell leukemias, 4 T-cell lymphomas, and 3 mixed-lineage leukemias (Kobayashi et al., 1997; Dreyling et al., 1998; Silliman et al., 1998; Kumon et al., 1999; Narita et al., 1999; Bohlander et al., 2000; Carlson et al., 2000; Salmon-Nguyen et al., 2000; Jones et al., 2001). In all *CALM-AF10* translocations, the breakpoints within the *CALM* gene lie in the 17th–19th introns (Silliman et al., 1998; Kumon et al., 1999; Narita et al., 1999; Bohlander et al., 2000; Carlson et al., 2000), resulting in *CALM-AF10* transcripts that include almost the entire 5′ *CALM* coding sequence, with only a small portion of 3′ *AF10* sequence. In contrast, the *CALM* breakpoint in the *MLL-CALM* translocation reported here falls in the 7th *CALM* intron, nearly 30 kb upstream. As a result, CALM contributes nearly 400 carboxy-terminal aas to MLL-CALM. The reciprocal *CALM-MLL* fusion transcript was not detectable, and genomic PCR failed to identify products indicative of a derivative *CALM-MLL* genomic translocation. However, the absence of *CALM-MLL* transcript/sequence might be attributed to a concomitant deletion of 5′ *CALM* genomic sequence; this remains to be definitively shown. Because reciprocal *AF10-CALM* transcripts are detected in only a minority of t(10;11) translocations, the conservation of carboxy-terminal CALM domains in both MLL-CALM and CALM-AF10 indicates that perturbation of functions dependent on motifs in this region likely plays a role in leukemogenesis.

As for nearly all other *MLL* translocations, the *MLL* breakpoint in the *MLL-CALM* translocation gene (in *MLL* intron 7) is within the 8.3-kb breakpoint cluster region encompassing exons 5 to 11. The *MLL-CALM* translocation results in a novel hybrid protein that contains MLL amino-terminal–derived AT-hook DNA binding and transcriptional repression motifs, but lacks MLL carboxy-terminal transcriptional activation and SET domains. CALM contributes nearly 400 aas to the carboxy terminal of MLL-CALM, containing the primary clathrin-binding domain, as well as the putative CALM transactivation domain. Nuclear localization of the hybrid MLL-CALM protein might disrupt normal MLL function by binding to DNA and misregulating the expression of downstream MLL targets that are important in hematopoietic cell development (Caslini et al., 2000). At the same time, cytoplasmic localization of the protein might perturb clathrin-dependent endocytic pathways because of the presence of CALM clathrin-binding motifs, in the absence of amino-terminal CALM regulatory domains. Thus, the retention of key MLL and CALM domains may result in novel functional

activities that potentially alter both nuclear and cytoplasmic processes.

The conservation of amino-terminal MLL transcriptional regulatory domains in all MLL fusion proteins indicates that gain of MLL function plays a critical role in leukemogenesis (Ayton and Cleary, 2001a,b). However, differences in the latency of different *MLL*-associated leukemias are likely related to disruption of partner function. MLL fusion proteins in leukemias with a short latency [e.g., *MLL-AF9* (Corral et al., 1996), *MLL-AF10* (Ayton and Cleary, 2001b), *MLL-ENL* (Lavau et al., 1997; Slany et al., 1998a,b)] are thought simultaneously to disrupt MLL-independent pathways through either loss or gain of partner protein function (DiMartino and Cleary, 1999; Ayton and Cleary, 2001b).

How might expression of the MLL-CALM fusion protein perturb clathrin-dependent endocytosis and contribute to leukemogenesis? Endocytosis of receptor-bound growth factors by clathrin-coated vesicles is one mechanism by which growth factor signaling is attenuated (reviewed in Floyd and De Camilli, 1998; Di Fiore and Gill, 1999). For example, epidermal growth factor (EGF)–dependent cell proliferation is enhanced in endocytosis-defective cells, suggesting that impaired clathrin-mediated endocytosis of EGF–EGF receptor (EGFR) complexes prolongs EGFR signaling (Vieira et al., 1996). Because MLL-CALM contains a clathrin-binding site, but lacks the amino-terminal CALM domains putatively involved in tethering to membrane inositol polyphosphates, the fusion protein might act in a dominant-negative fashion to interfere with endocytosis of proliferation-promoting hematopoietic growth factors. Disrupted clearance of receptor-bound factors could then result in persistent signaling and cell proliferation. The impaired attenuation of growth factor signaling caused by MLL-CALM protein expression, together with altered MLL function (as a result of loss of carboxy-terminal MLL domains), might have contributed to the aggressive leukemia seen in this infant.

In addition to *CALM*, other genes whose protein products participate in endocytosis have been identified as targets of chromosomal rearrangements in myeloid leukemia. The human *AF-1p* and *EEN* genes (homologs of murine *Eps15* and *SH3p8*, respectively) encode for components of clathrin-coated pits, and both genes are involved in translocations with *MLL* (Bernard et al., 1994; So et al., 1997, 2000). The *ABI-1* gene, whose protein product interacts with two other key components of clathrin-dependent vesicles (synaptojanin and dynamin), has also been described as an *MLL* translocation partner (Taki et al., 1998; So et al., 2000). In addition, other tumor-suppressor and cancer-predisposition genes are linked to endocytic pathways (Floyd and De Camilli, 1998; Di Fiore and Gill, 1999). The ataxia-telangiectasia (ATM) cancer-predisposition gene interacts with β-adaptin, a component of the AP2 clathrin adaptor (Lim et al., 1998). A member of the β-adaptin family, *BAM22* (Peyrard et al., 1994), is a candidate meningioma tumor-suppressor gene. Together with these observations, the current identification of *CALM* as an *MLL* fusion gene partner implicates clathrin-dependent vesicle pathways in the process of malignant transformation.

The extremely short latency and aggressive nature of overt leukemia in this patient suggest the likelihood that transforming events occurred in utero. Translocations involving *MLL* and *AF4* have been "backtracked" to birth (Gale et al., 1997; Rowley, 1998), and specific in utero exposures are thought to contribute to leukemogenesis (Ross, 1998, 2000; Strick et al., 2000; Alexander et al., 2001). Our demonstration that the *MLL-CALM* fusion was present in the neonatal Guthrie blood spot indicates a prenatal initiation of this patient's leukemia. Although PCR amplification is not quantitative, the reduced intensity of the *MLL-CALM* product relative to *MLL* in the Guthrie card sample, compared to that of the diagnostic leukemia specimen (Fig. 4C), suggests a relatively small number of cells harboring the translocation at birth. Whether subsequent transforming events (in addition to the *MLL-CALM* translocation) contributed to leukemogenesis remains unknown.

Finally, we have documented the human *CALM* genomic structure, and have identified a novel human *CALM* exon (17a). We have also identified an alternatively spliced *CALM* transcript that would result in a protein lacking 7 amino acids in the CALM carboxy-terminus. Previous studies of *CALM-AF10* have indicated alternative splicing of *CALM*. Specifically, *CALM* exons 13, 15, and 18 are spliced out of some *CALM* and *CALM-AF10* transcripts (Dreyling et al., 1996; Silliman et al., 1998; Kumon et al., 1999). The functional significance of *CALM* and *MLL-CALM* transcripts bearing or lacking exon 17a, or any of the other alternatively spliced transcripts, remains to be determined.

The significance of identifying an *MLL-CALM* fusion gene in infant AML is threefold. This is the first instance in which the *CALM* gene has been identified as a partner for the *MLL* gene. Although several defined *MLL* partners are transcription fac-

tors, a substantial number have other distinct functions. The fact that this patient's leukemia was rapidly progressive suggests that disruption of normal CALM function might contribute in a specific way to leukemogenesis. Second, the *CALM* gene was originally identified as a partner for the *AF10* gene in both myeloid and lymphoid leukemia. Because *AF10* was first identified as an *MLL* partner, the description of *MLL– AF10*, *AF10-CALM*, and now *MLL-CALM* translocations suggests that pathways involving all three of these genes are important in both leukemogenesis and normal hematopoiesis. Third, the specific role of the *CALM* gene product in clathrin-mediated endocytosis and vesicle transport suggests previously underappreciated mechanisms that might contribute to leukemogenesis. In particular, disruption of normal endocytosis and vesicle transport may result in aberrant signal transduction. Future studies will investigate the ability of the MLL-CALM fusion protein to transform hematopoietic progenitors, and elucidate the mechanisms by which this might occur.

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