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A Novel Transcript Encoding an N-Terminally Truncated AML1/PEBP2αB Protein Interferes with Transactivation and Blocks Granulocytic Differentiation of 32Dcl3 Myeloid Cells

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The gene *AML1/PEBP2αB* encodes the α subunit of transcription factor PEBP2/CBF and is essential for the establishment of fetal liver hematopoiesis. Rearrangements of *AML1* are frequently associated with several types of human leukemia. Three types of *AML1* cDNA isoforms have been described to date; they have been designated *AML1a*, *AML1b*, and *AML1c*. All of these isoforms encode the conserved-Runt domain, which harbors the DNA binding and heterodimerization activities. We have identified a new isoform of the *AML1* transcript, termed *AML1ΔN*, in which exon 1 is directly connected to exon 4 by alternative splicing. The *AML1ΔN* transcript was detected in various hematopoietic cell lines of lymphoid to myeloid cell origin, as revealed by RNase protection and reverse transcriptase PCR analyses. The protein product of *AML1ΔN* lacks the N-terminal region of AML1, including half of the Runt domain, and neither binds to DNA nor heterodimerizes with the β subunit. However, AML1ΔN was found to interfere with the transactivation activity of PEBP2, and the molecular region responsible for this activity was identified. Stable expression of AML1ΔN in 32Dcl3 myeloid cells blocked granulocytic differentiation in response to granulocyte colony-stimulating factor. These results suggest that AML1ΔN acts as a modulator of *AML1* function and serves as a useful tool to dissect the functional domains in the C-terminal region of AML1.

The transcription factor PEBP2 was originally identified as a polyomavirus enhancer-binding protein during efforts to identify factors involved in the early stages of mouse embryo development (21, 22). It is composed of two subunits, α and β (25). The α subunit binds to DNA, whereas the β subunit interacts with the α subunit to increase the affinity of the α subunit for DNA but shows no DNA binding by itself (23, 47, 49, 67). Three mammalian genes, *PEBP2αA* (49), *PEBP2αB* (3, 6), and *PEBP2αC* (4, 30, 69), are known to encode the α subunit, whereas only one gene, *PEBP2β*, is known to encode the β subunit (47, 67). The three species of α subunit share an evolutionarily conserved region of 128 amino acids, termed the Runt domain (24), with the product of the *Drosophila* segmentation gene, *runt* (26). Recently, another *Drosophila* gene, *lozenge*, which is essential for *Drosophila* eye development, was found to encode a protein with a Runt domain (11). The Runt domain-containing proteins from flies to humans also share a 5-amino-acid motif of unknown function, VWRPY, at their C-terminal end (21, 22). A core binding factor (CBF) which interacts with the core motif of murine retrovirus enhancers (67) is identical to PEBP2.

The *AML1* gene on human chromosome 21 was identified originally at the breakpoint of the 8 to 21 chromosome translocation, *t*(8;21), associated with acute myeloid leukemia (39). Subsequently, *AML1* was found to encode the α subunit of PEBP2 and correspond to *PEBP2αB* among three α-subunit-encoding genes (3, 6). Since then, *AML1* has been found to be involved in many different types of chromosome translocations including *t*(3;21), *t*(5;21), *t*(17;21), and *t*(12;21), and consequently, *AML1* is now recognized as one of the most frequent targets of the chromosome translocations associated with human leukemia (46). Furthermore, the breakpoint of another chromosome anomaly, inversion on chromosome 16, related to acute myeloid leukemia, occurs within the gene encoding the β subunit of PEBP2 (31). All these translocations result in chimeric proteins, at least some of which have been suggested to have leukemogenic potential (53, 60). In the case of the structurally altered α subunit, the Runt domain is always retained, and in most cases, it supplies the only DNA binding domain present in the chimeric proteins. Likewise, the structurally altered β subunit retains the region required for dimerization with the α subunit. Therefore, all these chimeric proteins could affect transcriptional regulation through the PEBP2 site (for reviews, see references 21 and 22). This notion implies that the chimeric proteins deregulate the expression of the target genes of normal PEBP2. This is why it is important to study how PEBP2 functions as a transcription factor and to identify the target genes of PEBP2. Such studies should lead to a better understanding of how these chromosome translocations lead to leukemogenesis.

Disruption of either *AML1/PEBP2αB* or *PEBP2β* in the mouse leads to exactly the same phenotype: hematopoiesis of all lineages in the fetal liver is impaired (44, 50, 51, 55, 65, 66). These and other results suggest that the two subunits actually function together and that they play pivotal roles in establishing hematopoietic stem cells, as well as inducing stem cell differentiation into some specific lineages including granulocytes and lymphocytes.

PEBP2 presents a number of unique features as a transcription factor. First, the Runt domain, which contains the DNA-binding activity, displays no homology to known DNA-binding motifs, suggesting the presence of a novel structure for interaction with DNA (24). Second, the two subunits display different subcellular locations when not expressed together. Although the α subunit is always a nuclear protein, the β subunit is found in the cytoplasm when not associated with the α subunit (33). One of the rate-limiting steps of transcriptional

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regulation by PEBP2 appears to be at the level of heterodimerization. Third, PEBP2 appears to function in conjunction with several other transcription factors, such as Ets-1 (15, 58, 70), Myb (18), and C/EBP (72). AML1 has been shown to physically interact and functionally cooperate with Ets-1 for transcription (15), probably because it stabilizes Ets-1 binding to its cognate site (15, 70). For the moment, however, it is not clear which of these proteins actually contributes to the stimulation of transcription in this system. Using the minimal T-cell receptor β enhancer, we and others have shown that the region of AML1 downstream of the Runt domain (referred to hereafter as the C-terminal region) is required for transcription stimulation (3, 36, 48, 61). However, it is not known if this region is required for the stabilization of Ets-1 binding to DNA, for direct or indirect interactions with the basal transcription machinery, or for modulation of the transactivation domain of Ets-1. It will be critical to answer these questions if we are to fully understand how PEBP2 functions. In this paper, we present an approach which goes part of the way toward answering some of these questions.

Our method relies on the observation that multiple transcripts of *AML1* are found in hematopoietic cells. While only two major *AML1* transcripts are found in NIH 3T3 cells (3), far more RNA species are detected in hematopoietic cells (reference 40 and references therein). Miyoshi et al. characterized these transcripts in a B-cell line and found three poly(A) addition sites at the 3′ region, generating three size class RNAs from one transcript (40). They identified nine *AML1* exons that spanned a region of more than 150 kb of genomic DNA. One class of the RNA species is initiated on exon 1, while the other class is initiated on exon 3, where it is presumably driven by a different promoter. They categorized the *AML1* transcripts into three basic types, termed *AML1a*, *AML1b*, and *AML1c*. The first two are initiated on exon 3, but *AML1a* is terminated prematurely. *AML1b* encodes a full-length AML1 (one that contains the Runt domain and the C-terminal 5 amino acids), whereas *AML1a* is truncated in the C-terminal region. *AML1c* is initiated on exon 1 and encodes essentially the same protein as *AML1b* except that the N-terminal 5-amino-acid sequence of *AML1b* is replaced by a different 33-amino-acid sequence in the product of *AML1c*. AML1c corresponds to AML1B described by Meyer et al. (36). In addition, three microsplice variants are synthesized from exon 1 (40).

Here, we describe a novel *AML1* transcript encoding an N-terminally truncated form of AML1 that lacks part of the Runt domain. The product of the new transcript interferes with the normal function of AML1 and should be useful for the future dissection of the functional domains of AML1.

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CATAAACAACCACAGAACCACAAGTTGGGTAGCCTGGCAGTGTCAGAAGTCTGAACCCAG    60
CATAGTGGTCAGCAGGCAGGACGAATCACACTGAATGCAAACCACAGGGTTTCGCAGCGT   120
GGTAAAAGAAATCATTGAGTCCCCCGCCTTCAGAAGAGGGTGCATTTTCAGGAGGAAGCG   180
ATGGCTTCAGACAGCATATTTGAGTCATTTCCTTCGTACCCACAGTGCTTCATGAGAGGT   240
GGTGGCCCTAGGGGATGTTCCAGATGGCACTCTGGTCACTGTGATGGCTGGCAATGATGA   300
                                          M  A  G  N  D  E      6
AAACTACTCGGCTGAGCTGAGAAATGCTACCGCAGCCATGAAGAACCAGGTTGCAAGATT   360
 N  Y  S  A  E  L  R  N  A  T  A  A  M  K  N  Q  V  A  R  F    26
TAATGACCTCAGGTTTGTCGGTCGAAGTGGAAGAGGGAAAAGCTTCACTCTGACCATCAC   420
 N  D  L  R  F  V  G  R  S  G  R  G  K  S  F  T  L  T  I  T    46
TGTCTTCACAAACCCACCGCAAGTCGCCACCTACCACAGAGCCATCAAAATCACAGTGGA   480
 V  F  T  N  P  P  Q  V  A  T  Y  H  R  A  I  K  I  T  V  D    66
TGGGCCCCGAGAACCTCGAAGACATCGGCAGAAACTAGATGATCAGACCAAGCCCGGGAG   540
 G  P  R  E  P  R  R  H  R  Q  K  L  D  D  Q  T  K  P  G  S    86
CTTGTCCTTTTCCGAGCGGCTCAGTGAACTGGAGCAGCTGCGGCGCACAGCCATGAGGGT   600
 L  S  F  S  E  R  L  S  E  L  E  Q  L  R  R  T  A  M  R  V   106
CAGCCCACACCACCCAGCCCCCACGCCCAACCCTCGTGCCTCCCTGAACCACTCCACTGC   660
 S  P  H  H  P  A  P  T  P  N  P  R  A  S  L  N  H  S  T  A   126
CTTTAACCCTCAGCCTCAGAGTCAGATGCAGGATACAAGGCAGATCCAACCATCCCCACC   720
 F  N  P  Q  P  Q  S  Q  M  Q  D  T  R  Q  I  Q  P  S  P  P   146
GTGGTCCTACGATCAGTCCTACCAATACCTGGGATCCATTGCCTCTCCTTCTGTGCACCC   780
 W  S  Y  D  Q  S  Y  Q  Y  L  G  S  I  A  S  P  S  V  H  P   166
AGCAACGCCCATTTCACCTGGACGTGCCAGCGGCATGACAACCCTCTCTGCAGAACTTTC   840
 A  T  P  I  S  P  G  R  A  S  G  M  T  T  L  S  A  E  L  S   186
CAGTCGACTCTCAACGGCACCCGACCTGACAGCGTTCAGCGACCCGCGCCAGTTCCCCGC   900
 S  R  L  S  T  A  P  D  L  T  A  F  S  D  P  R  Q  F  P  A   206
GCTGCCCTCCATCTCCGACCCCCGCATGCACTATCCAGGCGCCTTCACCTACTCCCCGAC   960
 L  P  S  I  S  D  P  R  M  H  Y  P  G  A  F  T  Y  S  P  T   226
GCCGGTCACCTCGGGCATCGGCATCGGCATGTCGGCCATGGGCTCGGCCACGCGCTACCA  1020
 P  V  T  S  G  I  G  I  G  M  S  A  M  G  S  A  T  R  Y  H   246
CACCTACCTGCCGCCGCCCTACCCCGGCTCGTCGCAAGCGCAGGGAGGCCCGTTCCAAGC  1080
 T  Y  L  P  P  Y  P  G  S  S  Q  A  Q  G  G  P  F  Q  A   266
CAGCTCGCCCTCCTACCACCTGTACTACGGCGCCTCGGCCGGCTCCTACCAGTTCTCCAT  1140
 S  S  P  S  Y  H  L  Y  Y  G  A  S  A  G  S  Y  Q  F  S  M   286
GGTGGGCGGCGAGCGCTCGCCGCCGCGCATCCTGCCGCCCTGCACCAACGCCTCCACCGG  1200
 V  G  G  E  R  S  P  P  R  I  L  P  P  C  T  N  A  S  T  G   306
CTCCGCGCTGCTCAACCCCAGCCTCCCGAACCAGAGCGACGTGGTGGAGGCCGAGGGCAG  1260
 S  A  L  L  N  P  S  L  P  N  Q  S  D  V  V  E  A  E  G  S   326
CCACAGCAACTCCCCCACCAACATGGCGCCCTCCGCGCGCCTGGAGGAGGCCGTGTGGAG  1320
 H  S  N  S  P  T  N  M  A  P  S  A  R  L  E  E  A  V  W  R   346
GCCCTACTGAGGCGCCAGGCCTGGCCCGGCTGGGCCACGCGGGCCGCCGCCTTCGCCTCC  1380
 P  Y  *                                                      348
GGGCGCGCGGGCCTCCTGTTCGCGACAAGCCCGCCCGGGATCCCGGGCCCTGGGCCCGGCC  1440
ACCGTCCTGGGGCCGAGGGCGCCCGACGGCCAGGATCTCGCTGTAGGTCAGGCCCGCGCA  1500
GCCTCCTGCGCCCAGAAGCCCACGCCGCCGCCGTCTGCTGGCGCCCCGGCCCTCGCGGAG  1560
GTGTCCGAGGCGACGCACCTCGAGGGTGTCCGCCGGCCCCAGCACCCAGGGGACGCGCTG  1620
GAAAGCAAACAGGAAGATTCCCGGAGGGAAACTGTGAATGCTTCTGATTTAGCAATGCTG  1680
TGAATAAAAAGAAAGATTTTATACCCTTGAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA  1740
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FIG. 1. Nucleotide sequence and predicted amino acid sequence of the *AML1ΔN* cDNA. The deduced amino acid sequence of the open reading frame is indicated in single-letter code under the DNA sequence. The partial Runt domain is underlined. The asterisk represents the termination codon. The arrowhead indicates the boundary of exons 1 and 4. The putative polyadenylation signal sequence (AATAAA) is indicated in boldface type.

MATERIALS AND METHODS

cDNA library screening. A cDNA library was prepared from cytoplasmic polyadenylated RNA of the human adult T-cell line KUT-2 by using the plasmid vector PSI4001 (49). The library was screened by the colony hybridization method under low-stringency conditions (54) with a probe containing the 1,348-bp *Sma*I fragment (nucleotides 314 to 1662) derived from mouse *PEBP2αA1* cDNA. The probe was labeled with [α-^{32}P]dCTP by using the Rediprime system (Amersham). Hybridization was performed at 42°C for 24 h with a mixture containing 100 mg of dextran sulfate per ml, 40% deionized formamide, 7 mM Tris-HCl (pH 7.4), 1× Denhardt's solution, 4× SSC buffer (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 100 μg of salmon sperm DNA per ml, and the labeled probe. Washing was performed twice with a solution containing 2× SSC and 0.1% sodium dodecyl sulfate (SDS) and then twice for 10 min at 42°C with a solution containing 0.5× SSC and 0.1% SDS. From 2×10^6 colonies, 7 positive clones were isolated.

DNA sequencing. A full-length cDNA insert of each positive clone was excised by digestion with *Eco*RI and *Mlu*I and subcloned into the *Eco*RV site of pBluescript II (KS-) (Stratagene). Deletions of each clone were generated with the exonuclease III/mung bean kit (Stratagene) and used for sequencing. DNA sequencing was performed by the dideoxy-chain termination method (Sequenase kit; U.S. Biochemicals) with double-stranded DNA.

Plasmid construction. The *Eco*RI-*Xho*I fragments from pSI4001-AML1b and pSI4001-AML1ΔN were subcloned into the compatible sites of the pBluescript II (KS-) vector, resulting in pKS-AML1b and pKS-AML1ΔN, respectively. pSK-PEBP2β2 was described previously (47). The bacterial expression plasmid pGEX2T-Ets-1 was constructed by inserting the *Bam*HI-*Bam*HI fragment from pGEX-Ets-HA (15) into the *Bam*HI site of pGEX2T (Pharmacia). For mammalian expression vectors, the blunt-ended *Ppu*MI-*Xho*I fragment from pKS-AML1b and the blunt-ended *Eco*RI-*Xho*I fragment from pKS-AML1ΔN were inserted into the blunt-ended *Bst*XI site of the pEF-Bos vector (41) in which the simian virus 40 ori sequence was removed, resulting in pEF-AML1b(1–453) and pEF-AML1ΔN(106–453), respectively. To construct N-terminal deletion mutants of AML1ΔN, the blunt-ended *Sma*I-*Sma*I, *Bam*HI-*Bam*HI, and *Bst*EII-*Bst*XI fragments derived from pKS-AML1ΔN were subcloned into the blunt-ended *Bst*XI site of pEF-Bos vector to make pEF-AML1ΔN(209–453), pEF-AML1ΔN(283–453), and pEF-AML1ΔN(341–453), respectively. For construction of C-terminal deletion mutants of AML1ΔN, pEF-AML1ΔN(106–448) was generated by inserting a double-stranded synthetic oligonucleotide containing the sequences 5′-ATGGCGCCCTCCGCGCGCCTGGAGGAGGCCTGAC GTC-3′ and 5′-TCAGGCCTCCTCCAGGCGCGCGGAGGGCGCCATGTTG -3′ into the compatible *Bst*XI site of pEF-AML1ΔN(106–453) after removal of the *Bst*XI fragment. pEF-AML1ΔN(106–340) was constructed by inserting a double-stranded oligonucleotide containing the sequences 5′-GTCACCTCGG GCATCGGCATCGGCTGA-3′ and 5′-CGCGTCAGCCGATGCCGATGCCC GAG-3′ into the compatible site of pEF-AML1ΔN(106–453) after removal of the *Bst*EII-*Bss*HII fragment. To construct pEF-AML1ΔN(106–208), a DNA fragment was generated by PCR with the primers 5′-TAGGGGATGTTCCAG

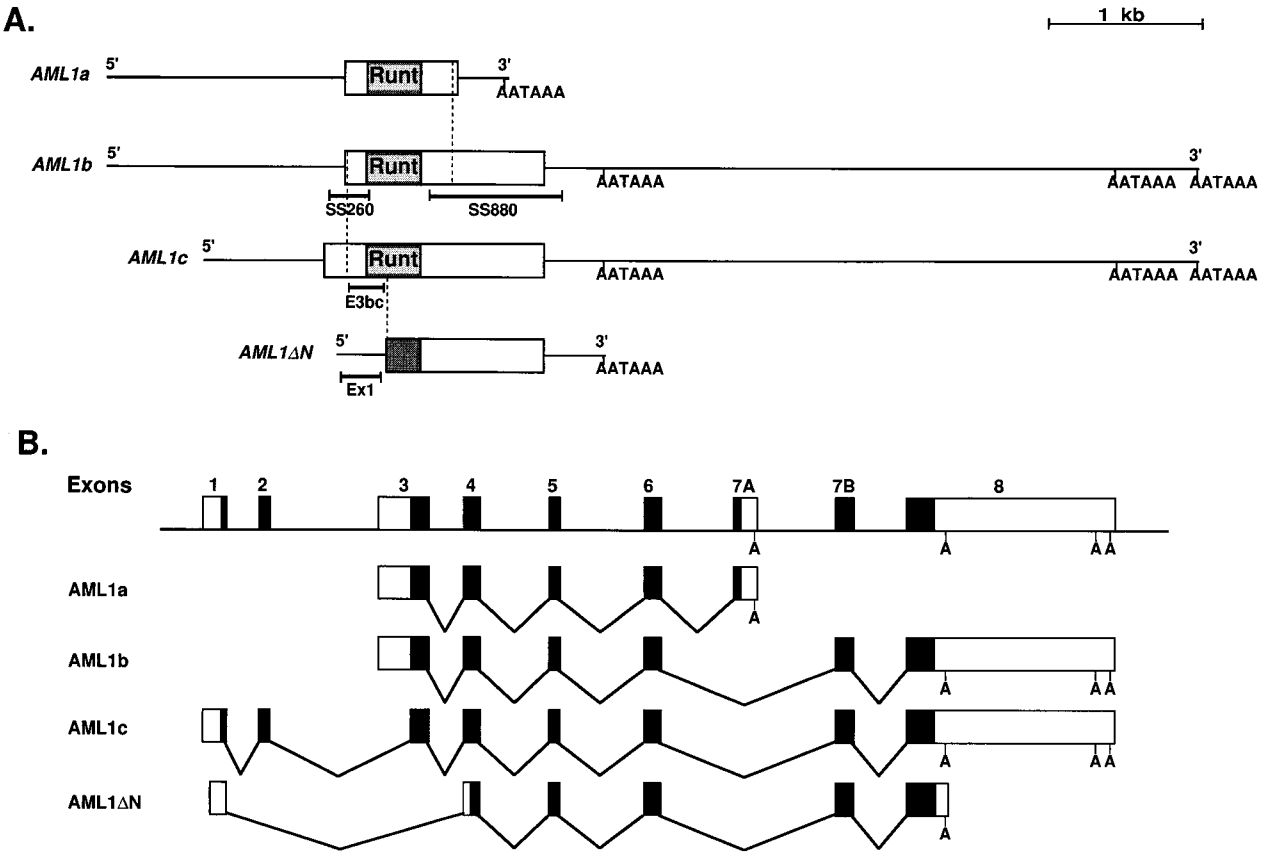

FIG. 2. (A) Structure of the *AML1ΔN* transcript compared to the known isoforms of *AML1* transcripts, *AML1a*, *AML1b*, and *AML1c*, described by Miyoshi et al. (40). The coding region is represented by boxes. The gray boxes indicate the Runt domain. Positions of polyadenylation signals are shown by AATAAA. The regions of the cDNA used to make probes SS880, SS260, E3bc, and Ex1 for Northern blot analysis are shown. (B) Alternative splicing pattern of the *AML1* transcripts. Exons are shown at the top. Solid boxes represent the coding regions, and open boxes represent the 5′ or 3′ untranslated regions. Positions of polyadenylation signals are shown by A. (C) The alternative splicing within exon 1. The *AML1ΔN* transcript corresponds to the type 3 form.

ATGGCACTC-3′ and 5′-CCTAGGCTGTGCGCCGCAGCT-3′ and pEF-AML1ΔN(106–453) as a template. The PCR fragment was then blunt-end ligated into the *Bst*XI site of the pEF-Bos vector. The sequences of these constructs were confirmed by the dideoxy-chain termination method. For the chloramphenicol acetyltransferase (CAT) assay, Tβ3W4W-tkCAT and Tβ3M4M-tkCAT, which have been described previously, were used as reporters (49). For the luciferase assays, the TCRβ-luc plasmid was constructed by insertion of the Tβ3-Tβ4 fragment derived from Tβ3W4W-tkCAT into the *Bgl*II site of the tk-luc reporter plasmid. The retrovirus construct pBabeneo-AML1ΔN was generated by inserting the blunt-ended *Eco*RI-*Xho*I fragment of pKS-AML1ΔN into the *Sna*BI site of the pBabeneo retroviral vector (42).

Cells and culture media. Cells from a variety of cell lines were grown in RPMI 1640 medium (GIBCO) supplemented with 10% fetal bovine serum. These included a Jurkat T-cell leukemia line (17, 63); KUT-2, an adult T-cell leukemia cell line (16); HBP-ALL, a T-lymphoblastic cell line (43); CCRF-HSB-2, a cell line derived from human acute lymphoblastic leukemia (1); Molt-4, a human acute T-lymphoblastic leukemia cell line (38); HL-60, a human myeloid leukemia cell line (10); K-562, a human chronic myelogenous leukemia cell line (32); Kasumi-1, a human acute myeloid leukemia cell line containing *t*(8;21) (2); ME-1, a human acute myelomonocytic leukemia containing *inv* (chromosome 16 [71]); P3HR-1, the Burkitt's lymphoma cell line (human B-cell origin) (20); and M12, a mouse B-lymphoblastoid cell line (28). A murine interleukin-3 (IL-3)-dependent myeloid cell line derived from normal bone marrow long-term culture, 32Dcl3 (64), was maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and 5% WEHI-3B (68) supernatant as a source of interleukin-3 (IL-3). P19 mouse embryonal carcinoma cells (35) and a murine fibroblast cell line for packaging recombinant murine retrovirus, ψ2 cells (34), were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum.

Northern blot analysis of *AML1* mRNA. Total RNA was isolated with TRIzol reagent (GIBCO BRL). Poly(A)$^+$ RNA was prepared with an oligo(dT) column mRNA purification kit (Pharmacia Biotech). Total RNA (20 μg) or poly(A)$^+$ RNA (2 μg) was separated on a 1% agarose–formaldehyde gel and transferred to a nylon membrane (Hybond N; Amersham). The probes were labeled with [α-^{32}P]dCTP by using the Rediprime system. Northern blot analysis was performed as specified by the manufacturer. The probes were generated as follows. The *Sma*I-*Sma*I 887-bp (probe SS880) (nucleotides [nt] 2146 to 3033) and the *Sma*I-*Sma*I 260-bp (probe SS260) (nt 1495 to 1754) fragments were from *AML1b* cDNA. The exon 3 region common to *AML1b* and *AML1c* cDNA (nt 1637 to 1846 of *AML1b*) (probe E3bc) or the exon 1 region (nt 9 to 235 of *AML1ΔN*) (probe Ex1) were prepared by PCR amplification.

RNase protection assay. The *Eco*RI-*Hin*dIII fragment from pSI4001-AML1ΔN was subcloned into pBluescript (SK-) and was used to prepare an RNA probe. An RNase protection assay was performed with a Riboprobe kit (Promega) by using 45 μg of total RNA per lane.

RT-PCR. The reverse transcriptase PCR (RT-PCR) analyses were performed with a Gene Amp RNA PCR core kit (Perkin-Elmer). First-strand cDNA was synthesized from 1 μg of total RNA prepared from P3HR-1 and ME-1 cells primed with random hexamer in 20 μl of solution. Then PCR amplification was

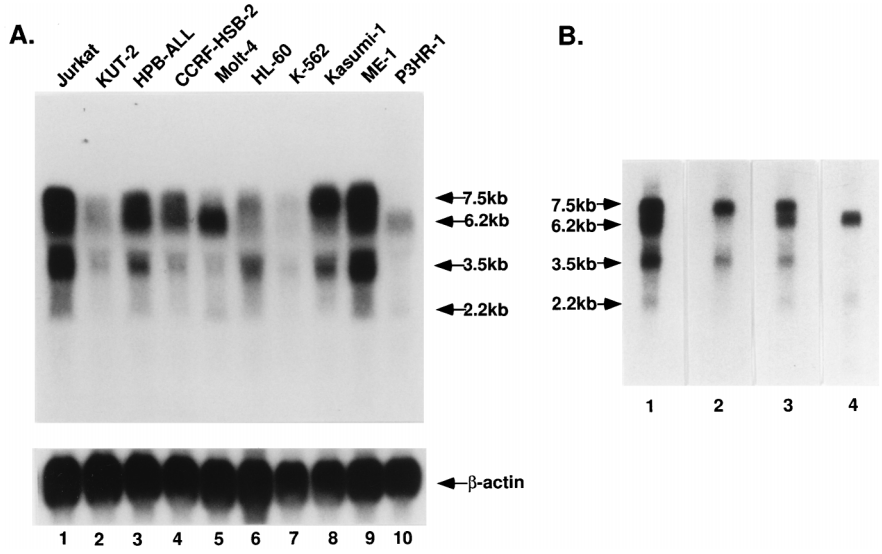

FIG. 3. Northern blot analysis of the AML1 transcripts in hematopoietic cell lines. (A) A 20-μg portion of total RNA from each of Jurkat (T cell), KUT-2 (T cell), HPB-ALL (T cell), CCRF-HSB-2 (T cell), Molt-4 (T cell), HL-60 (myeloid cell), K-562 (myeloid cell), Kasumi-1 (myeloid cell), ME-1 (myeloid cell), and P3HR-1 (B cell) was subjected to Northern blot analysis with probe SS880. The locations of four major RNA species (7.5, 6.2, 3.5, and 2.2 kb) are marked on the right. As an internal control, the expression of β-actin mRNA is also shown. (B) A 2-μg portion of poly(A)$^+$ RNA from Jurkat cells was hybridized with various probes shown in Fig. 2A. Lanes: 1, SS880; 2, SS260; 3, E3bc; 4, Ex1.

carried out for 30 cycles (denaturation at 95°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min) in 50 μl of solution containing 5 μl of first-strand cDNA. The primers used were as follows: primer A, 5′-GAAGTCT GAACCCAGCATAGTGGTCAGCAG-3′ (nt 46 to 75 of AML1ΔN); primer B, 5′-CACAGTGACCAGAGTGCCATCTGGAACATC-3′ (nt 254 to 283 antisense of AML1ΔN); primer C, 5′-GTGGACGTCTCTAGAAGGATTCATTCC AAG-3′ (nt 511 to 540 antisense of AML1c [40]); primer D, 5′-CGTGCACAT ACATTAGTAGCACTACCTTTG-3′ (nt 997 to 1026 of AML1b [40]); and primer E, 5′-CTTCCACGAATCTTGCTTGCAGAGGTTAAG-3′ (nt 1271 to 1300 antisense of AML1b).

In vitro transcription and translation. The [^{35}S]methionine-labeled, in vitro-translated proteins were prepared with the TNT system (Promega) by with pKS-AML1b, pKS-AML1ΔN, or pSK-PEBP2β2 as the template. Translated products were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) (12.5% polyacrylamide). After electrophoresis, the gel was fixed with 10% acetic acid–20% methanol, and an autoradiogram was made. For Western blot analysis and electrophoretic mobility shift assays (EMSA), nonradioactive in vitro-translated products were prepared.

Preparation of whole-cell extracts and Western blot analysis. P19 cells were transfected with expression plasmids by a modified version of the calcium phosphate-DNA precipitation method described by Chen and Okayama (8). Whole-cell extracts were prepared 40 h after transfection by a freeze-thaw method as described previously (52). The extracts were separated by SDS-PAGE (12.5% polyacrylamide), and Western blot analysis was performed as described previously (33), except that the ECL Western blot analysis system (Amersham) was used to visualize proteins.

EMSA. Synthetic oligonucleotides containing a PEBP2-binding site from ΔF9-5000 (25) were used as a wild-type probe by annealing 5′-GATCAACTGACC GCAGCTGGCCGTGCGA-3′ and 5′-GATCTCGCACGGCCAGCTGCGGTC AGTT-3′. The mutant probe was generated by annealing oligonucleotides 5′-G ATCAACTG**CTT**GCAGCTGGCCGTGCGA-3′ and 5′-GATCTCGCACGGC CAGCTGC**AAG**CAGTT-3′ (the mutation is shown by boldface type). EMSA was performed as described previously (56). Briefly, the binding assay was performed at room temperature for 30 min in a reaction mix (20 μl) containing 20 mM HEPES (pH 7.6), 4% (wt/vol) Ficoll, 2 mM EDTA, 2 μg of poly(dI-dC), 50 mM KCl, 1 mM dithiothreitol, and a ^{32}P-end-labeled probe (10^4 cpm). The competition assay was performed in the presence of a 50-fold molar excess of the unlabeled probe. The reaction mixtures were analyzed by electrophoresis on 5% nondenaturing polyacrylamide (60:1 acrylamide-bisacrylamide) gels.

IP. Immunoprecipitation (IP) was performed as described previously (54). Anti-αA1C17 and anti-PEBP2β2 are previously described rabbit polyclonal antibodies (33). Reaction mixtures containing ^{35}S-labeled in vitro-translated AML1b and PEBP2β2 or AML1ΔN and PEBP2β2 were incubated at 4°C for 1 h in a IP buffer containing 50 mM Tris-HCl (pH 7.4), 0.9% (wt/vol) NaCl, 0.1% (wt/vol) bovine serum albumin, 0.1% (vol/vol) Nonidet P-40, and 0.02% (wt/vol) sodium azide. The reaction products were then allowed to react with the anti-αA1C17 or anti-β2 antibody for 90 min at 4°C. Immune complexes were collected on protein A-Sepharose beads and washed three times with the IP buffer. Immune complexes were resuspended in 15 μl of 2× SDS buffer (100 mM Tris-HCl [pH 6.8], 200 mM dithiothreitol, 4% SDS, 20% glycerol, 0.2% bromophenol blue) and boiled for 5 min before being subjected to SDS-PAGE (12.5% polyacrylamide). The gels were fixed, and fluorography was performed.

Purification of recombinant proteins and in vitro binding assays. PEBP2β2 protein expressed in *Escherichia coli* was purified by a method described previously (47). For the glutathione *S*-transferase (GST)–Ets-1 protein, *E. coli* BL21 (DE3) cells containing the expression plasmids for GST fusion proteins were cultured in 500 ml of Luria-Bertani broth at 37°C for 3 h after 1:50 dilution of the overnight culture. Synthesis of the GST fusion protein was induced by culturing the cells at 37°C for 3 h in the presence of 2 mM isopropyl-β-D-thiogalactopyranoside, which was added to the culture cells at an optical density at 600 nm of 0.6. GST fusion proteins were prepared as described previously (57). A 2-μg portion of GST or GST–Ets-1 immobilized on glutathione-agarose beads was incubated with in vitro-translated ^{35}S-labeled AML1b or AML1ΔN for 2 h in 1 ml of IP buffer at 4°C with gentle rotation. The protein-GST beads were washed three times with the same buffer at 4°C and subjected to SDS-PAGE on a 12.5% gel.

Transient transfection and CAT and luciferase assays. For CAT assays, P19 cells were transfected by the method of Chen and Okayama (8). As an internal control, 1 μg of pRSVβ-gal plasmid was cotransfected. CAT and β-galactosidase activities were determined as described previously (54) with cell extracts prepared 48 h after transfection. CAT activities were normalized to the β-galactosidase activity of each extract. For luciferase assays, a luciferase reporter plasmid and an effector plasmid were cotransfected into Jurkat T cells or M12 B cells under electroporation conditions of 250 V and 960 μF (Gene Pulser; Bio-Rad). After 24 to 36 h of transfection, the cells were harvested and the luciferase activities were analyzed with a luciferase assay system (PicaGene; Toyo Inc.) and a luminometer (Bio-Orbit). The luciferase activities were normalized according to the protein content of each extract, which was determined by the Bradford method (Bio-Rad protein assay kit). The total amount of transfected plasmid DNAs was kept constant at 20 μg by adding the pEF-BOS backbone vector (41). All transfection experiments were performed at least three times.

Infection of recombinant retrovirus and establishment of 32Dcl3 stable transfectants. The retroviral construct containing the AML1ΔN cDNA was transfected into ψ2 cells by the calcium phosphate method (8) followed by selection in 1 mg of G418 per ml as described previously (12). 32Dcl3 cells, which had been recloned by us, were cocultivated for 48 h on virus-producing ψ2 monolayer cells with 8 μg of Polybrene per ml. After selection with G418 (800 μg/ml) for 1 week, 32Dcl3 subclones were isolated by limiting dilutions in 96-well plates and screened for expression of AML1ΔN by Western blot analysis. For the induction of granulocytic differentiation, 32Dcl3 cells were washed twice with phosphate-buffered saline and placed in RPMI 1640 medium supplemented with 10% fetal bovine serum and 500 U of recombinant human granulocyte colony-stimulating factor (rhG-CSF) (Kirin Beer, Japan) per ml.

Nucleotide sequence accession number. The nucleotide sequence of *AML1ΔN* has been deposited in the DNA Data Bank of Japan (DDBJ). The accession number is D89788.

RESULTS

Isolation of *AML1ΔN* cDNA, a new isoform of *AML1* cDNA. In an attempt to isolate human cDNAs encoding the α subunit, we screened a cDNA library prepared from human T cells, using a probe containing the Runt domain-encoding region of the mouse *PEBP2αA1* cDNA. Seven positive clones isolated represented one of the following three genes: human *PEBP2αA* (73), human *PEBP2αC*, and *AML1*. Of the five *AML1* cDNAs identified, one was found to have a distinct structure different from that of any previously reported isoforms of *AML1* cDNAs, i.e., *AML1a*, *AML1b*, or *AML1c*, all of which encode the entire Runt domain (40). Here, we designate the new isoform *AML1ΔN*.

Both exon 1 and exon 3 of *AML1* contain a 5′ untranslated region and an ATG initiation codon (40). The *AML1c* transcript begins with exon 1, which is joined to exon 2. Exon 2 is joined to exon 3 through an internal splice acceptor site in exon 3. On the other hand, *AML1a* and *AML1b* begin with exon 3, which implies the presence of an intronic promoter in intron 2. The Runt domain is encoded by a part of exon 3, exon 4, and exon 5. Sequence analysis of *AML1ΔN* cDNA (Fig. 1) revealed that *AML1ΔN* has exon 1 directly connected to exon 4 by alternative splicing (Fig. 2A and B). The sequence of the *AML1ΔN* cDNA within exon 1 shows a 208-bp deletion (nucleotides 178 to 385 of *AML1c*) which corresponds to the type 3 transcript described by Miyoshi et al. (40) (Fig. 2C). The 3′-untranslated region of *AML1ΔN* cDNA has a polyadenylation signal (AATAAA) followed immediately by a poly(A) tail. Other forms of *AML1ΔN* transcripts with a longer 3′ untranslated region may also exist.

Expression of *AML1ΔN* in various hematopoietic cell lines. To examine the expression of the *AML1ΔN* transcript, Northern blot, RNase protection, and RT-PCR analyses were performed. The probes used for Northern blot analysis are shown in Fig. 2A. To characterize the alternatively spliced transcripts of *AML1*, probe SS880, which contains a region common to *AML1b*, *AML1c*, and *AML1ΔN*, was hybridized to total RNAs isolated from several hematopoietic cell lines. As shown in Fig. 3A, four major transcripts of 7.5, 6.2, 3.5, and 2.2 kb were detected in most cell lines, except for Molt-4 and P3HR-1, in which the 7.5-kb species was not detected. To further analyze these four species, various probes were hybridized with poly(A)$^+$ RNA from Jurkat T cells in which the four major species of *AML1* transcripts were typically expressed at relatively high levels. As shown in Fig. 3B, probe SS260 detected both the 7.5- and 3.5-kb species clearly (lane 2). Probe SS260 is derived from *AML1b* cDNA and shares a partial overlap with *AML1c* but shows no sequence similarity to *AML1ΔN*. On the other hand, probe E3bc, containing sequences in common with both *AML1b* and *AML1c* but none in common with *AML1ΔN*, efficiently detected all four major transcripts (lane 3). When probe Ex1 was used, only the 6.2- and 2.2-kb species were detected (lane 4). Probe Ex1 is derived from exon 1 and is therefore specific for both *AML1c* and *AML1ΔN*. These results suggest that the 7.5- and 3.5-kb species belong to *AML1b* and that the 6.2- and 2.2-kb species belong to *AML1c*. Probe SS260 was also able to detect, albeit weakly, the 6.2- and 2.2-kb bands, as a result of the partial sequence overlap with *AML1c* DNA.

Since *AML1c* and *AML1ΔN* could not be distinguished by Northern blot analysis, we sought to examine the levels of *AML1ΔN* expression in more detail by the RNase protection assay. As shown in Fig. 4A, a 405-nt band specific for *AML1ΔN* was detected in all hematopoietic cell lines examined from lymphoid to myeloid origin. A 167-nt band was specific for

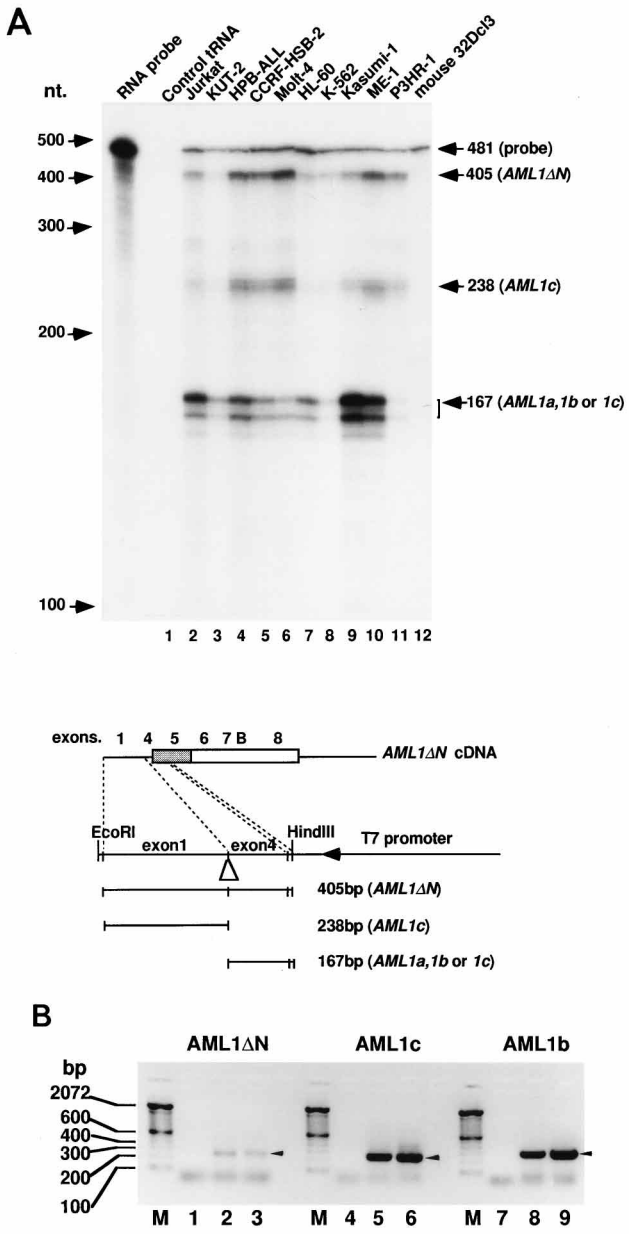

FIG. 4. Detection of the AML1ΔN transcript in several hematopoietic cell lines. (A) RNase protection assay. A 481-nt riboprobe was used, and the expected sizes of the protected bands are shown at the bottom (see also Fig. 2). The RNase protection reaction included 45 μg of control yeast tRNA and total RNA prepared from Jurkat, KUT-2, HPB-ALL, CCRF-HSB-2, Molt-4, HL-60, K-562, Kasumi-1, ME-1, or P3HR-1 cells. As a negative control, 45 μg of total RNA isolated from mouse 32Dcl3 cells was also included. One-twentieth of the full-length RNA probe used per assay shown in lanes 1 through 12 was loaded on the left. The positions of the protected bands of the corresponding transcripts are indicated on the right. The position of the undigested probe is also marked. Samples were analyzed by electrophoresis on a 6% polyacrylamide denaturing gel. The positions of the ^{32}P-end-labeled DNA fragments that were used as size markers are indicated on the left. (B) RT-PCR analysis. A 10-μl volume of each RT-PCR product amplified from P3HR-1 cells (lanes 2, 5, and 8) and ME-1 cells (lanes 3, 6, and 9) was separated by electrophoresis on 2% agarose gel and stained with ethidium bromide. Primers A and B were used to preferentially amplify 238-bp product of the AML1ΔN transcript. Negative controls without template are shown in lanes 1, 4, and 7. Primers A and C were used to preferentially amplify the 231-bp product of the AML1c transcript. Primers D and E were used to amplify the 304-bp product of the AML1b transcript. PCR products are indicated by arrowheads. M, DNA size markers.

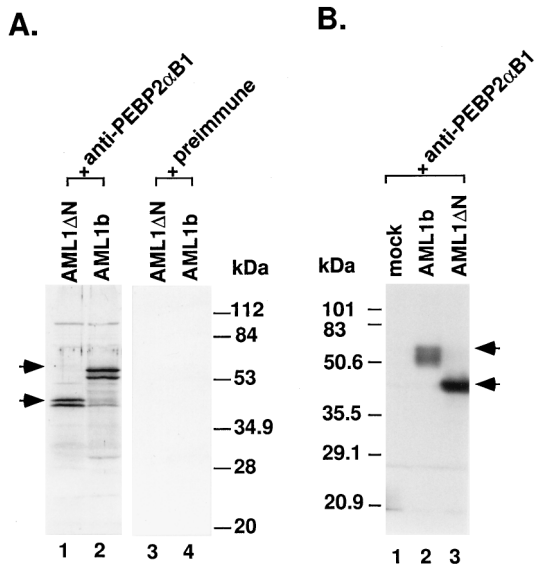

FIG. 5. Western blot analysis of AML1ΔN protein translated in vitro and in vivo. (A) Aliquots (5 μl) of in vitro-translated AML1ΔN or AML1b were seperated by SDS-PAGE (12.5% polyacrylamide). Western blot analysis was performed with anti-PEBP2αB1 antiserum or preimmune serum. (B) P19 cells were transfected with expression plasmids for AML1ΔN, AML1b, or an empty vector (lane mock). Whole-cell extract (20 μg) was analyzed by Western blotting with anti-PEBP2αB1 antiserum.

AML1a, *AML1b*, and *AML1c*, and a 238-nt band was specific for *AML1c*. Although a small fraction of the 481-nt free probe remained undigested, the results in the lanes with tRNA (Fig. 4A, lane 1) and mouse 32Dcl3 cell RNA (lane 12) suggest that the protected bands with human cell RNAs are authentic. We also detected *AML1ΔN* transcript as well as those of *AML1c* and *AML1b* by RT-PCR with corresponding primer sets in P3HR-1 and ME-1 (Fig. 4B). These data indicate that *AML1ΔN* is genuinely expressed in hematopoietic cells and that it represents a relatively small fraction of the total *AML1* transcripts. We also detected the *AML1ΔN* transcript in RNA from normal human thymus (data not shown).

***AML1ΔN* cDNA encodes an N-terminally truncated AML1.** The structure of the *AML1ΔN* transcript predicts that the reading frame, which begins with the initiation codon used for AML1c in exon 1, terminates after the synthesis of 38 amino acids. However, there are two ATG codons in exon 4 corresponding to amino acids 106 and 124, and the nucleotide sequences around them match well with the Kozak consensus (29) (the amino acid numbers of the AML1 protein used throughout this paper are of the AML1b isoform). Because these ATG codons are in frame with AML1, an N-terminally truncated AML1 protein of 348 or 330 amino acids would be produced if either of these methionine codons was used as a translation initiator.

To test this possibility, an *AML1ΔN* cDNA was translated in vitro and in vivo by transiently transfecting P19 cells with an expression plasmid. Western blot analysis with anti-PEBP2αB1 antiserum indicated that *AML1ΔN* cDNA was translated into a 43-kDa polypeptide both in vitro and in vivo (Fig. 5). The product of *AML1ΔN* cDNA also reacted with the anti-αA1C17 antiserum which was raised against the C-terminal 17-kDa portion of PEBP2αA1 and cross-reacts with the C-terminal region of AML1 (data not shown). Therefore, one of the methionine codons in exon 4 serves as a translation initiation site in *AML1ΔN* cDNA, even when the initiation codon in exon 1 is present. The product of *AML1ΔN* cDNA, therefore, is an N-terminally truncated AML1 protein which lacks roughly half of the N-terminal side of the Runt domain.

We have shown previously, that AML1 is a nuclear protein and that two regions in AML1 are responsible for its nuclear accumulation: one in the large C-terminal region, and the other in the Runt domain (33). Based on these observations, we predict that AML1ΔN is likely to be a nuclear protein. When NIH 3T3 cells transfected with an expression plasmid for AML1ΔN were labeled with anti-PEBP2αB1 antiserum, AML1ΔN was found to be localized mainly in the nucleus (data not shown).

AML1ΔN neither binds to DNA nor heterodimerizes with the β subunit. As shown in Fig. 6A, AML1b could bind to the PEBP2 recognition sequence with or without *E. coli*-expressed β protein (Fig. 6A, lanes 2 to 6 and 12 to 15), but AML1ΔN products were unable to bind under similar conditions (lanes 7 to 11 and 16 to 19). The band indicated by the arrowheads in Fig. 6A could be abolished by adding an excess of either unlabeled wild-type (lanes 4, 9, 14, and 18) or mutated-type (lanes 5, 10, 15, and 19) competitor. Therefore, it is nonspecific. Figure 6B shows the results of IP experiments. In vitro-translated ^{35}S-labeled AML1ΔN or AML1b was mixed with an equal amount of the in vitro-translated ^{35}S-labeled PEBP2β2 protein. Proteins were immunoprecipitated with anti-αA1C17 antiserum or anti-β2 antiserum. As expected, AML1b coprecipitated with β protein (lanes 7 and 12) whereas AML1ΔN did not (lanes 8 and 13).

AML1ΔN associates with Ets-1 almost as strongly as does AML1b. A physical interaction between Ets-1 and the Runt domain of PEBP2αA has been observed (15). Therefore, we wished to know whether AML1ΔN would also associate with Ets-1. We performed in vitro binding experiments in which GST–Ets-1 immobilized on glutathione-agarose beads was incubated with in vitro-translated ^{35}S-labeled AML1b or AML1ΔN. As shown in Fig. 7, AML1ΔN bound to GST–Ets-1 almost as strongly as AML1b did (Fig. 7, lanes 5 and 6) but not to GST-coated agarose beads (lanes 3 and 4). This result demonstrates not only that AML1ΔN physically interacts with Ets-1 but also that the Ets-1-interacting domain of AML1 resides somewhere toward the C-terminal side of the Runt domain.

AML1ΔN interferes with the transactivation activity of PEBP2/AML1. We tested the possibility that AML1ΔN interferes with the transcription-enhancing activity of AML1 through competition for potential AML1-binding proteins. As shown in Fig. 8A, exogenous expression of AML1b in P19 cells strongly activated the CAT activity of the reporter Tβ3W4W-tkCAT, which contains the PEBP2-binding sites. This activity was progressively reduced as increasing amounts of AML1ΔN were coexpressed. The reporter Tβ3M4M-tkCAT, which has mutations in PEBP2-binding sites, did not respond to AML1b either alone or in combination with AML1ΔN (Fig. 8A). This result indicated that the transactivation activity suppressed by AML1ΔN is PEBP2 site specific. Similarly, AML1ΔN also inhibited the TCRβ enhancer activity mediated by endogenous PEBP2/AML1 both in Jurkat T cells (Fig. 8B) and in M12 B cells (Fig. 8C).

To identify the region in AML1ΔN required for this effect, various deletion mutants of AML1ΔN were made (Fig. 9A) and tested in reporter assays. When transfected into P19 cells, all deletion constructs of AML1ΔN expressed proteins of the expected sizes (Fig. 9B). We observed that AML1ΔN(209–453), which has an N-terminal deletion up to amino acid 209, and AML1ΔN(106–340), which has a C-terminal deletion up to amino acid 340, retained their ability to inhibit the transcription reporter activities (Fig. 9C to E). Therefore, the region between amino acids 209 and 340 is mainly responsible for the

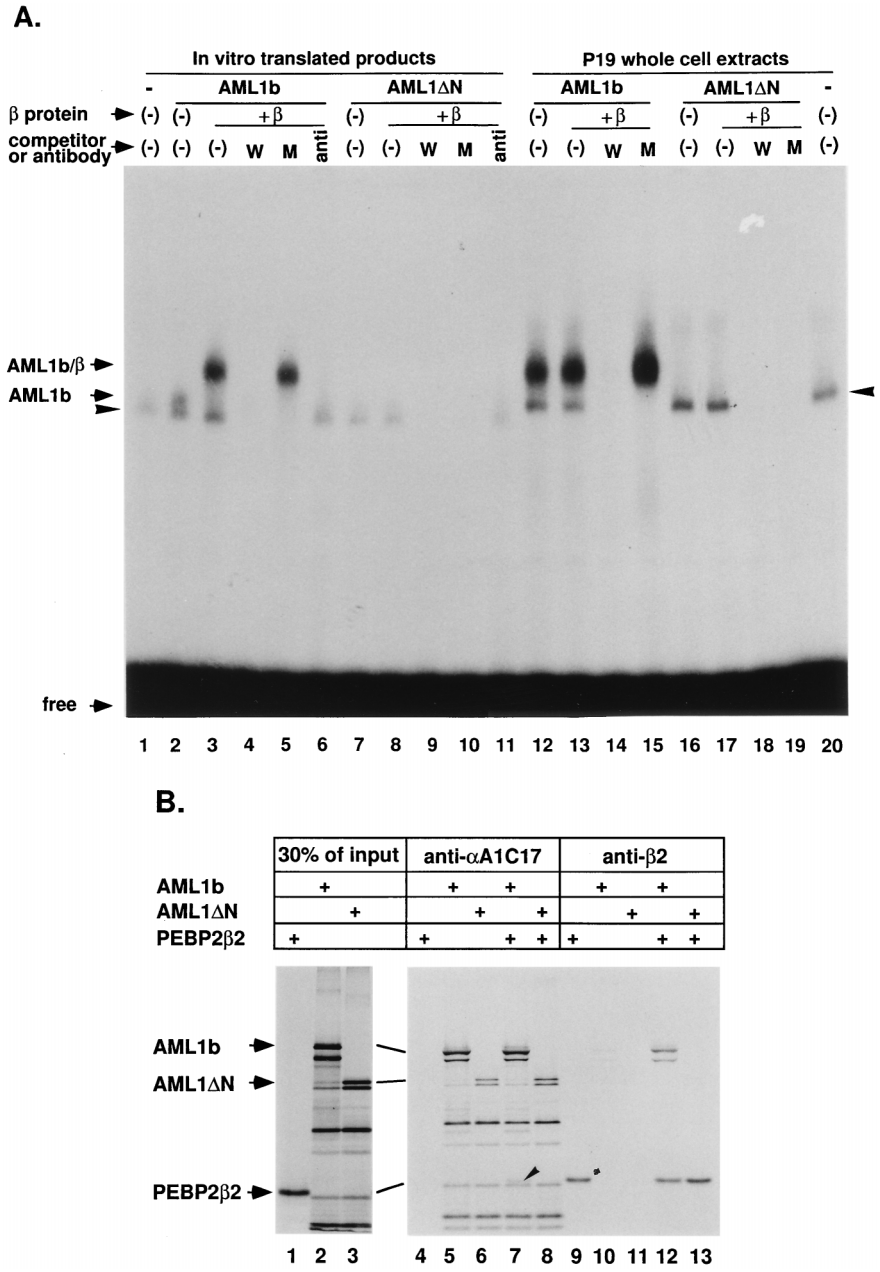

FIG. 6. Comparison of the DNA binding (A) and heterodimerization with β subunit (B) of AML1b and AML1ΔN. (A) EMSA. A 1-μl volume of in vitro-translated protein or 10 μg of protein from P19 whole-cell extracts was subjected to EMSA. The β2 protein produced in *E. coli* was added in lanes 3 to 6, 8 to 11, 13 to 15, and 17 to 19. Unlabeled wild-type (W) or mutant-type (M) competitor was added in a 50-fold molar excess. Reticulocyte lysate without template (lane 1) or P19 whole-cell extracts transfected with an empty vector (lane 20) are also included. A 1-μl volume of anti-PEBP2αB1 was added to the reaction mixture in lanes 6 and 11. The positions of AML1b, AML1b/β dimer, and free probe are indicated by arrows on the left. Nonspecific bands are indicated by arrowheads on both sides of the panel. (B) IP. A mixture of in vitro-translated, ^{35}S-labeled AML1b, AML1ΔN, and PEBP2β2, as indicated, was subjected to IP with anti-αA1C17 or anti-β2. Precipitated immunocomplexes were analyzed by SDS-PAGE (12.5% polyacrylamide). Thirty percent of the input was run on a separate gel (lane 1, β2; lane 2, AML1b; lane 3, AML1ΔN). The positions of each protein are indicated on the left. The position of the β2 band in lane 7 is marked by an arrowhead.

interference, implying that some positive factor interacts within this region. In contrast, the reporter activity induced by AML1 was enhanced by cotransfection with AML1ΔN(341–453), suggesting that a negative factor interacts with the region between amino acids 341 and 453. The mutant AML1ΔN(283–453) also enhanced the reporter activity in Jurkat cells but not in P19 or M12 cells, suggesting the presence of another positive factor in these cells which interacts with the region between amino acids 283 and 340. These results demonstrate that the region between amino acids 209 and 340 of AML1ΔN is mainly responsible for the inhibition of AML1 activity, most probably because this region serves as a sequestration site for one or more positive factors. We have evidence that the region between amino acids 209 and 340 does not affect the nuclear localization of AML1 (data not shown). The results also suggest that other multiple factors interact with the C-terminal region.

Overexpression of AML1ΔN blocks the granulocytic differentiation of 32Dcl3 cells in response to G-CSF. 32Dcl3 prolif-

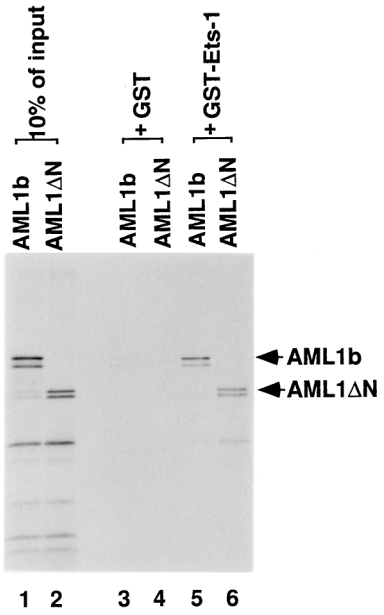

FIG. 7. Physical interaction between AML1-ΔN and Ets-1. In vitro-translated ^{35}S-labeled AML1b and AML1ΔN were subjected to coprecipitation with GST or GST-Ets-1 immobilized on glutathione-agarose beads. The bound protein complexes were analyzed by SDS-PAGE (12.5% polyacrylamide). Ten percent of the input was also run on the same gel (lanes 1 and 2). The positions of AML1b and AML1ΔN are indicated on the right.

erates in the presence of IL-3 and terminally differentiates into granulocytes in response to G-CSF (37). AML1 is thought to be required for the differentiation of 32Dcl3 cells (45, 61). Since AML1ΔN interfered with endogenous AML1 activity, we tested whether it would interfere with the differentiation and proliferation of 32Dcl3. We established 32Dcl3 clones that stably expressed AML1ΔN by using a recombinant retrovirus. Three AML1ΔN-expressing clones were analyzed for phenotypic changes. Parental 32Dcl3 cells required IL-3 for growth and survival. AML1ΔN-expressing clones also required IL-3 for growth and survival (data not shown). When parental 32Dcl3 cells or control clones were cultured in G-CSF, the cells underwent terminal differentiation and changed morphologically into mature granulocytes within 7 to 11 days and then progressively lost viability (Fig. 10B and C). In contrast, AML1ΔN-expressing clones kept growing in the presence of G-CSF (Fig. 10B). Morphologically, control cells displayed the mature granulocyte phenotype on day 8 after G-CSF treatment, whereas AML1ΔN-expressing cells still showed the immature phenotype on day 12 (Fig. 10C). We confirmed that the expression of myeloperoxidase, an early differentiation marker gene whose expression is specifically induced when the 32Dcl3 cells are committed to granulocytic differentiation (14), was very low in AML1ΔN-expressing cells (data not shown). These results demonstrate that the overexpression of AML1ΔN can significantly delay, if not block, the terminal differentiation of 32Dcl3 myeloid cells treated with G-CSF.

DISCUSSION

We have identified a novel *AML1* transcript, named *AML1ΔN*, that is generated by an alternative splicing of *AML1* mRNA. *AML1ΔN* encodes an N-terminally truncated AML1 protein which contains the complete C-terminal region but only about half of the Runt domain.

There exist many instances where it has been shown that a set of proteins with diverse functions can be generated from a single gene by alternative splicing (13). Three basic types of

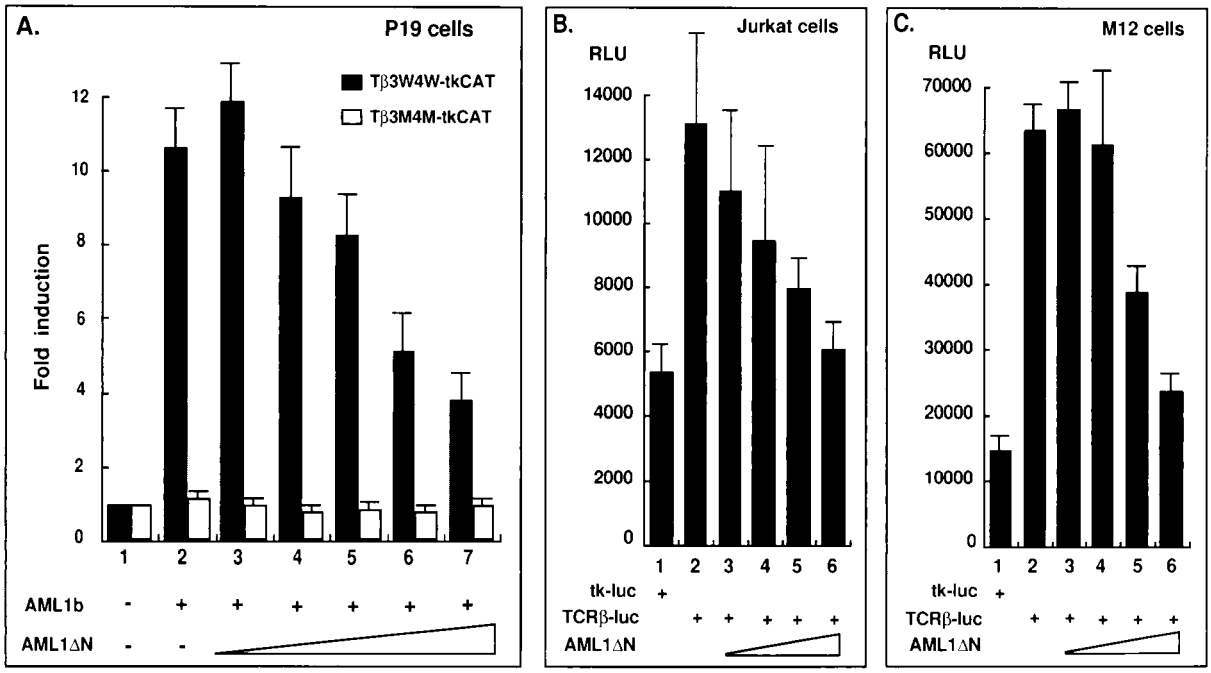

FIG. 8. Exogenous expression of AML1ΔN interferes with the transactivation activity of PEBP2/AML1. (A) P19 cells were transfected with 2 μg of Tβ3W4W-tkCAT or Tβ3M4M-tkCAT, with or without 0.5 μg of pEF-AML1b. Increasing amounts of pEF-AML1ΔN (lane 3, 1 μg; lane 4, 4 μg; lane 5, 8 μg; lane 6, 12 μg; lane 7, 16 μg) were cotransfected as indicated. (B and C) Jurkat cells (B) and M12 cells (C) were transfected with 5 μg of reporter plasmids tk-luc or TCRβ-luc in the absence (lane 2) or presence of increasing amounts of pEF-AML1ΔN (lane 3, 2.5 μg; lane 4, 5 μg; lane 5, 7.5 μg; lane 6, 10 μg). Each value represents the mean of three independent experiments. Standard deviations are indicated by error bars. RLU, relative luciferase unit.

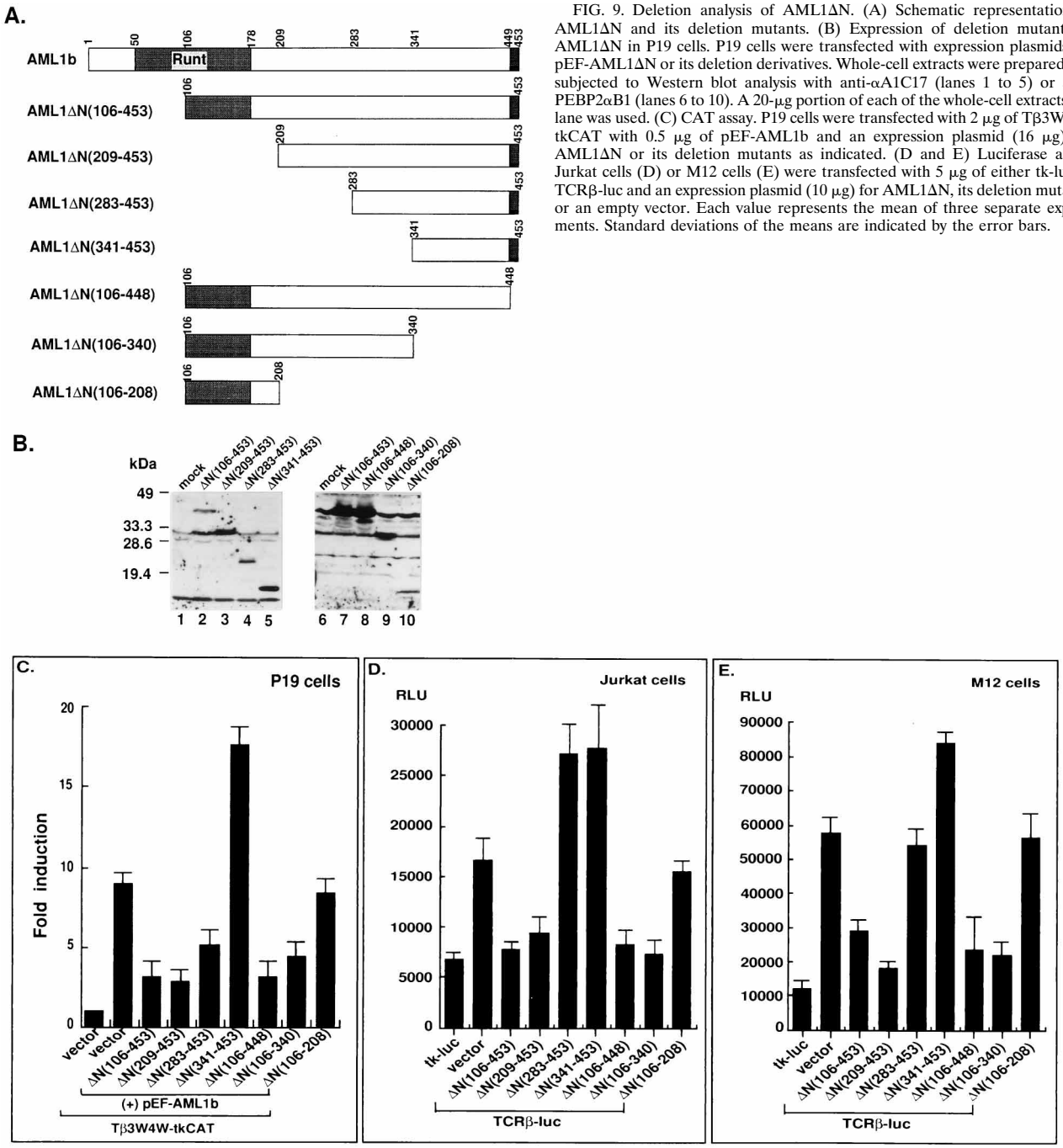

FIG. 9. Deletion analysis of AML1ΔN. (A) Schematic representation of AML1ΔN and its deletion mutants. (B) Expression of deletion mutants of AML1ΔN in P19 cells. P19 cells were transfected with expression plasmids for pEF-AML1ΔN or its deletion derivatives. Whole-cell extracts were prepared and subjected to Western blot analysis with anti-αA1C17 (lanes 1 to 5) or anti-PEBP2αB1 (lanes 6 to 10). A 20-μg portion of each of the whole-cell extracts per lane was used. (C) CAT assay. P19 cells were transfected with 2 μg of Tβ3W4W-tkCAT with 0.5 μg of pEF-AML1b and an expression plasmid (16 μg) for AML1ΔN or its deletion mutants as indicated. (D and E) Luciferase assay. Jurkat cells (D) or M12 cells (E) were transfected with 5 μg of either tk-luc or TCRβ-luc and an expression plasmid (10 μg) for AML1ΔN, its deletion mutants, or an empty vector. Each value represents the mean of three separate experiments. Standard deviations of the means are indicated by the error bars.

AML1 isoforms have been described and designated *AML1a*, *AML1b*, and *AML1c* (40). AML1b and AML1c stimulate transcription through the PEBP2/CBF-binding site (36, 61). In contrast, AML1a, which retains the Runt domain but lacks a large part of the C-terminal region, suppresses transcription by AML1b (36, 61). We propose that the two naturally occurring isoforms, AML1ΔN and AML1a, represent two contrasting types of negative regulators. AML1a possesses the DNA-binding domain but lacks the region required for transactivation. Presumably, it competes with AML1 for DNA binding and heterodimerization with the β subunit, thereby blocking transactivation by the functional PEBP2. While AML1ΔN is unable to bind to DNA or to heterodimerize with the β subunit, it retains the complete C-terminal region. Thus, it is conceivable that AML1ΔN competes with AML1 for factors interacting mainly with the C-terminal region of AML1. We have shown in this study that AML1ΔN interferes with the transcription activation function of AML1. The results suggest that AML1 associates with one or more limiting factors required for the stimulation of transcription and that AML1ΔN sequesters these factors. AML1 functionally cooperates with Ets-1 (15, 27, 58), and AML1ΔN contains the region of AML1 responsible for strong interaction with Ets-1 (27). Although it remains to be seen if AML1ΔN sequester Ets-1, mapping data obtained

with several deletion constructs of AML1ΔN (Fig. 9) suggests that it is unlikely to do so, because the major Ets-1 binding site is not found in the region of AML1ΔN(209–453) responsible for interference (27). One of the simplest interpretations of these deletion studies would be that a certain positive factor, most probably different from Ets-1, binds to AML1 between amino acids 209 and 340 and enhances the transcription activity of AML1. This region encompasses the region previously identified as the major mitogen-activated protein kinase phosphorylation site (62). It is possible, therefore, that mitogen-activated protein kinase is one of the factors sequestrated by AML1ΔN. Clearly, more detailed analysis will be required to reveal the true function of this region in transactivation by AML1. In addition to this region, it is worth noting that another region between amino acids 341 and 453 appears to interact with negatively functioning factor(s). Furthermore, the region between amino acids 283 and 340 appears to interact with a cell-type-specific factor. In a separate study, we identified the region of AML1 responsible for the stimulation of polyomavirus DNA replication. In this case, an interaction between the C-terminal region and a cellular factor(s) was also strongly suggested (9). In any event, an emerging picture is that the C-terminal region has a modular structure containing multiple sites responsible for interactions with positive and negative factors. To fully understand the mechanisms of transactivation by AML1, all these interacting factors must be characterized. For this purpose, AML1ΔN will be a very useful tool for the localization of the binding sites of these factors on AML1.

It has also been reported that AML1a blocks the differentiation and stimulates proliferation of 32Dcl3 myeloid cells treated with G-CSF (61), although conflicting results have also been reported (7). We demonstrated that overexpression of AML1ΔN also at least partly blocked the terminal differentiation into mature granulocytes. At present, it is not clear how AML1a and AML1ΔN, two contrasting negative regulators of AML1, induce similar effects in 32Dcl3 cells. More detailed analysis of this phenomenon is necessary. In any event, our data suggest that AML1ΔN sequestrates certain factors that must bind to AML1 for the induction of granulocytic differentiation. If this interpretation is correct, it should be possible to identify the protein by using AML1ΔN and its derivatives.

Accumulating evidence strongly suggests that AML1 plays important roles in the process of hematopoiesis (50, 51, 66). Alterations of AML1 structure by chromosomal rearrangements are closely related to leukemogenesis. The chimeric proteins, AML1-ETO(MTG8) (36), AML1-Evi1 (60), and TEL-AML1 (19), have been shown to repress the transactivation by AML1b through the TCRβ enhancer. Except for TEL-AML1, in which a potential dimerization motif of TEL is found fused to almost the full-length AML1, the chimeric proteins, AML1/ETO(MTG8) and AML1/Evi1, lose the C-terminal region of AML1 through replacement by ETO and Evi1, respectively. It is important to evaluate the loss of the C-terminal region of AML1 in these chimeric proteins in relation to leukemogenesis. In the case of TEL/AML1, contribution of the C-terminal region to leukemogenesis needs to be examined.

Finally, the possible physiological roles of *AML1ΔN* require some discussion. Levels of expression of the *AML1ΔN* transcript vary considerably among the cell lines tested. It is not clear whether any aspect of the phenotype of these cell lines is under the influence of AML1ΔN. However, it is conceivable that AML1ΔN is expressed in larger amounts to precisely regulate differentiation at specific stages of differentiation in some lineages of hematopoietic cells.

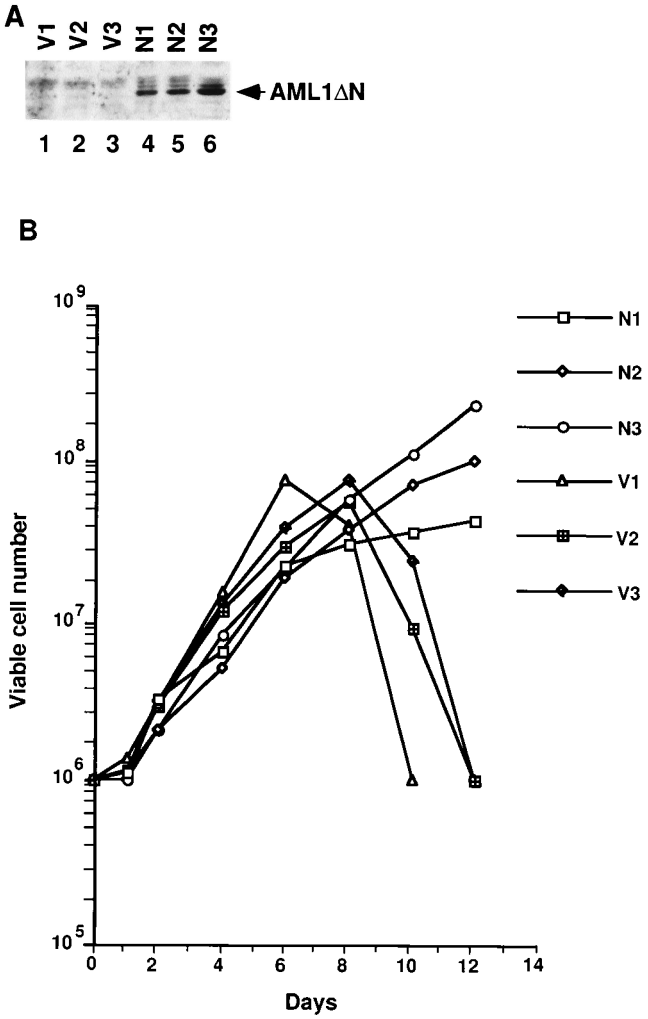

FIG. 10. Effect of AML1ΔN on the growth and differentiation of 32Dcl3 cells. (A) Expression of AML1ΔN in 32Dcl3 cells. A 20-μg portion of whole-cell extract prepared from pBabeneo vector-infected clones (V1, V2, and V3) and from pBabeneo-AML1ΔN-infected clones (N1, N2, and N3) was subjected to Western blot analysis with anti-PEBP2αB1 as a probe. (B) Cell growth curve in response to G-CSF. Control and AML1ΔN-expressing clones (10^6 cells each) growing in the presence of IL-3 were washed and cultured in medium containing 500 U of recombinant human G-CSF per ml. At each time point, viable cells excluding trypan blue were counted with a hemocytometer. Representative results from three independent experiments are shown. □, clone N1 of 32Dcl3 expressing AML1ΔN; ◇, clone N2 of 32Dcl3 expressing AML1ΔN; ○, clone N3 of 32Dcl3 expressing AML1ΔN; △, clone V1 of 32Dcl3 expressing vector alone; ⊞, clone V2 of 32Dcl3 expressing vector alone; ◆, clone V3 of 32Dcl3 expressing vector alone. (C) May-Grunwald-Giemsa staining of 32Dcl3 clones cultured in medium containing G-CSF was performed on each day indicated.

We reported previously an isoform of murine AML1, termed PEBP2αB2, that is encoded by a transcript lacking exon 6, a region encoding 64 amino acids immediately downstream of the Runt domain. The isoform was described as having a stronger DNA-binding activity and a lower transcription activation potential than did standard PEBP2αB1 (3). PEBP2αB1 and PEBP2αB2 were able to regulate *AML1/PEBP2αB* function in a positive and negative fashion, respectively (59). By changing the relative amounts of PEBP2αB1 and PEBP2αB2, levels of transcription could be precisely regulated. We have evidence that the *PEBP2αB2*-type transcript is also present in human T cells (5).

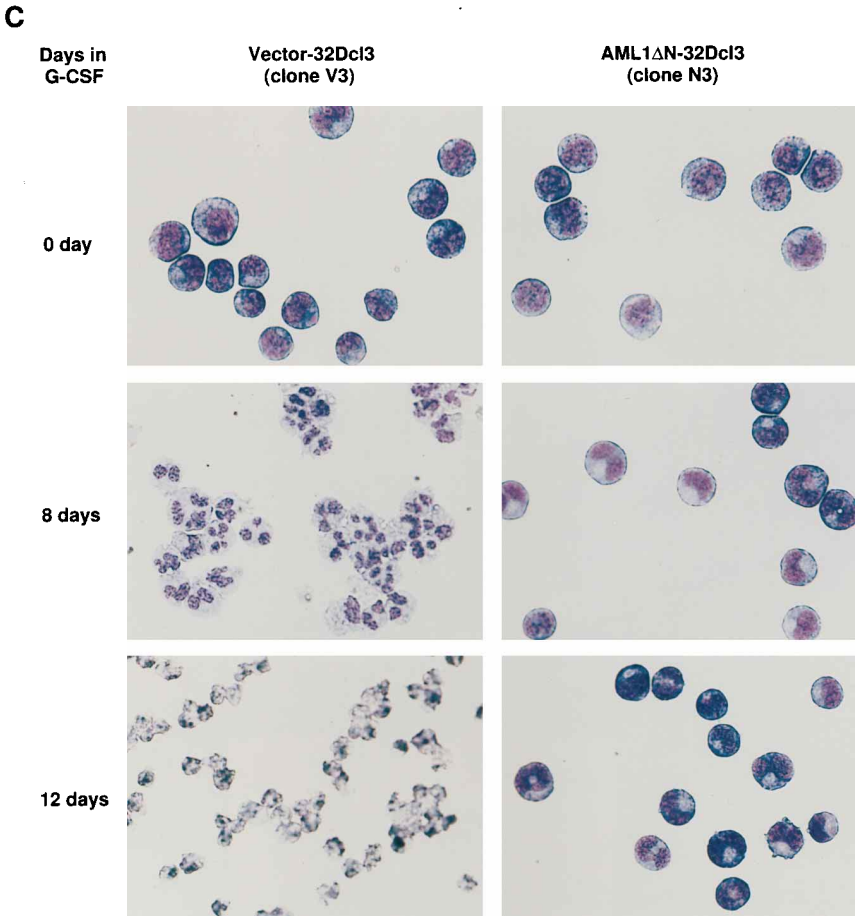

FIG. 10—*Continued.*

We have shown in this study an additional mechanism for the fine-tuning of *AML1* gene function. Thus, the expression of target genes of AML1 may be finely regulated by alterations in the relative amounts of PEBP2αB2, AML1a, and AML1ΔN.

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