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E2A-Pbx1, the t(1;19) Translocation Protein of Human Pre-B-Cell Acute Lymphocytic Leukemia, Causes Acute Myeloid Leukemia in Mice

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One-quarter of pediatric pre-B-cell leukemias contain the t(1;19) chromosomal translocation, which fuses 5′ exons encoding the transactivation domain of the E2A transcription factor gene to 3′ exons encoding the putative DNA-binding region of the unusual homeobox gene, PBX1. To test the leukemic potential of this fused gene, a cDNA encoding its major protein product, $p85^{E2A-Pbx1}$, was incorporated into a retrovirus construct and introduced into normal mouse marrow progenitors by infection. The cells were used in a bone marrow transplantation protocol to reconstitute the hematopoietic compartments of lethally irradiated recipients. After 3 to 8 months, reconstituted mice developed acute myeloid leukemias that expressed high levels of $p85^{E2A-Pbx1}$ and were readily transmissible to immunocompetent mice. Most acute myeloid leukemias also grew as granulocytic sarcomas and exhibited some neutrophilic differentiation. These results demonstrate a causative role for $p85^{E2A-Pbx1}$ in human acute leukemia and indicate that the oncogenic potential of Pbx1 is not limited to pre-B-cell malignancies.

The t(1;19)(q23;p13.3) chromosomal translocation is found in the leukemic blasts of 25% of children with pre-B-cell acute lymphoblastic leukemia (pre-B ALL) (3, 23). In more than 90% of cases (19), the translocation breakpoint occurs within the same introns of the E2A and PBX1 genes, joining exons of E2A that encode a transcriptional transactivator domain (9) to exons of PBX1 that encode a putative DNA-binding, homeobox domain (Fig. 1) (12, 17), and producing a family of five E2A-Pbx1 fusion proteins (11, 19). Differential splicing, which affects the carboxyl terminus of Pbx1, accounts for the expression of two major E2A-Pbx1 fusion proteins, designated $p85^{E2A-Pbx1}$ and $p77^{E2A-Pbx1}$ (12).

The oncogenicity of E2A-Pbx1 proteins has been tested thus far in NIH 3T3 fibroblasts. When assayed by focus formation, growth in agar, or contact inhibition in NIH 3T3 cells, the transforming activity of $p85^{E2A-Pbx1}$ is poor and that of $p77^{E2A-Pbx1}$ is moderate. However, NIH 3T3 cells expressing either $p85^{E2A-Pbx1}$ or $p77^{E2A-Pbx1}$ form tumors in nude mice (11), indicating that both proteins have malignant potential. Although NIH 3T3 transformation assays indicate that E2A-Pbx1 proteins are oncogenic, direct induction of acute leukemia, particularly of the B-cell lineage, in mice would be the strongest argument that expression of E2A-Pbx1 causes or contributes to the formation of human pre-B ALL containing t(1;19).

To determine the effects of human E2A-Pbx1 proteins on blood cell differentiation, we introduced the major form of E2A-Pbx1, $p85^{E2A-Pbx1}$, into normal mouse marrow cells by infection with a helper-free E2A-Pbx1 recombinant retrovirus (11). Infection of marrow progenitors with E2A-Pbx1 virus did not alter their growth properties in vitro, even when conditions favoring the growth of pre-B cells were used (22). However, reconstitution of lethally irradiated recipient mice with the same infected progenitors consistently resulted in the development of acute myeloid leukemia (AML). AMLs were monoclonal or biclonal, each expressed high levels of $p85^{E2A-Pbx1}$, and all grew in culture immediately after explant. Most were dependent on granulocyte-macrophage colony-stimulating factor (GM-CSF) or interleukin-3 (IL-3) for growth. These data prove that E2A-Pbx1 is a hematopoietic oncogene and reveal that its leukemic potential is not restricted to the pre-B lineage.

MATERIALS AND METHODS

High-titer, helper-free stocks of retrovirus. The cDNA encoding the 85-kDa form of E2A-Pbx1 (11) was cloned into the *BclI* site of the retroviral vector pGD (7). This vector was transfected into the packaging cell line Ψ-CRE, and a clone (Ψ-24L) expressing 5×10^5 infectious units/ml was identified by screening viral RNA preparations from G418-resistant colonies for maximal hybridization to a PBX1 probe.

Adoptive transfer (bone marrow reconstitution). Marrow isolation, infection with retrovirus, and reconstitution of lethally irradiated recipients were performed precisely as described by Daley et al. (7). Briefly, 5 mg of 5-fluorouracil (Sigma) was administered to donor BALB/c female mice by tail vein injection. Six days later, marrow was collected from femurs and tibias of 5-fluorouracil-treated mice. Progenitor cells were purified by centrifugation of marrow on discontinuous Ficoll-Paque (Pharmacia) gradients. Four million purified marrow cells were cocultivated with 10 million Ψ-24L fibroblasts for 48 h in the presence of 10% WEHI-3B supernatant as a source of IL-3 and 2 mg of Polybrene per ml. IL-3 stimulates the growth of most multipotent progenitors and is essential to maintain their viability. The Ψ-24L fibroblasts were treated with 5,000 rads of gamma irradiation prior to use to prevent their continued replication and prevent the subsequent injection of mice with transformed NIH 3T3 fibroblasts. After 48 h of cocultivation, infected donor marrow was collected by squirting the cells off the monolayer and rinsing them twice in cold phosphate-buff-

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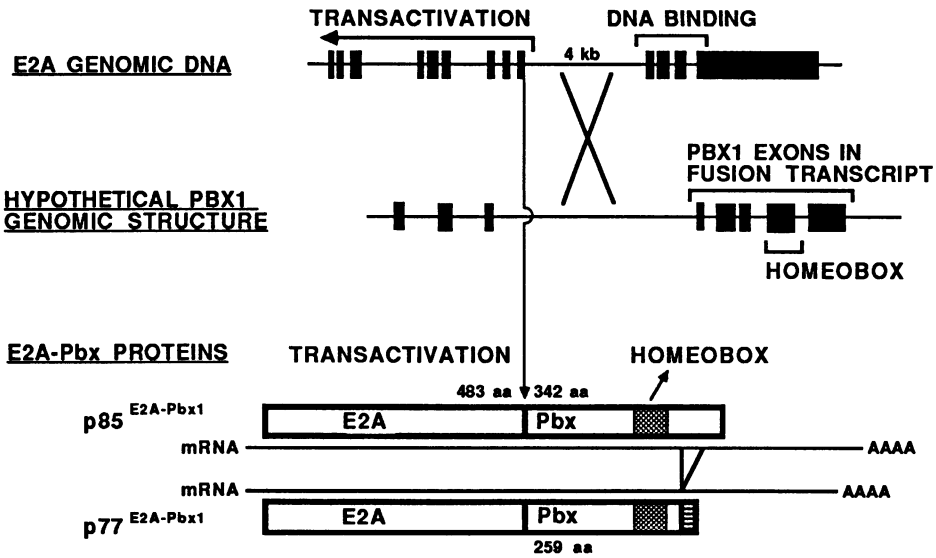

FIG. 1. Depiction of the t(1;19) chromosomal translocation and its effects at the mRNA and protein levels. Exons from E2A encoding an amino-terminal transactivation domain are fused with exons from PBX1 encoding a homeobox DNA-binding domain. At least two spliced variants of the E2A-Pbx1 fusion protein result from this DNA rearrangement. The larger one, designated $p85^{E2A\text{-}Pbx1}$, was used for our experiments because it is the most abundant form produced in pre-B ALL cells containing the t(1;19) translocation (11). aa, amino acids.

ered saline (PBS). Eight BALB/c mice were subjected twice, at 4-h intervals, to 455 rads of gamma irradiation to eliminate endogenous hematopoiesis, and each was then reconstituted with 2×10^6 E2A-Pbx1 virus-infected marrow cells by tail vein injection. Mice were maintained in microisolator cages and were fed sterile food and acid water for 6 weeks prior to reconstitution and forever thereafter.

Transfer of leukemic cells to secondary recipients. Secondary mice were subjected to a single dose of sublethal radiation (450 rads). A total of 2×10^6 cells derived from specified sources (see Table 2) in the primary leukemic mice were injected into the tail vein in a total volume of 200 μl. Mice were maintained in microisolator cages and fed normal food and water.

Clonal analysis of tumors. Samples of genomic tumor DNA were digested with *Eco*RI, which cuts between the E2A-Pbx1 and neomycin cDNAs in the retroviral vector and in unique sites in the host DNA, thereby producing size fragments that are diagnostic of a specific clone of cells. Digested DNA was analyzed on Southern blots, using the neomycin cDNA as a probe.

Cells and antisera. Leukemic cells from mouse AMLs were grown in RPMI 1640 containing 20% Ham's F12 medium, 10% fetal bovine serum, 50 μM 2-mercaptoethanol, and 16 U of GM-CSF per ml (1% supernatant from a B16 melanoma cell line secreting recombinant GM-CSF) and supplemented with glutamine, nonessential amino acids, and antibiotics. LyD9 pre-B cells were grown in RPMI 1640 containing 10% fetal bovine serum, 50 μM 2-mercaptoethanol, and 10% WEHI-3B supernatant as a source of IL-3. Antibodies to Pbx1 were raised in rabbits against a fusion protein between glutathione *S*-transferase and Pbx1 and were affinity purified against the same recombinant fusion protein as described by Kamps et al. (11).

Anti-Pbx1 immunoblotting. Samples of tissue containing leukemic cells were suspended in PBS (10 mM sodium phosphate [pH 7.2], 150 mM NaCl) in a 1.5-ml Eppendorf tube and dispersed by grinding with a Teflon pestle. Marrow was ejected from isolated femurs by cutting off the ends of the bone and forcing out the marrow with a stream of medium from a 25-gauge needle. Cells were washed twice in PBS, counted, and boiled in protein gel sample buffer at a concentration of 5×10^7/ml. Total cellular proteins in 20 μl of each sample were resolved by electrophoresis through 10% polyacrylamide gels and transferred to nitrocellulose. E2A-Pbx1 was stained by incubation of filters in affinity-purified, polyclonal rabbit antibodies to Pbx1 (10 μg/ml) followed by incubation in alkaline phosphatase-conjugated, goat anti-rabbit immunoglobulins and reaction with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate toluidinium as described previously (11).

Immunofluorescence. Binding of rat monoclonal antibodies to B220, Thy 1.2, and Mac-1 and of fluorescein isothiocyanate-conjugated goat anti-rat antibodies was performed on live cells in PBS, after which the cells were fixed in paraformaldehyde and analyzed by immunofluorescence.

RESULTS

E2A-Pbx1 causes an AML-like disease in mice that is accompanied by granulocytic sarcomas. Eight female BALB/c mice, designated HF1 to HF8 (helper-free mice 1 to 8), were transplanted with bone marrow from donor male mice that had been infected by a helper-free population of virus encoding $p85^{E2A\text{-}Pbx1}$. At 11 to 30 weeks posttransplant, mice HF1 to HF7 became sick, as evidenced by patchy fur, lethargy, hind limb paralysis, and enlarged palpable spleens. When mice became critically ill, usually within 7 days of exhibiting these symptoms, they were sacrificed, and their diseases were analyzed (Table 1). The control mouse was a nontransplanted littermate of the transplanted mice. Mouse HF8 was transplanted with E2A-Pbx1 virus-infected marrow, remained asymptomatic, and was sacrificed together with the control mouse at the end of the 33-week study. By comparison with the control, each diseased mouse exhibited an increased fraction of immature blasts in marrow (from 30 to 95% of total nucleated cells versus 10% in normal mice) (Fig. 2D and 2C; Table 1) and an

TABLE 1. Characteristics of E2A-Pbx1 disease in mice

Mouse[a]	Survival (wk)	Leukocyte count (10^3/ml)[b]	Hematocrit	Marrow blast fraction (%)[c]	Spleen (g)	Granulocytic sarcoma (g)[d]	Other sites of tumor involvement	Hind limb paralysis[e]
Control	33	6	49	8	0.08			
HF8	33	4	53	9	0.06			
HF1	11	185	38	76	0.80	0.06 (IP)	Liver	+++
HF1.1		12	22		0.80			
HF2	19	54	42	48	0.33	0.7 (IP)	Ascites fluid, liver, lung	
HF2.1		37	40		0.50	2.9 (PS)	Liver	+++
HF3	21	33	22	86	0.60	1.0 (PS)		+++
HF3.2		3	15		0.75	2.2 (PS)		+++
HF4	21	15	16	95	0.66		Nodes	
HF4.1		12	28		0.90			
HF5	24	8	30	70	0.60	2.7 (IP-PS)	Lung	
HF5.1		12	47		1.60		Liver	
HF6	29	11	50	30	0.20	1.4 (FM)		
HF7	30	11	18	70	1.00	0.18 (PS) 0.14 (IP)	Liver	

[a] HF1.1 to HF4.1 were injected with marrow from leukemic mice HF1 to HF4, respectively, and HF5.1 was injected with spleen cells from leukemic mouse HF5.
[b] Number of nonerythrocytes in whole blood at the time of sacrifice.
[c] Assessed after staining cytospins with Wright-Giemsa stain.
[d] Locations of masses: IP, intraperitoneal; PS, paraspinus; FM, femur.
[e] Absolute block of hind limb movement.

enlarged spleen (2.5- to 20-fold) (Fig. 2A and B; Table 1), and most mice contained proliferating blasts in liver or lung. All sick mice exhibited reduced hematocrits (packed erythrocyte volume) that paralleled the degree to which marrow was replaced by tumor cells (Table 1) and contained an increase in peripheral blood leukocytes (1.5- to 36-fold) due to large numbers of myeloblasts and immature neutrophils. Immunofluorescence analysis indicated that all proliferating blasts were myeloid (positive for the myeloid surface antigen Mac-1 and negative for the B- and pre-B-cell surface antigen B220 and the T- and pre-T-cell surface antigen Thy 1.2; data not shown). Clonal analysis based on unique retroviral integration sites demonstrated that all cells comprising each proliferative disorder were either monoclonal or biclonal and contained, at most, two integrated proviruses (unique sites designated A to L in Table 2; minor populations of distinct clones are designated in parentheses).

Six of the seven mice contained solid masses of leukemic cells, weighing up to 2.9 g, in nonhematopoietic tissue (Fig. 2B, G, I, K, and L), that were in two cases (HF3 and HF6) composed of a unique cell clone (Table 2). These masses appeared to be the mouse cognate of human granulocytic sarcomas (also designated chloromas or myeloblastomas) because they were composed, again, strictly of myeloid cells. In humans, granulocytic sarcoma is a rare myeloid malignancy, accompanying approximately 1% of AMLs, and most often associated with AML or the transition of chronic myeloid leukemia (CML) or myelodysplastic syndrome (MDS) to AML (16). The mouse granulocytic sarcomas exhibited a wide variation in degree of differentiation (Table 2), as do their human counterparts (16). As in human AML (16), they also grew in diverse locations in diseased mice, including the peritoneal cavity and regions adjacent to the spinal column (Table 1). Spinal cord compression resulting from their growth is likely to be responsible for the hind limb paralysis observed in mice HF1, HF2, and HF3 because both intradural and epidural spinal compressions resulting from granulocytic sarcomas have been reported to cause paralysis in humans (10, 15). The mouse granulocytic sarcomas also mimicked their human counterparts by sometimes arising before extensive hematopoietic progression of AML (e.g., mouse HF6).

On the basis of a 30 to 95% abundance of myeloblasts in marrow frequently accompanied by granulocytic sarcomas, these proliferative disorders were diagnosed as AML rather than CML or MDS. Expression of E2A-Pbx1 in tumor tissue was analyzed by Western immunoblotting with antibodies against Pbx1. Tumor cells from marrow, spleen, liver, granulocytic sarcomas, and ascites fluid contained amounts of $p85^{E2A\text{-}Pbx1}$ comparable to those present in the t(1;19)-containing human pre-B ALL cell line 697 (a subset is represented in Fig. 3A), strongly suggesting that retrovirus-induced expression of $p85^{E2A\text{-}Pbx1}$ performs an essential role in the genesis of these leukemias.

Consistent with the behavior of AML in mice, E2A-Pbx1-associated AML was transferred efficiently to secondary recipients (Table 1); each AML that developed in a secondary recipient was derived from one of the original leukemic donor clones (Table 2). (In Table 1, HF1.1 refers to the secondary recipient of 2×10^6 cells from the primary diseased mouse, HF1.)

E2A-Pbx1 disease is marked by factor dependence and blocked differentiation. Each AML exhibited variable degrees of differentiation in vivo. This variation was most strongly demonstrated by analyzing granulocytic sarcomas because these solid masses contain essentially only tumor cells. The granulocytic sarcomas exhibited different degrees of differentiation; some consisted almost solely of myeloblasts (Fig. 2K; Table 2), whereas others contained mostly myelocytes and metamyelocytes (Fig. 2I). Partial differentiation of leukemic cells was also evident in the peripheral blood of mouse HF1 (20-fold-elevated leukocyte count; Fig. 2E) and in the ascites fluid of mouse HF2 (Fig. 2F). Other AMLs exhibited a phenotype indicative of a stronger block in differentiation at the promyelocyte (HF4; Fig. 2J) or myeloblast stage (HF3, HF5, and HF7; Fig. 2H, K, and L, respectively).

Cell lines representing original AML clones from each mouse (Table 2) were readily established by growth in GM-CSF (final concentration, 16 U/ml). All clones main-

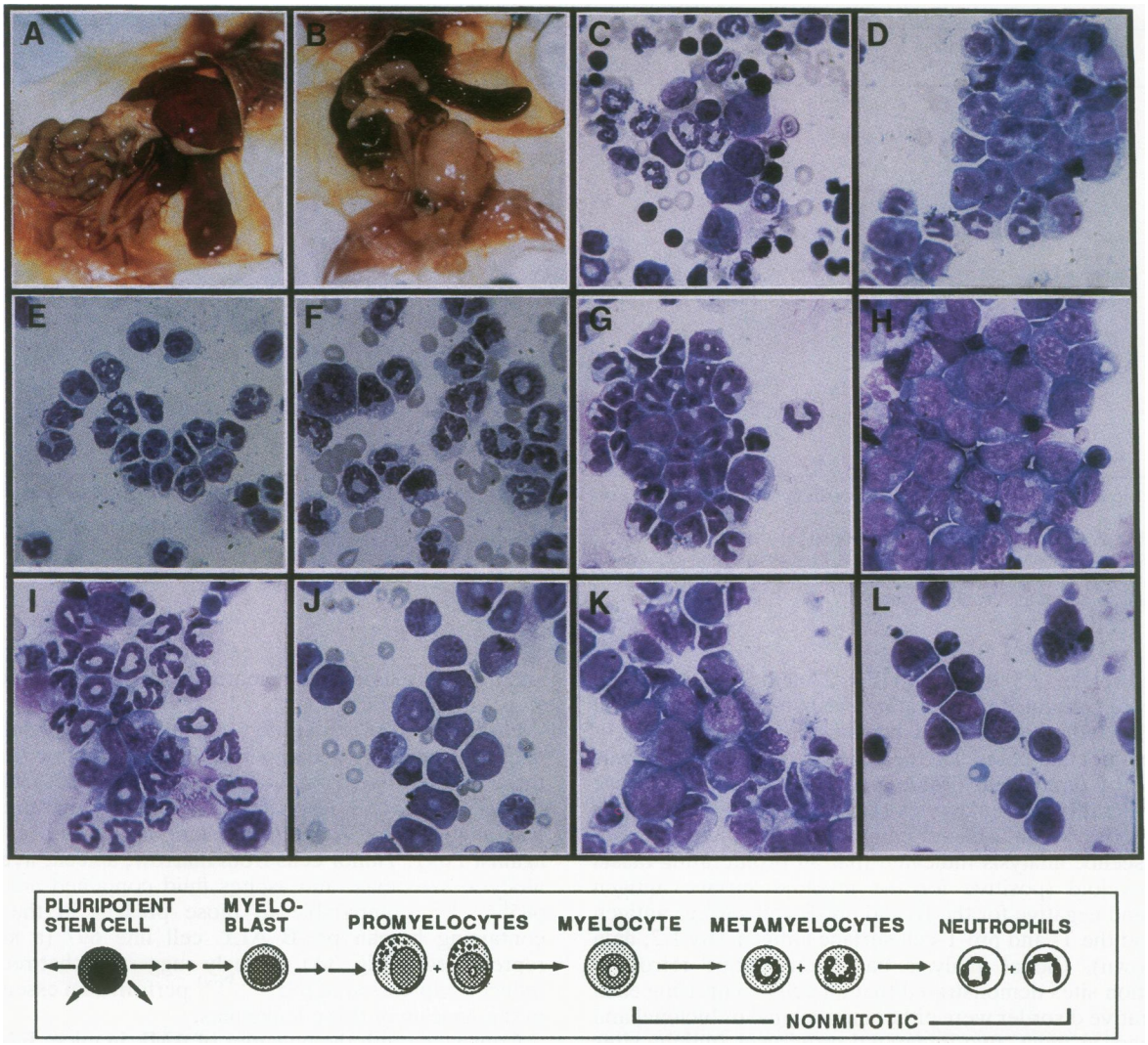

FIG. 2. (A to L) Morphology of AML cells resulting from disease induced in mice by E2A-Pbx1. Seven of the eight reconstituted mice, designated HF1 to HF7, developed AML, each of which was clonal or pauciclonal (Table 2) and all of which contained significant amounts of $p85^{E2A\text{-}Pbx1}$ (Fig. 3). (A and B) Diseased mice at sacrifice. (A) Mouse HF1, exhibiting tumor nodules in the spleen and tumor growth (lighter zones) in the liver (cells pictured in panels D to E); (B) mouse HF5, containing a large granulocytic sarcoma (white mass just below spleen; see panel K). (C to L) Wright-Giemsa stains of cells from normal (C) and leukemic (D to L) tissues at the time of sacrifice. (C) Normal mouse marrow, containing a large myeloblast (upper center) and cells exhibiting properties of normal neutrophil differentiation (a circular nucleus, condensed chromatin, and transparent cytoplasm); (D) marrow from HF1, containing predominantly blasts with limited neutrophil maturation; (E) Ficoll-purified blood cells from HF1; (F) ascites fluid from HF2, containing a large population of myelocytes and neutrophils exhibiting abnormal chromatin condensation, and blue-staining cytoplasm; (G) granulocytic sarcoma from HF2, containing the same clone of cells as represented in ascites fluid (F); (H) myeloblasts from HF3 spleen; (I) granulocytic sarcoma from HF3, representing a clone of malignant cells different from those depicted in panel H; (J) HF4 spleen, exhibiting a promyelocytic phenotype; (K) granulocytic sarcoma from HF5; (L) HF7 spleen, exhibiting a myeloblastic phenotype. Bottom panel, the myeloid differentiation pathway. Myeloid progenitors remain mitotic until the myelocyte stage. Mouse myelocytes develop a distinctive "donut" morphology, which facilitates their easy identification. As chromatin condenses, the cytoplasm becomes virtually transparent to staining.

tained their myeloid phenotype in culture (Mac-1 positive, Thy 1.2 negative, B220 negative) with the exception of clone G from mouse HF4, which also became strongly Thy 1.2 positive. Each line was tested for its dependence on GM-CSF for growth. In the absence of GM-CSF, clones A, G, and L continued to proliferate whereas clones D, E, F, and J died (Fig. 4). These data suggest that one of the target cells for transformation by E2A-Pbx1 is a GM-CSF-dependent myeloid progenitor. The results also demonstrated that $p85^{E2A\text{-}Pbx1}$ does not substitute fully for the viability or growth-stimulatory functions of GM-CSF. Expression of either $p85^{E2A\text{-}Pbx1}$ or $p77^{E2A\text{-}Pbx1}$ in LyD9 pro-B cells (18) also did not alter their IL-3 dependence, demonstrating that the function of E2A-Pbx1 is distinct from that of v-Abl or Bcr-Abl, both of which abrogate IL-3 dependence (5, 6).

A major defect in each AML clone was its inability to differentiate in response to GM-CSF. When cultured in 16 U of GM-CSF per ml, a clone from each AML grew out as a cell line. All cell lines except clone G consisted of myeloblasts, 1 to 20% of which resembled myelocytes or metamyelocytes (Fig. 2, bottom panel). In contrast to the behavior of the AML cell lines, normal GM-CSF-responsive progenitors

TABLE 2. Analysis of clonality and degree of differentiation of primary and secondary AMLs

Mouse	Tissue	% Maturing neutrophils[a]	Integration site designation(s)[b]	Site(s) in cell line[b]
HF1	Blood	73	A, (B)	
	Spleen	47	A, (B)	A
	Liver	50	A, (B)	
HF1.1	Spleen	21	A	
HF2	Spleen	67	C	
	Granulocytic sarcoma	78	C, (D)	D, (C)
	Ascites fluid	89	C	
HF2.1	Spleen	43	C	
	Granulocytic sarcoma	53	C	
HF3	Spleen	7	E	E
	Granulocytic sarcoma	93	F	E
HF3.1	Spleen	0.2	E	
	Granulocytic sarcoma	1	E	
HF4	Spleen	2[c]	G	G
HF4.1	Spleen	2	G	
HF5	Spleen	14	H	
	Granulocytic sarcoma	6	H, (I)	H, (I)
HF5.1	Spleen	4	H	
HF6	Marrow	—[d]	J	J
	Granulocytic sarcoma	33	K	
HF7	Spleen	4	L	L

[a] Fraction of cells comprising the nonmitotic fraction of tumor cells (metamyelocytes, banded forms, and mature neutrophils; see Fig. 2C).
[b] Minor populations of distinct clones are indicated in parentheses.
[c] Of the 98% of immature cells, all of which were heavily granulated (Fig. 2J), 50% contained nuclei exhibiting the "donut" morphology.
[d] Normal myelopoiesis precluded an accurate determination of differentiation of the malignant clone.

isolated from marrow proliferate and undergo terminal differentiation to neutrophils and macrophages within 4 weeks of being cultured in 16 U of GM-CSF per ml. None of the AML clones were induced to differentiate by the addition of G-CSF to media containing GM-CSF, nor did G-CSF alone substitute for GM-CSF or induce differentiation in GM-CSF-independent clones.

After cultivation in GM-CSF-containing medium for 5 months, each AML maintained expression of E2A-Pbx1 at levels comparable to that found in the human pre-B ALL line 697 (Fig. 3C). Although cells from HF5 initially expressed only $p85^{E2A\text{-}Pbx1}$ (Fig. 3A, lanes 10 and 11), they expressed both full-length $p85^{E2A\text{-}Pbx1}$ and a shorter Pbx1-containing protein after cultivation in vitro for 4 months (Fig. 3C, lane 3). It is possible that the mutation in this form of E2A-Pbx1 contains an activating carboxyl-terminal deletion similar to that which enhances the transforming potential of $p77^{E2A\text{-}Pbx1}$ (11).

DISCUSSION

E2A-Pbx1-induced disease was diagnosed as AML on the basis of its (i) myeloid immunologic and histologic phenotype, (ii) high fraction of marrow blasts, (iii) presentation with granulocytic sarcoma, (iv) variable level of circulating myeloid progenitors, (v) ease of transmission to secondary recipients, and (vi) ease of establishment as GM-CSF-dependent cell lines in culture. E2A-Pbx1 disease was not diagnosed as CML or MDS. In humans, CML is consistently accompanied by a large increase in the abundance of mature and maturing neutrophils in the periphery. In three cases, HF1, HF2, and HF3, E2A-Pbx1 disease was accompanied by substantial increases in circulating neutrophil progenitors that typify CML; however, the rapidly rising leukocyte count in these mice occurred coincident with the growth of granulocytic sarcomas, a high marrow blast fraction, and efficient transplantability of the leukemic clone in secondary mice, arguing that the disease represented AML. In addition, some human AMLs, specifically the M2 subgroup containing the t(8;21) translocation, exhibit substantial neutrophilic differentiation of circulating leukemic cells (1). The transplantability of E2A-Pbx1 disease distinguishes it from the CML-like diseases induced by retrovirus-mediated expression of $p210^{Bcr\text{-}Abl}$ or $gp120^{v\text{-}Fms}$, which are characterized by a dramatic increase in mature, circulating neutrophils and which are nontransplantable (8, 24). E2A-Pbx1 disease was distinct from MDS, which in humans is characterized by an enlarged spleen in only 20% of cases, a suppressed leukocyte count, and less than 30% blasts in marrow, with cells exhibiting morphologic evidence of abnormal myeloid cell development. In addition, MDS itself is not accompanied by the growth of granulocytic sarcomas, which are observed in five AML-associated contexts: (i) preceding AML in apparently normal individuals, (ii) accompanying AML, (iii) accompanying blast crisis in CML, (iv) accompanying conversion of MDS to AML, or (v) fully independent of other detectable AML, CML, or MDS (4, 13, 16, 21, 24–26). Finally, it is possible that E2A-Pbx1-associated AMLs were preceded either by MDS or by a very brief CML-like phase.

Expression of E2A-Pbx1 alone appears insufficient to produce AML. Because E2A-Pbx1 itself did not induce factor independence, the lymphokine-independent growth of clones A, G, and L indicates that secondary mutations abrogating factor dependence have probably occurred. These mutations may complement the function of E2A-Pbx1 in producing overt AML in these cases. The hypothesis for secondary mutation is also supported by analysis of mouse HF8, which remained asymptomatic 8 months posttransplantation even though its marrow and spleen expressed moderate amounts of $p85^{E2A\text{-}Pbx1}$ (Fig. 3B; marrow appeared normal as assessed from Wright-Giemsa stains). The clonality of each leukemia also suggests that an additional mutation(s) might complement E2A-Pbx1 expression to produce dominant outgrowth of a single cell. Secondary mutations

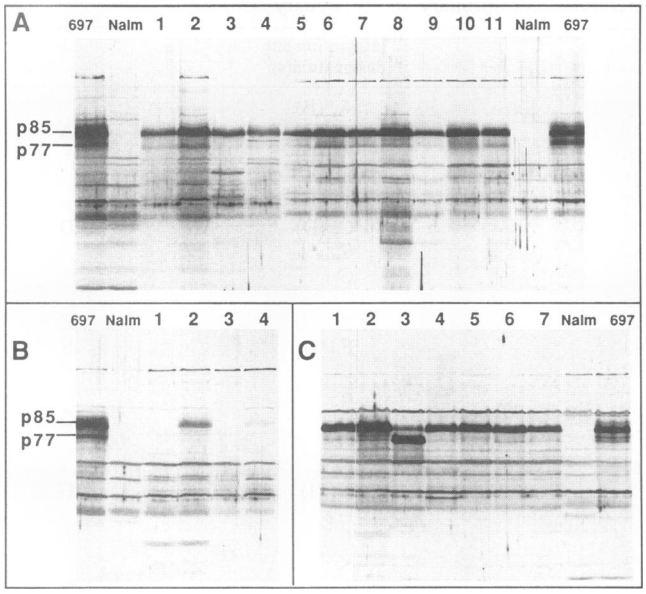

FIG. 3. Immunoblot analysis of E2A-Pbx1 proteins in mouse AMLs. Samples of leukemic cells were isolated from tissue, rinsed in PBS, and subjected to anti-Pbx1 immunoblotting as described in Materials and Methods. Lanes 697 contain extracts from human 697 pre-B cells, which contain the t(1;19) translocation; lanes Nalm contain extracts from human Nalm-6 pre-B cells, which do not contain t(1;19). p85 and p77 indicate the positions of native $p85^{E2A\text{-}Pbx1}$ and $p77^{E2A\text{-}Pbx1}$ from 697 cells. (A) Lanes: 1 to 4, HF1 marrow, spleen, Ficoll-purified peripheral blood leukocytes, and liver, respectively; 5 to 7, HF7 mass, spleen, and marrow, respectively; 8 and 9, HF6 mass and spleen, respectively; 10 and 11, HF5 mass and spleen, respectively. (B) Immunoblot performed on tissues from a nontransplanted mouse and from a mouse that was transplanted with marrow infected by the $p85^{E2A\text{-}Pbx1}$ virus yet remained asymptomatic after 8 months (HF8). Lanes: 1, normal marrow; 2, HF8 marrow; 3, normal spleen; 4, HF8 spleen. (C) Anti-Pbx1 immunoblot containing extracts from cell lines L, J, H, G, E, D, and A (lanes 1 to 7, respectively), as defined in Table 2.

might account for the wide variance in differentiation exhibited by each AML.

One mechanism for the relatively long latency of disease in transplanted mice might be the activation of endogenous helper virus or recombination of the E2A-Pbx1 retroviral vector with an endogenous host provirus to generate a myelotrophic helper virus. If this were to happen, one might argue that the AMLs observed in this model were induced by helper virus alone. Two experiments strongly discount this possibility. First, if any of the mouse AMLs contained helper virus, they should produce virions containing the recombinant E2A-Pbx1 genomic RNA that could confer G418 resistance upon infected NIH 3T3 cells. Samples (6 ml) of culture supernatants from clones A, E, G, H, J, and L were tested for this activity, and none were positive. Second, we have now demonstrated directly that helper-free virus encoding $p85^{E2A\text{-}Pbx1}$ will reproducibly immortalize primary marrow GM-CSF-dependent progenitors within 4 weeks of infection of primary marrow only when these cells are cultivated in the presence of GM-CSF, whereas infection by an equivalent titer of the parental neomycin virus, or helper virus alone, does not result in the outgrowth of myeloblasts (10a). These results support the suggestion that $p85^{E2A\text{-}Pbx1}$ is essential for the genesis of AML in this mouse model and demonstrate that insertional mutagenesis by a helper provirus is an unlikely mechanism for generating complementary oncogenic mutations.

By what molecular mechanisms does $p85^{E2A\text{-}Pbx1}$ cause AML? One mechanism would be competition with other Pbx homeobox proteins for a single specific DNA recognition sequence that regulates transcription of a family of Pbx-responsive genes. Three PBX genes (PBX1 to PBX3), encoding a distinct family of homeobox proteins, have been sequenced thus far (14; our unpublished observations) and are widely expressed in both fetal and adult tissue (11, 14). The proteins that they encode contain almost identical homeoboxes, exhibit extensive amino acid identity in nonhomeobox sequences, and diverge significantly in only small amino-terminal and carboxyl-terminal domains. Therefore,

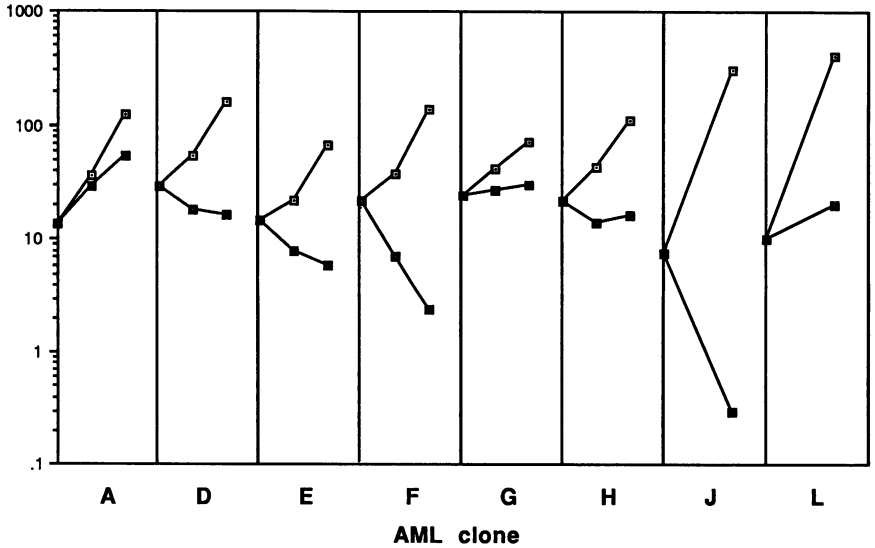

FIG. 4. Growth factor dependence of cell lines derived from AMLs. Approximately 100,000 cells from cell lines representing each AML (Table 2) were grown in the presence (open squares) or absence (filled squares) of GM-CSF for 5 days. The vertical axis represents cell populations in thousands. Cell clones were derived from each mouse as follows: clone A (HF1), clone D (HF2), clone E (HF3 spleen), clone F (HF3 mass), clone G (HF4), clone H (HF5), clone J (HF6), and clone L (HF7).

it is possible that all Pbx proteins bind the same DNA sequence motif and that a minimum threshold of $p85^{E2A-Pbx1}$ is required to compete with normal Pbx proteins and interfere with their regulation of Pbx-responsive genes. Alternatively, overexpression of $p85^{E2A-Pbx1}$ might titrate out an E2A- or Pbx-binding protein that is required for normal differentiation. Answers to these mechanistic questions are the subject of current investigations aimed at determining how the structural and biochemical properties of E2A-Pbx1 govern its transforming abilities.

The association of $p85^{E2A-Pbx1}$ with AML in mice raises the possibility that Pbx1 as well as Pbx2 and Pbx3 has oncogenic roles in other malignancies. However, the lack of pre-B disease in mice engrafted with $p85^{E2A-Pbx1}$-expressing marrow is puzzling. The dominant AML phenotype might arise if the population of myeloid progenitors susceptible to transformation by $p85^{E2A-Pbx1}$ vastly outnumbered that of B-lymphoid progenitors or if the transforming function of E2A-Pbx1 is more pronounced in myeloid progenitors than it is in B-lymphoid progenitors. It is also possible that the correct pre-B target cell is not efficiently infected when marrow is cultured with virus-producing cells prior to introduction into recipients; however, because experiments using the same retroviral vector encoding $p210^{Bcr-Abl}$ led to pre-B ALL in some mice (7), we would argue that expression of $p85^{E2A-Pbx1}$ does occur at least in this pre-B-cell progenitor. Alternatively, the need for mutation of a second gene, such as N-*ras*, which is activated in a low frequency of childhood ALLs and AMLs (2, 20), might occur more frequently in the myeloid lineage than it does in the lymphoid lineage in mice. Finally, marrow from 6-week-old mice may not contain a target cell equivalent to that which undergoes the t(1;19) chromosomal translocation in the generation of pre-B ALL in humans. However, regardless of the reasons that account for the absence of pre-B-cell disease in this mouse model, we postulate that the transforming function of E2A-Pbx1 in human pre-B ALL is likely to parallel its transforming function in mouse AML.

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