

Biological Characteristics of the Leukemia-Associated Transcriptional Factor AML1 Disclosed by Hematopoietic Rescue of *AML1*-Deficient Embryonic Stem Cells by Using a Knock-in Strategy

TSUKASA OKUDA,^{1*} KIYOSHI TAKEDA,^{2†} YASUKO FUJITA,^{1,3} MOTOHIRO NISHIMURA,^{1,4}
SHIGEKI YAGYU,⁵ MAKIE YOSHIDA,⁵ SHIZUO AKIRA,^{2†} JAMES R. DOWNING,⁶
AND TATSUO ABE¹

Departments of Hygiene,¹ Internal Medicine,³ and Surgery,⁴ Kyoto Prefectural University of Medicine, Kyoto,⁵ and
Department of Biochemistry, Hyogo Medical College, Nishinomiya,² Japan, and Department of Pathology,
St. Jude Children's Research Hospital, Memphis, Tennessee⁶

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***AML1* is one of the most frequently mutated genes associated with human acute leukemia and encodes the DNA-binding subunit of the heterodimerizing transcriptional factor complex, core-binding factor (CBF) (or polyoma enhancer binding protein 2 [PEBP2]). A null mutation in either *AML1* or its dimerizing partner, *CBF β* , results in embryonic lethality secondary to a complete block in fetal liver hematopoiesis, indicating an essential role of this transcription complex in the development of definitive hematopoiesis. The hematopoietic phenotype that results from the loss of *AML1* can be replicated in vitro with a two-step culture system of murine embryonic stem (ES) cells. Using this experimental system, we now demonstrate that this hematopoietic defect can be rescued by expressing the *PEBP2 α B1* (*AML1b*) isoform under the endogenous *AML1*-regulatory sequences through a knock-in (targeted insertion) approach. Moreover, we demonstrate that the rescued *AML1*^{-/-} ES cell clones contribute to lymphohematopoiesis within the context of chimeric animals. Rescue requires the transcription activation domain of *AML1* but does not require the C-terminal VWRPY motif, which is conserved in all *AML1* family members and has been shown to interact with the transcriptional corepressor, Groucho/transducin-like Enhancer of split. Taken together, these data provide compelling evidence that the phenotype seen in *AML1*-deficient mice is due solely to the loss of transcriptionally active *AML1*.**

Hematopoietic development in mammals is supported by two discrete cellular populations which are believed to be derived from distinct cellular origins (5, 22). In the mouse, for example, the first wave of primitive hematopoiesis emerges around day 7.5 postcoitus (E 7.5) in the yolk sac and consists primarily of large nucleated primitive erythrocytes, which diminish midgestation. By contrast, around E 9.5, the second wave of hematopoiesis emerges in the fetal liver and consists of so-called definitive hematopoietic cells, including enucleated erythrocytes containing adult-type globin molecules, myeloid cells, and lymphoid progenitors. The site of this second wave of hematopoiesis is subsequently shifted to the bone marrow and spleen prior to birth.

Recent studies have revealed that a number of transcriptional factors play critical roles in regulating the fate of the hematopoietic stem cell populations. These factors are divided into two groups: one contains factors which regulate both primitive and definitive hematopoiesis, and the other contains factors whose activities are required for the development of some or all of the definitive hematopoietic lineages (reviewed in reference 44). The acute myeloid leukemia (AML) 1 gene, *AML1*, belongs to the latter group and is unique in that loss of this gene in mice results in the complete absence of all definitive hematopoietic lineages (37, 51).

AML1 was originally cloned from the breakpoint of chro-

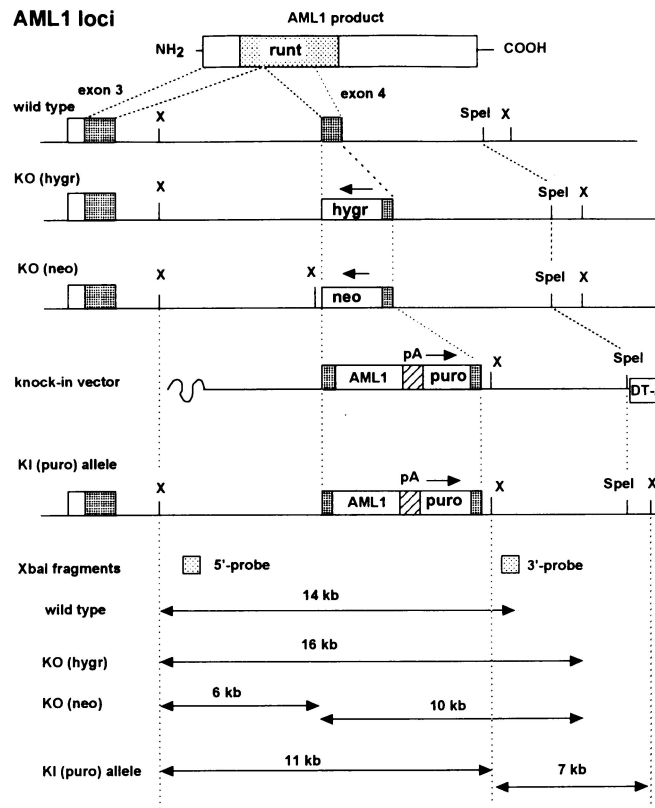
mosome 21 in t(8;21)(q22;q22), which is associated with 40% of the AML cases of the French-British-American classification M2 subtype (6, 27, 29). *AML1* has subsequently been shown to be one of the most frequent targets of leukemia-associated gene aberrations, including t(3;21), which is found in the blastic crisis of chronic myelocytic leukemia and myelodysplastic syndromes (26, 31), t(12;21), which is observed in pediatric B-lineage acute lymphocytic leukemia (9, 40), and t(16;21), which is associated with secondary leukemias (7). Together, these translocations comprise ~12% of AML cases and ~20% of acute lymphocytic leukemia in pediatric and young adult populations (20). Moreover, recent studies have revealed that *AML1* is also disrupted by several other rare chromosomal translocations (41) and is inactivated by point mutations in occasional cases of adult AML (39), thus further documenting the high prevalence of *AML1* alterations in human leukemia.

AML1 encodes the DNA-binding subunit of the heterodimerizing transcriptional factor complex, core-binding factor (CBF) (or polyoma enhancer binding protein 2 [PEBP2]) (12, 33), and binds to the DNA sequence TGT/cGGT, which is essential for the transcriptional regulation of a number of hematopoietic specific genes, including those for granulocyte-macrophage colony-stimulating factor (GM-CSF), M-CSF receptor, myeloperoxidase, neutrophil elastase, and each of the subunits of the T-cell antigen receptor genes (reviewed in references 16 and 47). *AML1*'s DNA binding is mediated through a central 128-amino-acid domain with 69% identity to the product of the *Drosophila* pair-rule gene, *runt* (3, 13) (Runt domain), and its DNA-binding affinity is increased by binding to its heterodimeric partner *CBF β* (or *PEBP2 β*) (32, 53) through the Runt domain (12, 23). Interestingly, *CBF β* has

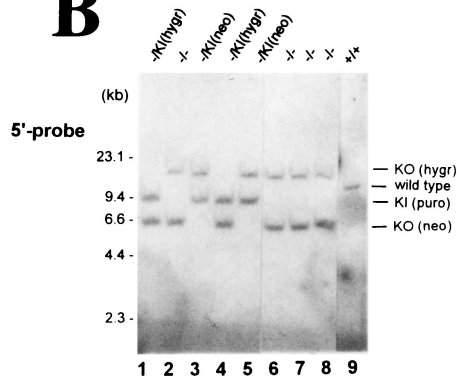
* Corresponding author. Mailing address: Department of Hygiene, Kyoto Prefectural University of Medicine, Kawaramachi-Hirokoji, Kamigyo-ku, Kyoto 602-8566, Japan. Phone: 81-75-251-5335. Fax: 81-75-251-5334. E-mail: okuda@basic.kpu-m.ac.jp.

† Present address: Research Institute for Microbial Diseases, Osaka University, Suita, Japan.

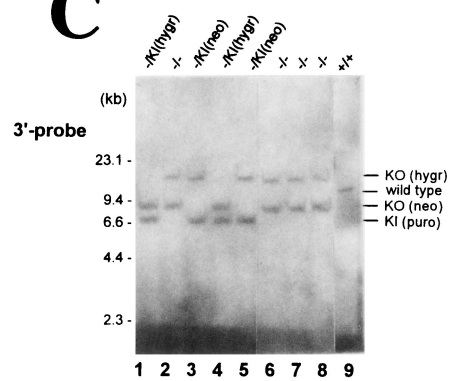
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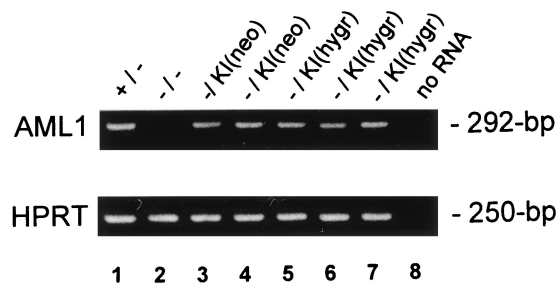


FIG. 1. Rescue strategy of *AML1*-deficient ES cells by expressing *PEBP2 α B1* (*AML1b*) cDNA from a knock-in allele. (A) The *AML1* loci of the ES cell lines were disrupted by targeting exon 4, which encodes the middle of the Runt domain; one allele was replaced by a hygromycin resistance cassette [KO(hygr)] and the other by a neomycin resistance cassette [KO(neo)] (37). To rescue the *AML1*-deficient ES cell phenotype, we designed a replacement-type vector (knock-in vector) which

been demonstrated to be the target of the leukemia-associated chromosomal abnormalities *inv(16)(p13q22)* and *t(16;16)(p13;q22)* (19). Homozygous disruptions in either *AML1* or *CBF β* result in an embryonic lethal phenotype secondary to a complete block in fetal liver hematopoiesis, thus demonstrating an essential role for this transcription factor complex in normal definitive hematopoiesis (30, 37, 42, 51, 52). Moreover, recent studies have demonstrated that mice expressing the *t(8;21)*-encoded *AML1-ETO* (eleven twenty-one, or myeloid translocation gene on chromosome 8 [*MTG8*]) (6, 27) leukemic gene through a knock-in approach die from a similar phenotype, suggesting that this fusion protein functions at least in part as a dominant negative repressor of normal *AML1*-mediated transcription (34, 55). Importantly, however, fetal liver cells from *AML1-ETO*-expressing embryos showed abnormal differentiation and an increased self-renewal capacity, suggesting that *AML1-ETO* directly contributes to the promotion of early steps in leukemic transformation of the hematopoietic stem cells (34; reviewed in references 4 and 46).

In an attempt to directly define the structural features of *AML1* required for its biological role in establishing definitive hematopoiesis, we examined the ability of wild-type or mutant *AML1* genes to rescue the hematopoietic defect that results from the loss of *AML1*. Using an in vitro hematopoietic differentiation assay with murine embryonic stem (ES) cells, we now demonstrate that the C-terminal transactivation domain of *AML1* is required for its biological activity. By contrast, the five C-terminal amino acids that mediate interaction with the Groucho/transducin-like Enhancer of split corepressor are not necessary for its role in early definitive hematopoiesis.

MATERIALS AND METHODS

Construction of the plasmids. The full-length mouse cDNA for the *AML1* gene, *PEBP2 α B1* (2), equivalent to human *AML1b* (28), was kindly provided by Yoshiaki Ito, Institute for Viral Research, Kyoto University. The deletion mutations of *AML1b*, at codons 293 (*AML1b Δ 293*), 320 (*AML1b Δ 320*), and 390 (*AML1b Δ 390*), were introduced by cleaving the *PEBP2 α B1* cDNA with the restriction endonucleases *Sall*, *SphI*, and *NcoI*, respectively, followed by the insertion of double-stranded oligonucleotides containing stop codons in all three frames, TAATTAATTAATTA, surrounded by corresponding cohesive overhangs. To construct an *AML1* mutant, *AML1b Δ 446*, that lacks the genetic code for the C-terminal VWRPY motif, a GT-to-TA nonsense mutation was introduced at codon 447 by PCR with the primer pair 5'-TCCTACCATCTACTACGG-3' (sense) and 5'-GCCACTAGGCCTCCTCCAGG-3' (antisense). The *NcoI*-restricted fragment of the resultant PCR product, which contained an artificial stop codon at residue 447, was inserted into the corresponding site of the *PEBP2 α B1* cDNA.

To produce replacement-type vectors for creating knock-in alleles, a genomic DNA fragment of ca. 12 kb encompassing exon 4 of the mouse *AML1* gene, which corresponds to the middle of the Runt domain, was cloned into a pBlue-script II plasmid vector (Stratagene, La Jolla, Calif.), followed by an insertion of a diphtheria toxin-A suicide cassette (34) at the 3' end of the insert in the sense orientation. Next, a *SacII*-restricted fragment from either the full-length or each of the C-terminal deletion mutants of *PEBP2 α B1* cDNA, accompanied by a DNA fragment of the polyadenylation signal from the rabbit globin gene, was inserted into the corresponding *SacII* site of exon 4 of the *AML1* genomic DNA fragment so as to create an artificial exon which then contained the entire cDNA sequence of mouse *AML1b* (*PEBP2 α B1*) or one of the designed mutations downstream from exon 4. Finally, a puromycin resistance cassette was inserted just downstream of the polyadenylation signal sequence for sense orientation. This cassette was constructed by replacing the neomycin phosphotransferase coding sequence of a neomycin resistance cassette (37) with the puromycin *N*-acetyltransferase coding sequence. The vectors were named pRES-*AML1b*,

pRES-*AML1b*(Δ 293), pRES-*AML1b*(Δ 320), pRES-*AML1b*(Δ 390), and pRES-*AML1b*(Δ 446) and were linearized at a unique *ScaI* site within the cloning vector just prior to being transfected into ES cells.

To construct the eukaryotic expression vectors of hemagglutinin (HA)-tagged *AML1b* or its mutants, a site-directed mutagenesis was performed to introduce a T-to-G substitution at nucleotide 253 and a G-to-C substitution at nucleotide 257 of *PEBP2 α B1* cDNA (2) to convert the initiation codon into an isoleucine codon followed by an artificial *BamHI* site. The *BamHI*-cleaved mutant cDNA was fused to a cohesive double-stranded oligonucleotide encoding an HA peptide (sense, 5'-AGCTTGCCGCCACCATGATGATGTTCTGATTATGCTAGCCTCTT-3'; antisense, 5'-GATCAAGAGGCTAGCATAATCAGGAACA TCATACATGTTGGCGGCA-3') and then subcloned into the multiple-cloning site of a cytomegalovirus promoter-based eukaryotic expression vector, pRc/CMV (Invitrogen, Carlsbad, Calif.). A DNA fragment downstream from the unique *SacII* site of *PEBP2 α B1* was then replaced with the corresponding fragments of the *AML1* mutants to construct expression vectors for each of the mutants. The resulting plasmids were named pRc:HA-*AML1b*, pRc:HA-*AML1b*(Δ 293), pRc:HA-*AML1b*(Δ 320), pRc:HA-*AML1b*(Δ 390), and pRc:HA-*AML1b*(Δ 446) to indicate the name of the replacing mutant.

All ligated and mutated sites of the constructs were sequenced for both strands to confirm their integrity prior to use for the subsequent experiments.

Transient expression of *AML1* proteins in COS-7 cells. One million COS-7 cells were transfected by the DEAE-dextran method (21, 48) with 5 μ g of the supercoiled plasmid DNA of each pRc construct (mentioned above). Cells were lysed for Western blot analysis after a 72-h culture.

Isolating the knock-in clones. E14 cell clones which had undergone homologous recombination for both *AML1* alleles at exon 4, with a neomycin cassette replacement vector for one allele and a hygromycin B-resistance cassette vector for the other, were cultured as previously described (37) so as to preserve the undifferentiated phenotype. Twenty micrograms of the linearized targeting vector for each of the above mentioned pRES constructs was electroporated into 10⁷ ES cells, and the subsequent clones were selected in the presence of puromycin (Sigma, St. Louis, Mo.) at 1.3 μ g/ml. Resistant clones for the targeted integration were serially evaluated by Southern blot analysis under conventional conditions (36) with 5' and 3' outside probes (see Fig. 1) as previously described (37). Single-copy integration of the vector DNA to the clones was then confirmed by Southern blot analysis with an internal probe corresponding to the puromycin *N*-acetyltransferase sequence. ES clones showing successful homologous recombination at either of the disrupted alleles were cultured for expansion and then kept frozen until further analyses.

Reverse transcriptase (RT)-PCR analysis for expression of the *AML1* gene. Total RNA was isolated from undifferentiated ES cells or embryoid bodies (EBs), and cDNA was subsequently synthesized with random hexamers as described elsewhere (35). A part of the *AML1* messages was amplified by PCR with a pair of oligonucleotide primers which bracketed the targeted site within the Runt domain and corresponded to the respective sequences in exons 3 and 4 (37). Parallel reactions were performed with hypoxanthine phosphoribosyltransferase primers (15) to ensure the integrity of the RNA samples. Amplified PCR products were size fractionated by electrophoresis through an agarose gel and detected on a UV transilluminator after staining with ethidium bromide.

Western blot analysis for expressed *AML1* proteins. Cells were lysed in boiling radioimmunoprecipitation assay buffer, electrophoretically separated on a 10% denaturing polyacrylamide gel, and transferred to a nitrocellulose membrane. Proteins were detected with an *AML1* N-terminal peptide (34) or the monoclonal antibody against HA peptide (BAbCO, Berkeley, Calif.) with ECL detection substrate (Amersham, Little Chalfont, United Kingdom) as described previously (34).

In vitro differentiation of EBs. The procedure for the in vitro differentiation of EBs was based on a previously described method (15, 54), with some modifications (37). In brief, ES cells were adapted to grow without a feeder cell layer. Triplicated cell suspensions of 3 \times 10² adapted ES cells were plated in 1-ml mixtures of 1.2% methylcellulose in Iscove's modified Dulbecco's medium containing 15% fetal calf serum, 2 mM glutamine, and 450 μ M monothioglycerol. The cultures were supplemented with 2 U of human erythropoietin per ml and 50 ng of human G-CSF (both purchased from Kirin Brewery Co., Tokyo, Japan) per ml and 10 ng of murine GM-CSF per ml, 20 ng of murine stem cell factor per ml, and 5 ng of murine interleukin-3 (IL-3) (all from R & D Systems, Minneapolis, Minn.) per ml for hematopoietic differentiation of EBs, which were scored by visual examination with an inverted microscope for the presence of primitive erythrocytes on day 9 and of surrounding macrophages on day 14. The

creates an artificial allele [KI(puro)] that expresses the *PEBP2 α B1* isoform of the *AML1* protein in place of the disrupted exon 4 by means of homologous recombination. Clones which had undergone targeted insertion at either of the hygromycin or neomycin alleles were detected by Southern blot analyses with a 5' outside probe (B) and 3' outside probe (C), in which the knock-in alleles were detectable as *XbaI*-restricted fragments of ca. 11 and 7 kb, respectively. Abbreviations: hygr, hygromycin B resistance cassette; neo, neomycin resistance cassette; puro, puromycin resistance cassette; DT-A, diphtheria toxin-A suicide cassette. Arrows above selection cassettes indicate the orientation of the transcription. (D) Expression of the *AML1* message in the knock-in ES clones was confirmed by RT-PCR analysis, which amplified the 292-bp fragment of the cDNA with a primer pair corresponding to exons 3 and 4 of the gene locus (top panel). Parallel amplification for the *HPRT* gene (bottom panel) demonstrates the integrity of the RNA samples obtained from the undifferentiated ES cell clones.

presence of individual hematopoietic cell lineages was confirmed by microscopic examination of cytocentrifuge preparations of single EBs.

Second-step in vitro cultures for hematopoietic progenitors. Second-step cultures for hematopoietic progenitors were performed according to the methods described by Keller et al. (15). EBs, which were grown under the culture conditions described earlier (supplemented with 20 ng of murine stem cell factor per ml), were recovered on day 6 of culture for the examination of erythroid precursors of primitive origin and on day 10 for that of hematopoietic progenitors of definitive origin. The EBs were disrupted by collagenase treatment, and 10^3 recovered single cells were then recultured in triplicate in 1-ml mixtures of Iscove's modified Dulbecco's medium containing 1.2% methylcellulose, 30% fetal calf serum, 2 mM glutamine, 1% bovine serum albumin, and 0.1 mM β -mercaptoethanol. Cultures for the replated primitive erythroid colonies were supplemented with 2 U of human erythropoietin per ml, and cultures for definitive progenitors were supplemented with the same combination of CSF as described for the EB cultures above (see Fig. 3A). Colonies were scored by visual examination with an inverted microscope on days 4 and 12 for primitive erythroid progenitors (Ery-P) and colonies of definitive origin, respectively. The hematopoietic lineages of the colonies were confirmed by microscopical examination of the May-Gruenwald-Giemsa-stained cytospin preparations.

Chimeric mice production. The generation of chimeric mice was essentially as previously described (49). In brief, ES cell clones of normal ploidy and with a single vector insertion were injected into C57BL/6 blastocysts. Manipulated blastocysts were then transferred into the uterines of pseudopregnant mother mice, and the chimerism of the subsequently born mice was evaluated on the basis of the coat color.

GPI isoenzyme analysis. Glucose phosphate isomerase (GPI) isoenzyme analysis was performed as described elsewhere (37). Briefly, frozen tissue samples were homogenized in 50 mM Tris-HCl containing 0.1% Triton X-100, and aliquots of the appropriately diluted supernatant were subjected to electrophoretic fractionation on cellulose acetate membranes (Helena Laboratories, Beaumont, Tex.). The bands of the enzyme were visualized by treating the membranes with a 10-ml mixture of 0.9% agarose containing 5 mM magnesium acetate, 15 mg of fructose-6-phosphate, 2 mg of methylthiazolium tetrazolium, 0.36 mg of phenazine methosulfate, 2 mg of NADP, and 10 U of glucose-6-phosphate dehydrogenase (all from Sigma) for 10 to 15 min.

RESULTS

Creating a knock-in allele which expresses *PEBP2 α B1* (*AML1b*) cDNA in *AML1*-deficient ES cells. *AML1* is known to be expressed in a cell-lineage-specific and developmental-stage-specific manner in hematopoietic cells (17, 28, 43, 45). Interestingly, an analysis of *AML1*'s own transcriptional regulatory sequences revealed multiple *AML1*-binding sites in both its proximal and distal promoters, suggesting that its actual transcription may be autoregulated (8). Consistent with this interpretation, our preliminary attempts to rescue the *AML1*-deficient phenotype by expressing *PEBP2 α B1* (murine *AML1b*) from a ubiquitous promoter, the *cis* element of the mouse phosphoglycerate kinase 1 gene, failed (not shown). In order to overcome the inability of ubiquitously expressed *AML1* to rescue the phenotype, we chose to use a knock-in approach that would achieve the expression of the exogenous gene from the endogenous *AML1* regulatory elements. We designed a replacement-type vector which created an artificial exon 4 that generates a full-length transcript for the *PEBP2 α B1* (*AML1b*) isoform of the murine *AML1* protein, followed by a polyadenylation signal and puromycin resistance cassette (Fig. 1; see Materials and Methods). We examined 110 single *AML1*^{-/-} ES cell clones which became resistant to puromycin after electroporation of the linearized replacement vector, pRES-*AML1b*, by Southern blot analysis (Fig. 1B and C) and found that the homologous recombination of the vector had occurred within the hygromycin allele in 28 single-cell clones and at the neomycin allele in 21 single-cell clones (45% targeting efficiency). Expression of the *AML1b* gene from the resultant knock-in allele was confirmed by RT-PCR analysis with primers corresponding to the genomic sequence for *AML1* exons 3 and 4. As shown in Fig. 1D, the mRNA for the knocked-in *PEBP2 α B1* gene was expressed in all knock-in clones regardless of which allele was targeted.

Knock-in clones restore the ability of ES cells to undergo hematopoietic differentiation of definitive origin in vitro. To determine if the knock-in *AML1* allele could restore the ability of these ES cell clones to differentiate into hematopoietic cells of definitive origin, the clones were assessed by an in vitro differentiation assay system. As reported by us and others, homozygous mice deficient in *AML1* die in midgestation as a result of a complete block in definitive fetal liver-derived hematopoiesis, thus pointing to a pivotal role for *AML1* in the establishment of definitive hematopoiesis (37, 51). This in vivo phenotype can be replicated in vitro by the ES cell system (37). When *AML1*^{+/+} or *AML1*^{+/-} ES cells were cultured in semi-solid media without leukemia inhibitory factor, they formed EBs, which consisted of tight cell aggregates that contained all three germ cell layers (15, 54). The *AML1*^{+/-} clone-derived EBs developed primitive erythrocytes inside them after 8 to 10 days in culture as well as surrounding definitive macrophages (Fig. 2A, upper left panel) by 12 to 14 days (15, 54). Microscopic examination of the cytospin preparations showed that most of the hematopoietic cells within these EBs were mature macrophages, while a few erythroid and granulocytic cells were also observed (Fig. 2A, lower left panel). EBs derived from *AML1*^{-/-} clones, in contrast, failed to develop any definitive hematopoietic cells (Fig. 2A, center panel), thus replicating the *AML1*-deficient phenotype in vitro. When knock-in ES clones (*AML1*^{-/KI}) were examined, cultured EBs were surrounded by macrophages and were similar in appearance to those formed with wild-type or *AML1*^{+/-} cells, as expected (five clones examined, representative results are shown) (Fig. 2A, upper right panel). Cytospin preparations of these EBs demonstrated the cells to be mostly macrophages with occasional granulocytes and erythrocytes (Fig. 2A, lower right panel). The morphology of the cells derived from the knock-in clones was indistinguishable from that of cells developed from control *AML1*^{+/+} or *AML1*^{+/-} clones. Moreover, as shown in Fig. 2B, the plating efficiencies and the incidence of visible macrophage differentiation within EBs of *AML1*^{+/-} and knock-in clones were similar. In addition, knock-in clones with a targeted insertion of the cDNA either at the hygromycin allele (three clones analyzed) or the neomycin allele (two clones analyzed) showed a similar capability of hematopoietic differentiation (Fig. 2B).

We then used a two-step culture system to analyze the frequency of individual progenitors developing within EBs. To assess Ery-P, day 6 EBs were disrupted and recultured in the presence of erythropoietin, as schematically outlined in Fig. 3A. As shown in Fig. 3B, comparable numbers of Ery-P were detected in all analyzed clones, including *AML1*^{+/-}, *AML1*^{-/-}, and *AML1*^{-/KI}, with representative colonies shown in Fig. 3C. As expected, no definitive progenitors were detected in day 10 EBs derived from *AML1*-deficient ES cell clones (Fig. 3B). In contrast, hematopoietic progenitors of definitive origin were identified in EBs derived from ES cells containing the knock-in allele (Fig. 3B). Moreover, both the number of colonies (Fig. 3B) and the morphology of the cells of the developing colonies (Fig. 3D) of *AML1*^{+/-} and knock-in clones were similar although there was a tendency for the formation of fewer colonies in cultures from the knock-in EBs. Thus, the expression of the *PEBP2 α B1* (*AML1b*) isoform through a knock-in strategy restored the ability of the *AML1*-deficient ES cells to differentiate into hematopoietic lineages in vitro.

Knock-in clones can contribute to both myeloid and lymphoid tissues in vivo. To determine whether the knock-in clones have the capacity to differentiate into hematopoietic lineages in vivo, these ES cells were injected into blastocysts and the resultant chimeric mice were analyzed for the contribution of the ES cells to hematopoietic tissues. The relative

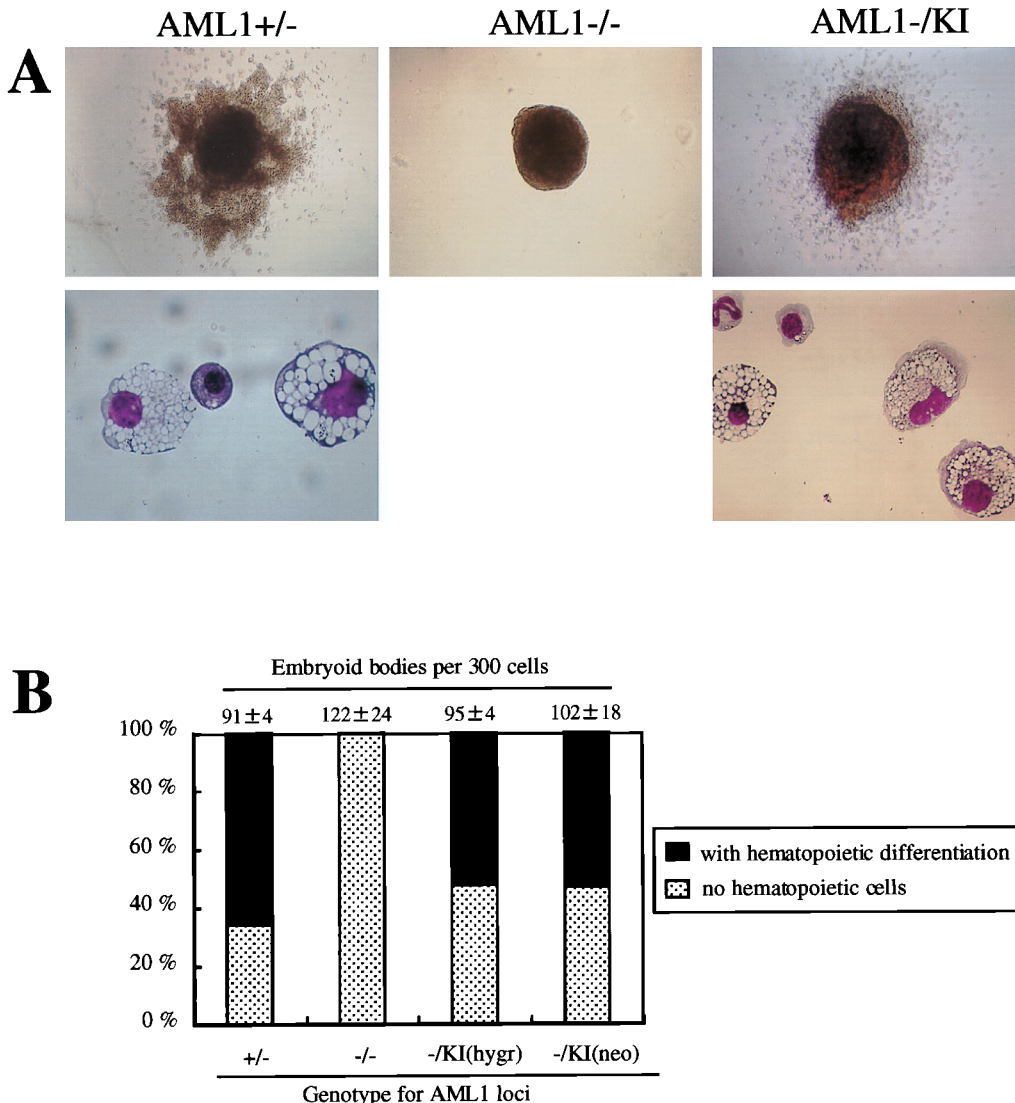


FIG. 2. Results of the EB differentiation experiments of knock-in clones. (A) Appearance of the representative day 14 EBs derived from the ES cell clones of *AML1*^{+/-} and *AML1*^{-/-} and knock-in with wild-type *AML1b* (*AML1*^{-/KI}) genotypes. *AML1*^{+/-} ES cell clones formed EBs, the majority of which was surrounded by hematopoietic cells (upper left panel) consisting primarily of mature macrophages and a few erythroid and granulocytic cells (lower left panel). EBs derived from *AML1*^{-/-} clones developed no such cells (center). In contrast, knock-in ES clones (*AML1*^{-/KI}) formed hematopoietic cells around the EBs (upper right panel), consisting of macrophages with occasional granulocytes and erythrocytes (lower right panel). The morphology of the cells derived from the knock-in clones was indistinguishable from that of cells developed from control *AML1*^{+/-} clones. Original magnifications: upper panels ×20, lower panels, ×132. (B) Incidence of hematopoietic differentiation of the day 14 EBs derived from ES cell clones of *AML1*^{+/-}, *AML1*^{-/-}, *AML1*^{-/-} with a knock-in *AML1b* at the hygromycin allele [*AML1*^{-/KI(hygr)}], and *AML1*^{-/-} with a knock-in *AML1b* for the neomycin allele [*AML1*^{-/KI(neo)}] genotypes in a representative experiment. Dark bars indicate the percentage of the EBs with visible hematopoietic cells whereas light bars represent those without a hematopoietic element. Actual numbers of the EBs grown per 300 ES cells are given above each bar as the mean ± standard deviation of triplicate cultures.

contribution of *AML1*^{+/-}, *AML1*^{-/-}, and *AML1*^{-/KI} cells to the chimeric animals, as estimated by an agouti coat color, was similar and typically ranged from 30 to 50%. The extent of contribution of the injected ES cells to individual organs was determined by means of GPI isoform analysis (37). The E14 ES cells contain the GPI-A isoform, whereas the C57BL/6 of the host strain are GPI-B isoform specific. We examined 11 different tissues from 1-month-old chimeric mice, and representative results are illustrated in Fig. 4. As described previously (37), *AML1*^{-/-} ES cells failed to contribute to hematopoietic tissues, whereas they made significant contributions to nonhematopoietic tissues. In contrast, the knock-in clones, regardless of which allele underwent a targeted insertion of the

cDNA, made a major contribution to all tissues, including sites of lymphohematopoiesis, i.e., peripheral blood, bone marrow, spleen, and thymus. These results clearly indicate that the expression of the *AML1b* (*PEBP2αB1*) isoform from the knock-in allele is sufficient to enable the *AML1*-deficient ES cells to develop into lymphoid and myeloid-erythroid lineages within the context of the animal.

Hematopoietic rescue depends on the transactivation domain of the PEBP2αB1 (*AML1b*), but not on the C-terminus, including the VWRPY motif. As an initial attempt to dissect the subdomains of the *AML1* molecule required for its biological role in hematopoietic development, we expressed a series of C-terminal deletion mutants of *PEBP2αB1* (*AML1b*) in

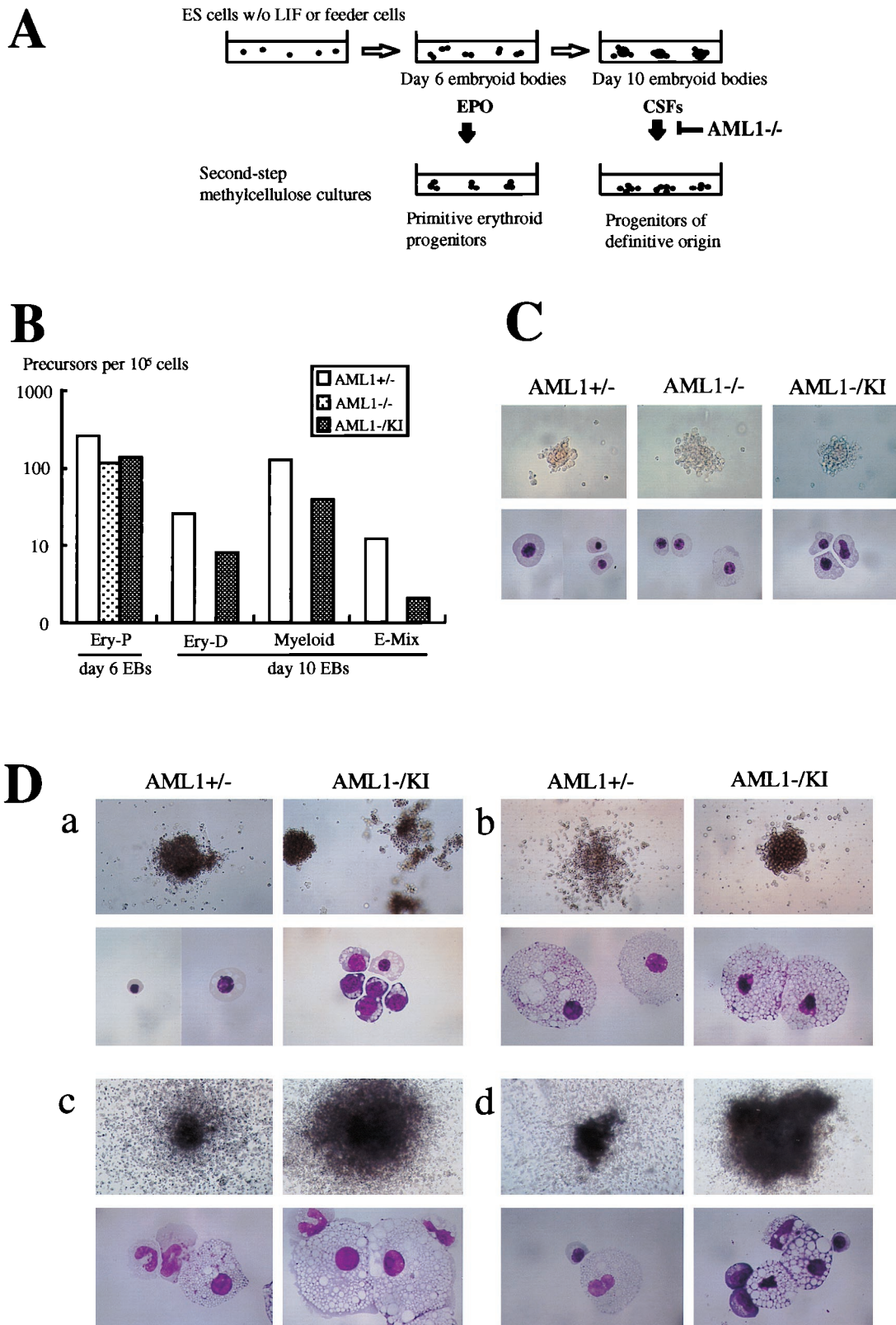


FIG. 3. Results of the two-step differentiation experiments of the knock-in clones. (A) Schematic representation of the method used in the present study to induce the hematopoietic differentiation of ES cells. When ES cells are cultured to form EBs in semisolid media without feeder cells or leukemia inhibitory factor, they develop hematopoietic cells of primitive and definitive origins, which are detectable by second-step cultures in the presence of appropriate CSF. In contrast, when *AML1*^{-/-} ES cells were subjected to this analysis, they failed to develop hematopoietic cells of definitive origin. (B) In vitro differentiation of ES cell clones of each of the *AML1* genotypes into hematopoietic precursors in a representative experiment. Colonies of Ery-P, definitive erythroid (Ery-D), and granulocyte-macrophage and macrophage (Myeloid), as well as mixed lineages including definitive erythroid (E-Mix), were scored in a two-step replating assay. ES cells were cultured to form EBs and then

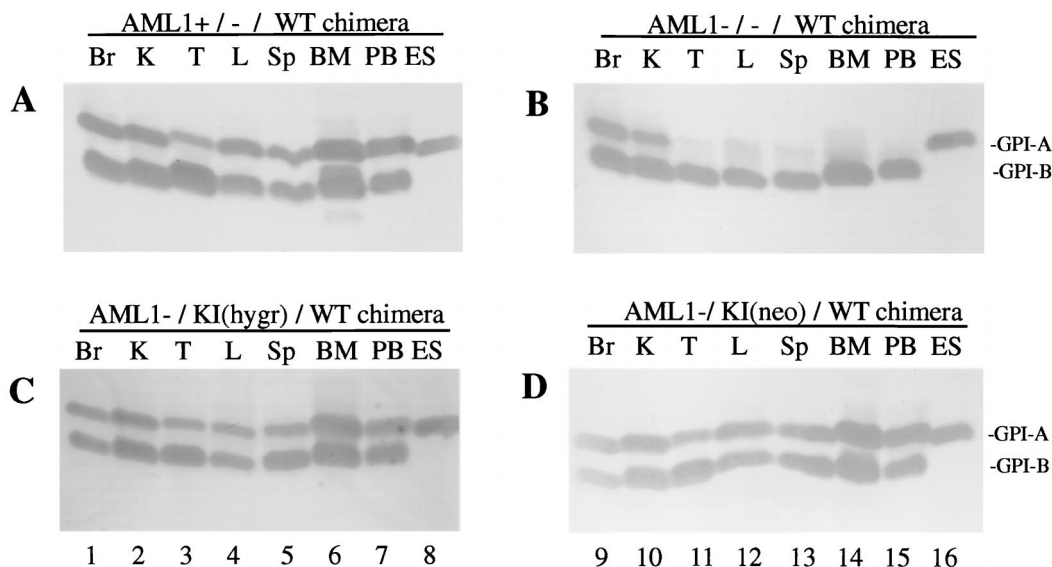


FIG. 4. Contribution of *AML1*^{-/-}, *AML1*^{+/-}, and *AML1*^{-KI} ES cells to tissues in chimeric mice. Representative results for the GPI analysis of chimeric mice derived from *AML1*^{+/-} (A) and *AML1*^{-/-} (B) and knock-in clones for the hygromycin allele [*AML1*⁻/*KI*(hygr)] (C) and neomycin allele [*AML1*⁻/*KI*(neo)] (D). ES cells contain the GPI-A isoform as indicated in lanes 8 and 16, whereas the host contains the GPI-B isoform. Results from the following tissues are shown: brain (Br), kidney (K), thymus (T), liver (L), spleen (Sp), bone marrow (BM), and peripheral blood (PB), and ES cells (ES) were controls. WT, wild type.

AML1^{-/-} ES cells by using the knock-in approach (see Materials and Methods). In each transfection, 20 to 38% of the puromycin-resistant clones underwent homologous recombination at either allele, and two to six clones for each of the knock-in constructs, whose expression was confirmed by RT-PCR analysis (Fig. 5C), were assessed for hematopoietic differentiation, and representative results are illustrated in Fig. 5. When the *AML1b* Δ 446 mutant, which lacks the five most C-terminal amino acids of the VWRPY motif, was expressed, the *AML1*-deficient ES cell clones showed a restored ability to differentiate into definitive hematopoietic lineages (Fig. 5D). Similarly, the clones with a knock-in allele for the *AML1b* Δ 390 mutant, which lacks the 61 C-terminal amino acid residues, retained the ability to rescue the *AML1*-deficient phenotype. The phenotype was reproducible when the procedure was repeated with multiple clones regardless of which allele was targeted. The morphology of the hematopoietic cells that developed from these clones was similar to those observed in *AML1*^{+/-} clones (not shown). In contrast, *AML1b* Δ 320 and *AML1b* Δ 293 failed to rescue the hematopoietic defect (Fig. 5D). Thus, the in vitro rescue assay revealed that the 390 N-terminal amino acid residues of *AML1b* (451 full-length residues) are sufficient to maintain its biological activity in definitive hematopoiesis. By contrast, the 61 C-terminal residues, including the VWRPY motif at the terminus, were not required for this function.

DISCUSSION

In this study, we established an in vitro ES cell experimental system that replicates the hematopoietic defect resulting from the loss of *AML1*. Using this system, we demonstrated that the hematopoietic defect can be rescued by expressing a *PEBP2 α B1* (*AML1b*) isoform under the control of the endogenous *AML1*-regulatory sequences through a knock-in approach. The ES cell clones containing the *AML1* knock-in allele showed a restored ability to differentiate into all lineages of definitive hematopoiesis. These results provide direct evidence that the hematopoietic phenotype found in *AML1*-deficient mice is due solely to the lack of this gene, thus excluding any possibility that the phenotype of the mice might have been influenced by so-called positional artifacts (38). Importantly, the knock-in clones could participate in lymphohematopoiesis in vivo as demonstrated in the chimera mouse analysis, thus suggesting that the behavior observed in the in vitro ES cell experimental system should correspond to their behavior within the context of an intact animal.

We successfully adopted a knock-in strategy to rescue the *AML1*-deficient phenotype in the present study. The experimental strategy that we used targeted *AML1* exon 4, thus resulting in the generation of an artificial allele that retained the ability to be alternatively spliced to produce *AML1b*, *AML1c*, and *AML1* Δ N (17, 28, 56). These *AML1* isoforms contain the presumably full-length C terminus, including the

disrupted on day 6 for Ery-P and on day 10 for definitive precursors, after which 10⁵ cells were subjected to second-step cultures with appropriate CSF (see Materials and Methods). Open bars, grey bars, and dark bars represent *AML1*^{+/-}, *AML1*^{-/-}, and *AML1*^{-/-} with knock-in clones, respectively. (C) Representative colonies of the primitive erythroid lineage observed in the two-step replating assay with EBs on day 6 of culture. The morphology of the colonies in situ (top panels) and of composing primitive erythroid cells stained with May-Gruenwald-Giemsa staining (lower panels) was almost identical among the clones. Magnifications: top panels, \times 50; bottom panels, \times 330. (D) Representative hematopoietic colonies of definitive origin observed in the second-step cultures of day 10 EBs derived from *AML1*^{+/-} and knock-in clones. As shown, the appearance of the colonies and the morphology of the composing cells of definitive erythroid (a), macrophage (b), granulocyte-macrophage (c), and mixed lineages including definitive erythroid (d) of the rescued knock-in clones (*AML1*^{-KI}) were indistinguishable from those observed in the cultures of the control heterozygous clones (*AML1*^{+/-}). Magnifications: upper panels of a, \times 50; upper panels of b to d, \times 20; lower panels, \times 198.

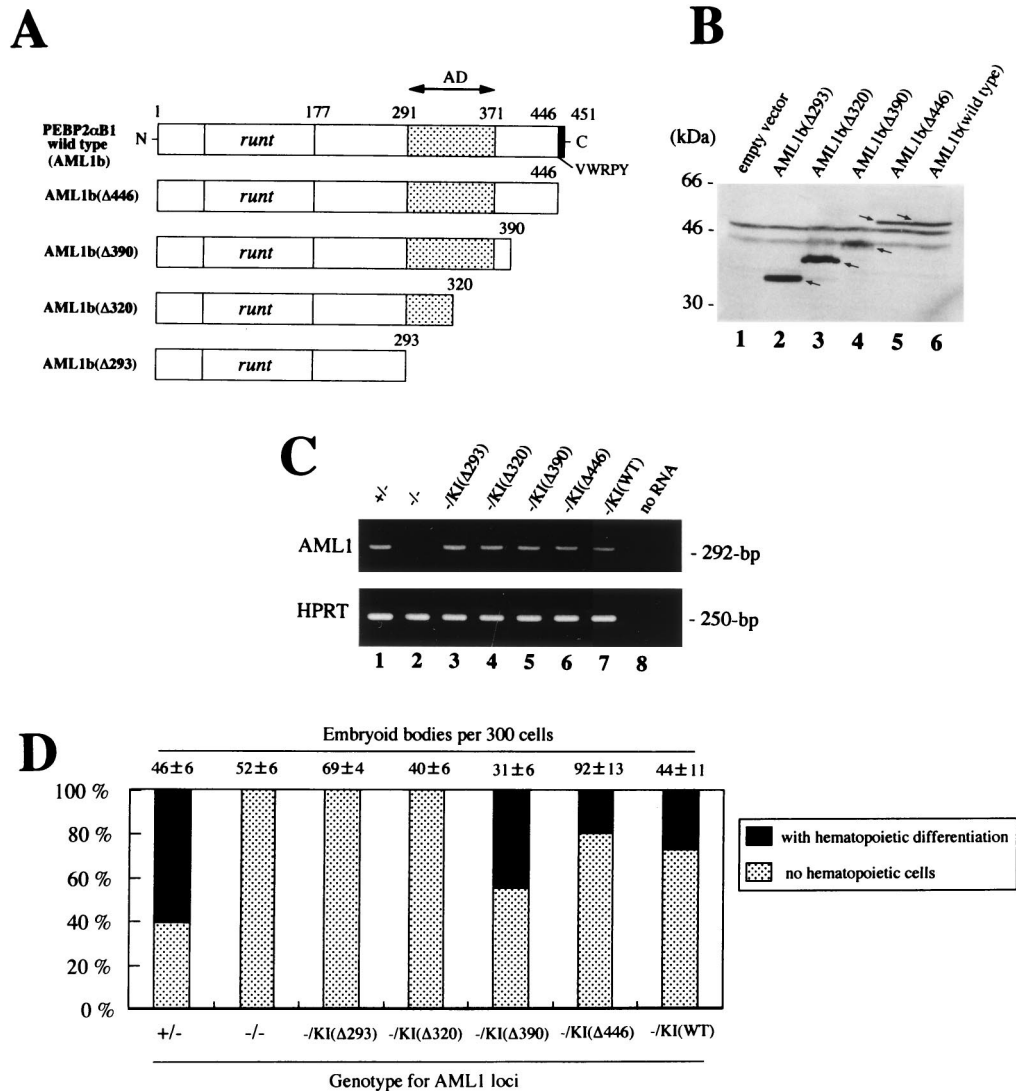


FIG. 5. Biological properties of the C-terminal deletion mutants of AML1b. (A) Schematic representation of the module structure according to Kanno et al. (14) of the wild-type PEBP2 α B1 (AML1b) and each mutant analyzed. (B) The integrity of the mutant constructs was confirmed by Western blot analysis of transiently transfected COS-7 cells. (C) Expression of the exogenous genes in ES cells were confirmed by RT-PCR analysis with primers from exons 3 and 4 (see the legend for Fig. 1D). (D) Hematopoietic differentiation of *AML1*^{-/-} ES knock-in clones expressing the different *AML1b* mutants in a representative experiment. Dark bars indicate the percentage of the EBs with visible hematopoietic cells whereas light bars represent those without a hematopoietic element. Actual numbers of the EBs grown per 300 ES cells are given above each bar as the mean \pm standard deviation of triplicate cultures.

conserved VWRPY motif at the end, and are differentially produced by alternative splicing in their 5' sequences upstream to exon 4 (17, 28, 56). Therefore, our results do not directly assess differences in the biological activities of these isoforms. However, our results demonstrate that the *AML1* isoform(s) which has the longer C terminus is responsible for the biological activity of this gene and that the isoform(s) which lacks the C terminus, such as that encoded by *AML1a* (29), is not required for stem cells to undergo hematopoietic differentiation into definitive lineages. Consistent with these observations, Northern blot analyses revealed that *AML1b* and *AML1c*, which encode polypeptides of 453 and 480 amino acid residues, respectively, are the most common forms expressed in hematopoietic cells in humans in contrast to the minor amount of the *AML1a* message (28). Furthermore, by mobility shift assay analysis with AML1-binding oligonucleotide probes and specific antibodies raised to the AML1 protein, it has been re-

ported that only a full-length AML1 protein was consistently detected in a panel of hematopoietic cell lines (25). While AML1b and AML1c isoforms have been shown to activate transcription in reporter gene assays with *cis* elements of putative target genes, including the M-CSF receptor, GM-CSF, and T-cell antigen receptor genes (1, 24, 50), the *AML1a* isoform, which contains 250 residues and lacks most of the carboxyl terminus downstream from the Runt domain, lacks the ability to activate transcription although it retains the ability to bind to CBF sites and to associate with CBF β (1, 12, 23). Therefore, AML1a is believed to interfere, in a dominant manner, with normal AML1 (or CBF/PEBP2) activity (50), and thus regulation of alternative splicing has been proposed to function in the modulation of the biological activity of AML1. It will be of interest to define the biological role, if any, of the *AML1a* isoform in later stages of hematopoiesis or in other nonhematopoietic cell lineages through the production of

genetically engineered mice that homozygously carry the knock-in *AML1b* allele in their germ line.

Conventional biochemical studies suggest that the transactivation domain of AML1 resides within its C-terminal portion (1, 24, 50). Specifically, a classic transactivating domain is localized to the region between residues 291 and 371, whereas a transcription repression domain is located between 371 and 411 (Fig. 5A) (14). In addition, the C-terminal VWRPY motif (Fig. 5A) was conserved throughout evolution among all known AML1-related molecules and is believed to mediate binding to the transcriptional corepressor Groucho/transducin-like enhancer of split (10, 11, 18), but deletion of this region has little effect on transcriptional activation (14). We used the *AML1*-deficient ES cell rescue system to dissect the functional domains in the C-terminal region. Our results indicate that the transcription activation domain is required for AML1's role in early hematopoiesis. By contrast, the C-terminal region of the protein appears to be dispensable for this biological function. Thus, the biological significance of the conserved C-terminal VWRPY motif remains to be clarified.

In summary, our data demonstrate a critical role for AML1-mediated transcriptional activation in establishing definitive hematopoiesis. These findings, together with the experimental system established in the present study, should contribute to further clarification of the molecular mechanisms of normal and leukemic hematopoiesis.

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