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E2a/Pbx1 Induces the Rapid Proliferation of Stem Cell Factor-Dependent Murine Pro-T Cells That Cause Acute T-Lymphoid or Myeloid Leukemias in Mice

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Oncoprotein E2a/Pbx1 is produced by the t(1;19) chromosomal translocation of human pre-B acute lymphoblastic leukemia. E2a/Pbx1 blocks differentiation of primary myeloid progenitors but, paradoxically, induces apoptosis in established pre-B-cell lines, and no transforming function of E2a/Pbx1 has been reported in cultured lymphoid progenitors. Here, we demonstrate that E2a/Pbx1 induces immortal proliferation of stem cell factor (SCF)-dependent pro-T thymocytes by a mechanism dependent upon both its transactivation and DNA-binding functions. E2a-Pbx1 cooperated with cytokines or activated signaling oncoproteins to induce cell division, as inactivation of conditional E2a/Pbx1 in either factor-dependent pro-T cells or pro-T cells made factor independent by expression of Bcr/Abl resulted in pro-T-cell quiescence, while reactivation of E2a/Pbx1 restored cell division. Infusion of E2a/Pbx1 pro-T cells in mice caused T lymphoblastic leukemia and, unexpectedly, acute myeloid leukemia. The acute lymphoblastic leukemia did not evidence further maturation, suggesting that E2a/Pbx1 establishes an early block in pro-T-cell development that cannot be overcome by marrow or thymic microenvironments. In an E2a/Pbx1 pro-T thymocyte clone that induced only pro-T acute lymphoblastic leukemia, coexpression of Bcr/Abl expanded its leukemic phenotype to include acute myeloid leukemia, suggesting that unique functions of cooperating signaling oncoproteins can influence the lymphoid versus myeloid character of E2a/Pbx1 leukemia and may cooperate with E2a/Pbx1 to dictate the pre-B-cell phenotype of human leukemia containing t(1;19).

The t(1;19) translocation is found predominantly in pediatric pre-B-cell acute lymphocytic leukemias (9) but can also be identified in a small number of acute T-lymphoid and myeloid leukemias (43). This translocation produces E2a/Pbx1, a chimeric oncoprotein containing the transcriptional activation domains of E2a fused with the majority of the Pbx1 protein, including its DNA-binding homeodomain. An alternate recurring t(1;19) translocation fuses the same region of E2a with Pbx1 sequences immediately upstream of the homeodomain, eliminating approximately half of the Pbx1 N-terminal sequences retained by the major translocation protein. The topography of E2a/Pbx1 oncoproteins therefore suggests the importance of activation by E2a and DNA binding by Pbx1.

No leukemic property of E2a/Pbx1 has been identified in pre-B cells. Transgenic expression of E2a/Pbx1 from the immunoglobulin kappa light chain promoter does, however, cause a late pre-T-cell acute leukemia (11), though no cognate function of E2a/Pbx1 has been observed in cultured thymocytes. Two observations prompt caution in assuming that the mechanism of pre-T leukemia in transgenic mice is similar to that of pre-B leukemia in humans. First, the pre-T-cell disease does not require the majority of Pbx1, including the homeodomain. Second, because the E2a/Pbx1 disease in the transgenic model is similar to the pro-T-cell leukemias that arise in E2a knockout mice, it may proceed via a dominant-negative mechanism in which the chimeric E2a oncoproteins sequester coactivators bound to the two E2a activation motifs, thereby disrupting the function of endogenous E2a proteins. In contrast to the forms of E2a/Pbx1 that lack the homeodomain yet are active in the transgenic model, the fact that both forms of E2a/Pbx1 found in human pre-B acute lymphoblastic leukemia contain the Pbx1 homeodomain suggests that direct gene targeting by E2a/Pbx1 is critical for the development of human pre-B-cell acute lymphoblastic leukemia.

In mice, E2a/Pbx1 also induces acute myeloid leukemia with an average latency of 5 months following adoptive transfer of primary marrow transduced with E2a/Pbx1 retrovirus (21). This myeloid phenotype is paralleled in culture by the ability of E2a/Pbx1 to block the differentiation of granulocyte-macrophage colony-stimulating factor- or interleukin (IL)-3-dependent myeloid progenitors, resulting in the rapid outgrowth of immortal, factor-dependent myeloid progenitor cell lines (22). When conditional, estrogen-dependent forms of E2a-Pbx1 are used in this myeloid immortalization assay, their inactivation results in cell cycle arrest and maturation to neutrophils or monocytes (40). In this assay, differentiation arrest by E2a/Pbx1 requires the Pbx1 homeodomain and may be a more accurate model of the requirements for human pre-B-cell acute lymphoblastic leukemia. E2a/Pbx1-immortalized myeloid progenitors are incapable of initiating acute myeloid leukemia in irradiated recipient mice, suggesting that the immortalized myeloblast is not the cell that initiates myeloid leukemia in the adoptive transfer model, which as yet remains undefined.

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E2a/Pbx1 also induces proliferation of NIH 3T3 fibroblasts, and sequences required to induce proliferation can be segregated from sequences required to block myeloid differentiation based on mutational analysis (5). The fact that both forms of E2a/Pbx1 encoded by human translocations retain mitotic function suggests that mitogenesis is likely an important oncogenic function of E2a-Pbx1 required for human pre-B acute lymphoblastic leukemia.

Here we report the identification of a new single-hit function of E2a/Pbx1 in murine lymphoid progenitors, the induction of primary pro-T thymocyte proliferation. This observation was further supported by conditional forms of E2a/Pbx1, which reversibly induced pro-T thymocyte proliferation. Both forms of E2a/Pbx1 involved in human pre-B acute lymphoblastic leukemia induced pro-T thymocyte proliferation, while those nonmitotic variants still capable of blocking myeloid differentiation failed to induce pro-T thymocyte outgrowth. This suggests that E2a/Pbx1 may contribute to human pre-B-cell acute lymphoblastic leukemia by stimulating pre-B-cell proliferation and that the genesis of pre-B acute lymphoblastic leukemia in humans likely requires both mitotic and differentiation arrest functions.

In mice, E2a-Pbx1 pro-T thymocytes could cause pro-T-cell leukemias, suggesting that E2a/Pbx1 also establishes T lineage differentiation arrest. Other E2a/Pbx1-immortalized pro-T thymocytes could cause acute myeloid leukemia, suggesting that an immature pro-T cell may represent the target cell that initiates myeloid leukemia in the retroviral/adoptive transfer model. The possibility of transdifferentiation of the E2a-Pbx1 pro-T-cell in vivo suggests that secondary mutations can alter lineage definition. Indeed, coexpression of $p190^{Bcr/Abl}$ consistently shifted the phenotype of leukemias initiated by an E2a-Pbx1 pro-T-cell clone from pro-T-cell acute lymphoblastic leukemia to acute myeloid leukemia. These observations suggest that lineage specificity of human leukemias may not simply be the passive consequence of random oncogene formation followed by selective outgrowth of the committed progenitor but may actually be the active consequence of specific oncoproteins that have the ability to determine cell fate.

MATERIALS AND METHODS

Expression vectors. The E2a/Pbx1, E2a/Pbx1Δ623 (Δ487-623), and E2a/Pbx1Δ623ER constructs were described previously (5, 40). The cDNA encoding $p190^{Bcr/Abl}$ was the kind gift of Richard Van Etten and was subcloned into the MSCVneo retroviral vector (18).

Cell culture. Cells were maintained in a 37°C humidified incubator with 5% CO_2. 293T embryonic kidney cells were grown in Dulbecco's modified Eagle's medium (Cellgro) supplemented with 4.5 g of glucose per liter, 10% fetal bovine serum (Gemini, Woodland, Calif.), and penicillin-streptomycin-L-glutamine (PSQ; Invitrogen, Carlsbad, Calif.). BL3 cells (48) were maintained in RPMI 1640 medium (Cellgro) supplemented as above. Scid.adh cells (8) were maintained in Iscove's modified Dulbecco's medium (Invitrogen) supplemented with 10% fetal bovine serum, PSQ, 50 μM β-mercaptoethanol (M7522, Sigma, St Louis, Mo.), nonessential amino acids (Invitrogen), and sodium pyruvate (Invitrogen). The cytokine-producing CHO (stem cell factor [SCF]) and J558 (IL-7) cell lines were maintained in OptiMem (Invitrogen) supplemented with 10% fetal bovine serum, PSQ, and β-mercaptoethanol. Where applicable, β-estradiol (E2758, Sigma) was added to the culture medium at a final concentration of 1 μM from a 10,000× stock in 100% ethanol.

Retroviral infection of primary murine marrow progenitors. Total bone marrow was harvested from the femurs and tibia of 8 to 12 week old female BALB/c mice, and bone marrow mononuclear cells were isolated on a Ficoll-Paque gradient (Pharmacia, Piscataway, N.J.). Mononuclear cells were cultured for 2 days in OptiMem medium (Invitrogen) supplemented with 10% fetal bovine serum, PSQ, 50 μM β-mercaptoethanol, SCF, and IL-7. SCF and IL-7 were added as 1:100 dilutions of conditioned medium from CHO (SCF) and J558 (IL-7) cells. Helper-free retrovirus was prepared by calcium phosphate transfection (Invitrogen, Carlsbad, Calif.) of 293T cells with murine stem cell virus (MSCV) retroviral constructs and an ecotropic proviral packaging vector. Following the 2-day stimulation in SCF and IL-7, 250,000 cells in 250 μl of complete medium were infected with 1 ml of retroviral supernatant supplemented with Lipofectamine (final 1:1,000; Invitrogen). Spinoculation was performed in nontissue culture plates coated for 2 h at 37°C with 10 μg of fibronectin (F0895, Sigma) per ml in phosphate-buffered saline (PBS). The PBS was aspirated, and cells and retroviral supernatant were added. The plates were spun for 2 h at 2,500 × *g* at 22°C. Following spinoculation, 2 ml of medium was added to each well. The cells were fed and passaged to a fresh plate every 3 to 5 days.

Leukemia assays. Female BALB/c mice, 8 to 12 weeks old, were sublethally irradiated (450 rads). Cells were thoroughly washed in PBS and resuspended at 2×10^7/ml. The mice received 100 μl of either test cells or PBS by tail vein injection. Mice exhibiting a leukemic phenotype (lethargy, scruffy fur, hind limb paralysis, splenomegaly) were sacrificed, and samples were taken of peripheral blood, marrow, spleen, thymus, lymph node, and extramedullary tumors, such as granulocytic sarcomas. Wright-Giemsa staining (3-min Wright's, 9-min 20% Giemsa; Sigma) was performed on cytocentrifuged (Thermo Shandon, Pittsburgh, Pa.) preparations of cells.

Immunoblot analysis of E2a/Pbx1 proteins. Protein from 5×10^4 cells was resolved by sodium dodecyl sulfate–7.5% polyacrylamide gel electrophoresis (SDS–7.5% PAGE) and transferred to a polyvinylidene difluoride membrane (Pall). Membranes were blocked in Tris-buffered saline plus 0.1% Tween 20 containing 5% nonfat milk, and immunolocalization was performed with monoclonal mouse anti-human E2a (E12/E47) antibody (G193-86, PharMingen) and detected by horseradish peroxidase-linked rabbit anti-mouse immunoglobulin (NEB) and chemiluminescence (Pierce).

Flow cytometric analysis. All fluorescently labeled monoclonal antibodies (Thy1.2^{FITC}, CD3^{PE}, CD25^{APC}, CD44^{PE}, CD117^{PE}, GR-1^{FITC}, Mac-1^{APC}, Mac-1^{FITC}, CD19^{PE}, B220^{FITC}, Sca-1^{FITC}, and CD135^{PE}, where FITC is fluorescein isothiocyanate, PE is phycoerythrin, and APC is allophycocyanin) were purchased from BD PharMingen (San Diego, Calif.). Cells isolated from the organs of leukemic and control mice were subjected to red blood cell lysis prior to analysis. Antibody binding was performed on 10^6 cells for 30 min at 4°C in PBS–1%fetal bovine serum–0.1%NaN_3. Cells were washed and resuspended in the same buffer with 2 μg of propidium iodide per ml. Flow cytometry data were acquired with the program Cellquest on either a FACScan (Becton Dickinson, San Jose, Calif.) or FACScalibur bench top flow cytometer. Live cells were gated for analysis by forward and side scatter signals and lack of propidium iodide staining.

Northern blot analysis. Cells (5×10^7 to 10^8) were grown in the presence or absence of 1 μM β-estradiol as indicated. Cytoplasmic RNA was purified (RNEasy, Qiagen, Valencia, Calif.), 15 μg was resolved by formaldehyde–1% agarose gel electrophoresis, and capillary transferred to a positively charged nylon membrane (GeneScreen Plus, NEN, Boston, Mass.). Membranes were dried for 2 h at 80°C in a vacuum oven prior to hybridization. [^{32}P]dCTP-labeled DNA probes were prepared from 100 ng of DNA subjected to random hexamer oligolabeling (Pharmacia). Hybridization in Ultrahyb (Ambion, Austin, Tex.) and washing were carried out at 42°C according to the manufacturer's protocols.

Southern blot analysis of retroviral integration. Genomic DNA was isolated from 5×10^7 cells subjected to overnight sodium dodecyl sulfate-proteinase K digestion, phenol-chloroform extraction, and ethanol precipitation. Then 10 μg of genomic DNA was digested with either *Eco*RI or *Bam*HI and resolved on a 0.7% agarose gel. The DNA was fragmented, capillary transferred to a positively charged nylon membrane (GeneScreen Plus, NEN), and dried for 2 h at 80°C in a vacuum oven. DNA probes corresponded to the neomycin resistance cassette in the MSCVneo vector or to a portion of the *Abl* gene found in Bcr/Abl.

Caspase assays. Caspase activity was assayed on fluorescent substrates with protein extracts harvested over 24 h.

RESULTS

E2a/Pbx1 but not Pbx1 induces rapid, immortal proliferation of an SCF-dependent pro-T1/T2 progenitor. To test the hypothesis that pre-B-cell cytokines might permit a transforming activity of E2a/Pbx1 to be evidenced in cultured immature B cells, primary marrow was infected with retrovirus encoding

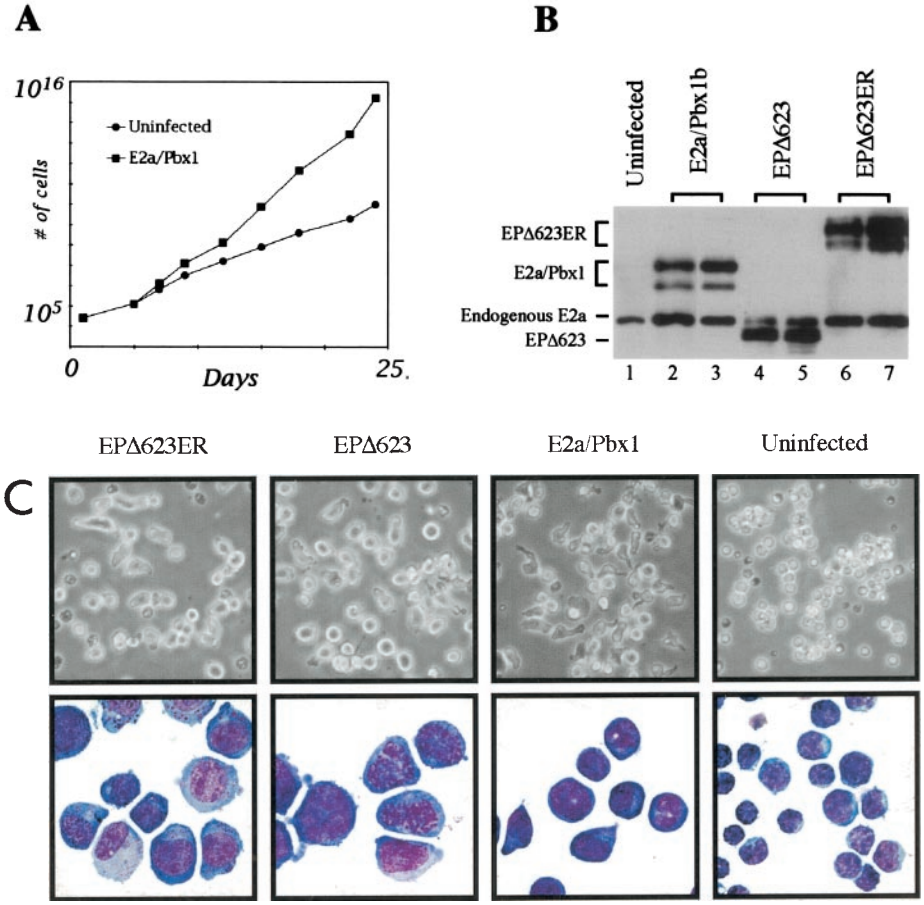


FIG. 1. E2a/Pbx1 immortalizes early T-cell progenitors from primary marrow cultured in SCF and IL-7. (A) Proliferation of normal marrow (circles) and marrow infected with E2a/Pbx1 retrovirus (squares), both cultured in SCF plus IL-7. (B) Anti-E2a immunoblot demonstrating the presence of E2a/Pbx1, EPΔ623, and EPΔ623ER in cell outgrowths. (C) Light micrographs and Wright-Giemsa staining of E2a/Pbx1-induced outgrowths, which evidence a large progenitor phenotype and a high nuclear-cytoplasmic ratio, compared with uninfected cultures, which are comprised of pre-B lymphocytes. EPΔ623 designates the alternative version of the E2a/Pbx1 translocation of human pre-B acute lymphoblastic leukemia, and EPΔ623ER designates a conditional form of the same protein produced by fusion with the estrogen-binding domain of the estrogen receptor. (D) Flow cytometric analysis of the primary marrow target cells cultured in SCF and IL-7 for 3 days, uninfected cells cultured in SCF and IL-7 for 56 days, and a population of E2a/Pbx1-immortalized cells cultured in SCF and IL-7 for 21 days. Though there is some variability between clones in CD44 staining, the majority of clones are strongly CD44 positive. (E) An abbreviated schematic depicts the stages of T-lymphoid maturation and accompanying cell surface staining.

Pbx1a, Pbx1b, E2a/Pbx1a, or E2a-Pbx1b and cultured in medium containing SCF and IL-7. Control retrovirus as well as retrovirus encoding the myeloid oncoprotein Hoxb8, Hoxa9, or Nup98-Hoxa9 were used to infect parallel cultures to determine E2a-Pbx1-specific effects.

All oncoproteins were expressed efficiently, as evidenced by immunoblot analysis of lysates from infected NIH 3T3 fibroblasts (data not shown). Uninfected cultures evidenced proliferation of $CD19^+$ pre-B progenitors, and infection with retrovirus encoding Hoxb8, Hoxa9, or Nup98-HoxA9 did not alter the identity or proliferation rate of $CD19^+$ pre-B progenitors. In contrast, retrovirus expressing E2a/Pbx1a or E2a/Pbx1b induced the outgrowth of a distinct population of large, rapidly proliferating progenitors. This population constituted 50% of cultures by day 7 and 90% of cultures by day 10 (Fig. 1A), quickly outcompeting the $CD19^+$ pre-B progenitors. The outgrowths strongly expressed E2a/Pbx1 (Fig. 1B, lanes 2 and 3), were SCF dependent for proliferation and viability, and exhibited a "tennis racket/hand mirror" morphology characteristic of T-cell progenitors (Fig. 1C, panel 2 versus control pre-B cells in panel 1).

Ten independent E2a/Pbx1-induced outgrowths (populations) and 15 clonal derivatives were evaluated by flow cytometry. E2a/Pbx1-induced populations were c-kit$(CD117)^+$, $CD44^+$, Sca-1^-, $CD3^-$, $CD4^-$, $CD8^-$, $B220^-$, $CD19^-$, $MacI^-$, Thy$1.2^{variable}$, and $CD25^{variable}$ (Fig. 1D), a pattern of surface antigen expression corresponding to the pro-T1 and pro-T2 stages of T-cell differentiation (Fig. 1E). Clonal variability in CD25 was stable during long-term passage. These experiments demonstrate that pro-T-cell proliferation is an activity associated specifically with E2a-Pbx1 but not with normal Pbx1.

E2a-Pbx1 T-cell progenitors exhibited immortal proliferation. Uninfected CD19 pre-B-cell progenitors entered "crisis" after approximately 45 generations (approximately 6 weeks postexplant), characterized by coexisting proliferation and apoptosis. This "crisis" lasted 6 to 8 weeks and was followed by

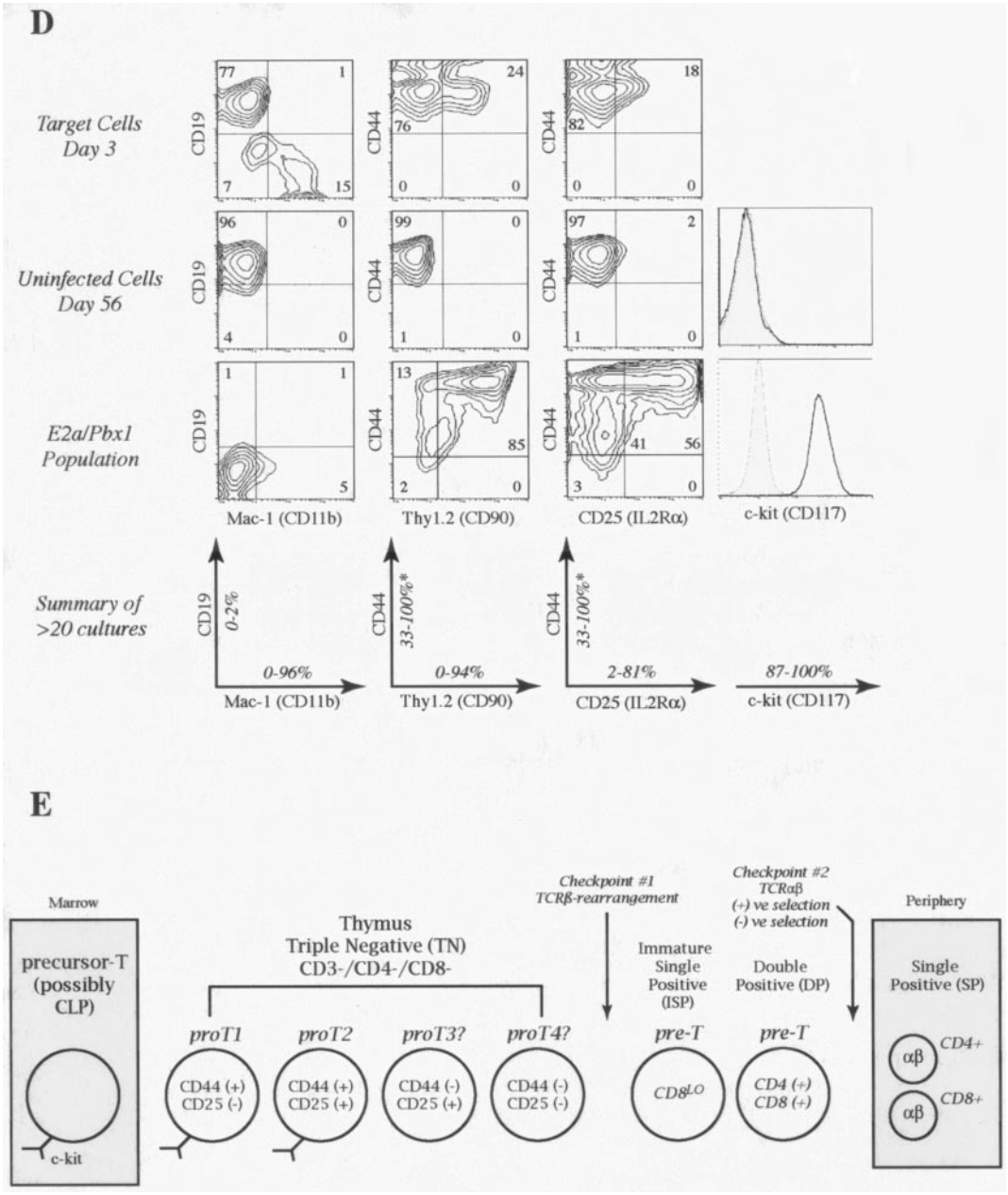


FIG. 1—*Continued*.

the emergence of stable, apoptosis-resistant pre-B-cell lines in most control cultures. By contrast, all populations of pro-T cells expressing E2a-Pbx1 have retained rapid proliferation and cytokine dependence for over 1.5 years now.

The biochemical functions of E2a-Pbx1 required for pro-T-cell immortalization were tested with well-characterized mutants of E2a-Pbx1 lacking DNA-binding or transactivation functions. Use of E2a-Pbx1 mutants demonstrated that all defined biochemical functions of E2a-Pbx1 (DNA binding, transactivation through homodimer and Hox heterodimer elements) are essential for pro-T1/2 immortalization. A naturally occurring alternate t(1;19) translocation in human pre-B acute lymphoblastic leukemia, which encodes an E2a/Pbx1 protein that eliminates Pbx1 sequences upstream of the homeodomain (designated E2a/Pbx1Δ487-623) and retains efficient transactivation through Pbx-Hox or Pbx-Pbx motifs (5), also induced pro-T1/2 outgrowths (Fig. 1B, lanes 4 and 5; Fig. 1C, panel C).

By contrast, E2a-Pbx1 deletion mutants defective in specific biochemical properties that are never observed in t(1;19) pro-B-cell human leukemias failed to induce pro-T1/2 outgrowths. These included an internal deletion of Pbx1 sequences (E2a/Pbx1Δ487-578) that exhibits poor transactivation (10% of wild type) through Pbx-Hox or Pbx-Pbx motifs and does not stimulate NIH 3T3 proliferation, yet blocks myeloid differentiation efficiently (5); a C-terminal truncation of Pbx1 sequences downstream of the homology domain (HD) (E2a/

Pbx1Term691) that activates transcription through Pbx-Hox but not through Pbx-Pbx motifs, blocks myeloid differentiation, and does not stimulate NIH 3T3 proliferation (5); a DNA-binding mutant of E2a-Pbx1N682S that fails to activate transcription through either Pbx-Pbx or Pbx-Hox motifs, does not effectively block myeloid differentiation, and yet induces fibroblast proliferation efficiently (23); a C-terminal truncation of E2a-Pbx (E2a-Pbx1627Term) that lacks the Pbx1 HD and fails to immortalize myeloid progenitors but retains fibroblast transforming ability (23); and an E2a transactivation mutant (E2a-Pbx1Δ16-220) that binds DNA cooperatively with Hox proteins but does not cause either fibroblast or myeloid transformation (23).

The pro-T1/2-immortalizing function of E2a-Pbx1 also required Pbx1 as a fusion partner, as expression of the pro-B-cell acute lymphoblastic leukemia oncoprotein E2a-Hlf (38) failed to induce pro-T1/2 outgrowths despite its ability to immortalize myeloid progenitors (unpublished observation). Collectively, these data demonstrate that pro-T1/2 immortalization is induced uniquely by E2a fusions with Pbx1 and that it requires retention of the same biochemical functions that contribute to human pre-B-cell acute lymphoblastic leukemia.

E2a/Pbx1 pro-T target cell is a unique marrow progenitor unrelated to the pre-B cell. Two types of E2a-Pbx1 target cells initiating pro-T-cell outgrowths were considered: an SCF-responsive pre-B cell that undergoes transdifferentiation and a lymphoid progenitor that normally does not proliferate rapidly in SCF plus IL-7 and hence is outcompeted by the proliferating pre-B cells. These different targets could be distinguished by their rates of depletion in marrow continuously cultured in SCF plus IL-7; the pre-B targets should persist and expand to form the homogeneous $CD19^+$ population, while the abundance of a less mitotic pro-T progenitor should decrease. Marrow was typically cultured for 2 days in SCF and IL-7 prior to infection, and E2a-Pbx1-expressing pro-T outgrowths were readily apparent after 5 days. Marrow progenitors cultured for 19 days in SCF plus IL-7 prior to infection with E2a/Pbx1 retrovirus required an additional 9 days before evidencing immortalized pro-T cell outgrowths (14 versus 5 days), while those progenitors cultured for 80 days in SCF plus IL-7 (comprised exclusively of $CD19^+$ pre-B cells; Fig. 1D) failed to yield pro-T-cell outgrowths. Thus, it seems that E2a/Pbx1 is capable of inducing proliferation in a progenitor of low mitotic index, which is distinct from the $CD19^+$ pre-B cell.

E2a/Pbx1 pro-T clones express early thymocyte genes. Gene expression in E2a/Pbx1-induced pro-T thymocytes was compared to that of primary pre-B cells cultured under the same SCF plus IL-7 conditions (Fig. 2). Controls included NIH 3T3 fibroblasts, BL3 cells ($Thy\text{-}1^+$, $Sca\text{-}1^+$, Lin^- lymphoid leukemia [48] that exhibits stem cell properties capable of reconstituting lethally irradiated mice), EML.C1 cells (SCF dependent, $Sca\text{-}1^+$, $B220^+$ cell line established by expression of a dominant negative retinoic acid receptor α (RARα403) that are capable of lymphoid, myeloid, and erythroid differentiation (44), and Scid.adh cells ($CD44^{lo}$, $CD25^+$ thymic lymphoma [8] phenotypically similar to a immature thymocytes just prior to β-selection). Consistent with their proposed identity as pro-T cells, the E2a/Pbx1 outgrowths expressed the pro-T-cell genes c-*kit* (SCF receptor), *Scl/tal1*, IL-7 receptor α, E2A, Ikaros, PU.1, GATA3, and ID2 (Fig. 2). E2a/Pbx1 pro-T-cell proliferations also expressed c-*myb* and *Egr2* (not shown), genes required for all early stages of thymocyte development (2, 20). Neither of the the pre-T-cell genes Aiolos (required for thymocyte differentiation) or *Rag-1* (required for T-cell receptor gene rearrangement; Fig. 2) nor the early B-cell differentiation genes Pax5, λ5, or VpreB (data not shown) were expressed in E2a/Pbx1-induced pro-T-cell proliferations.

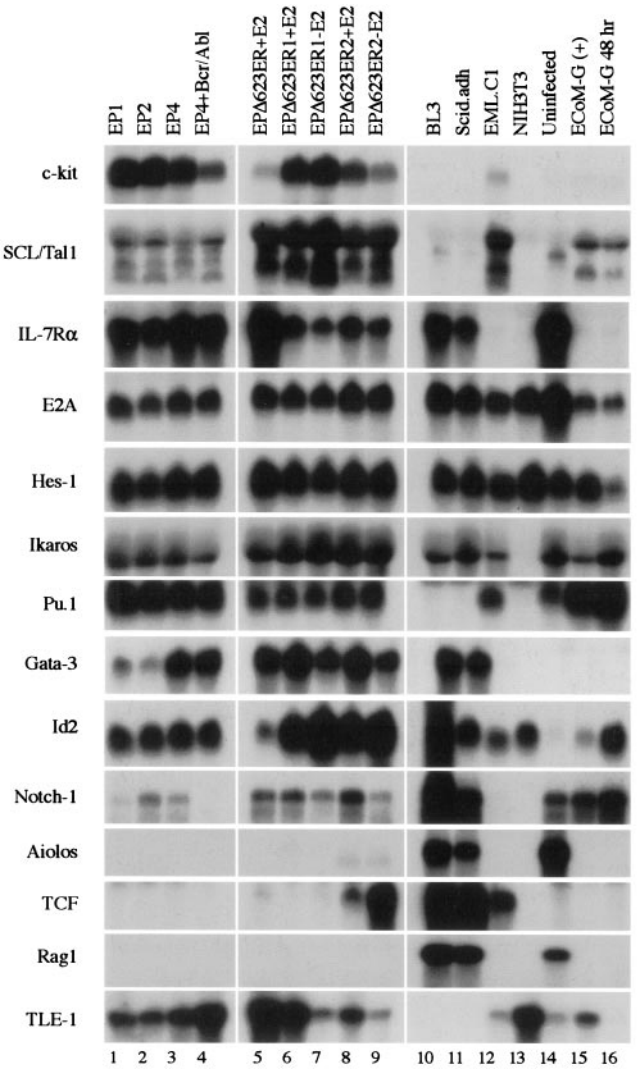


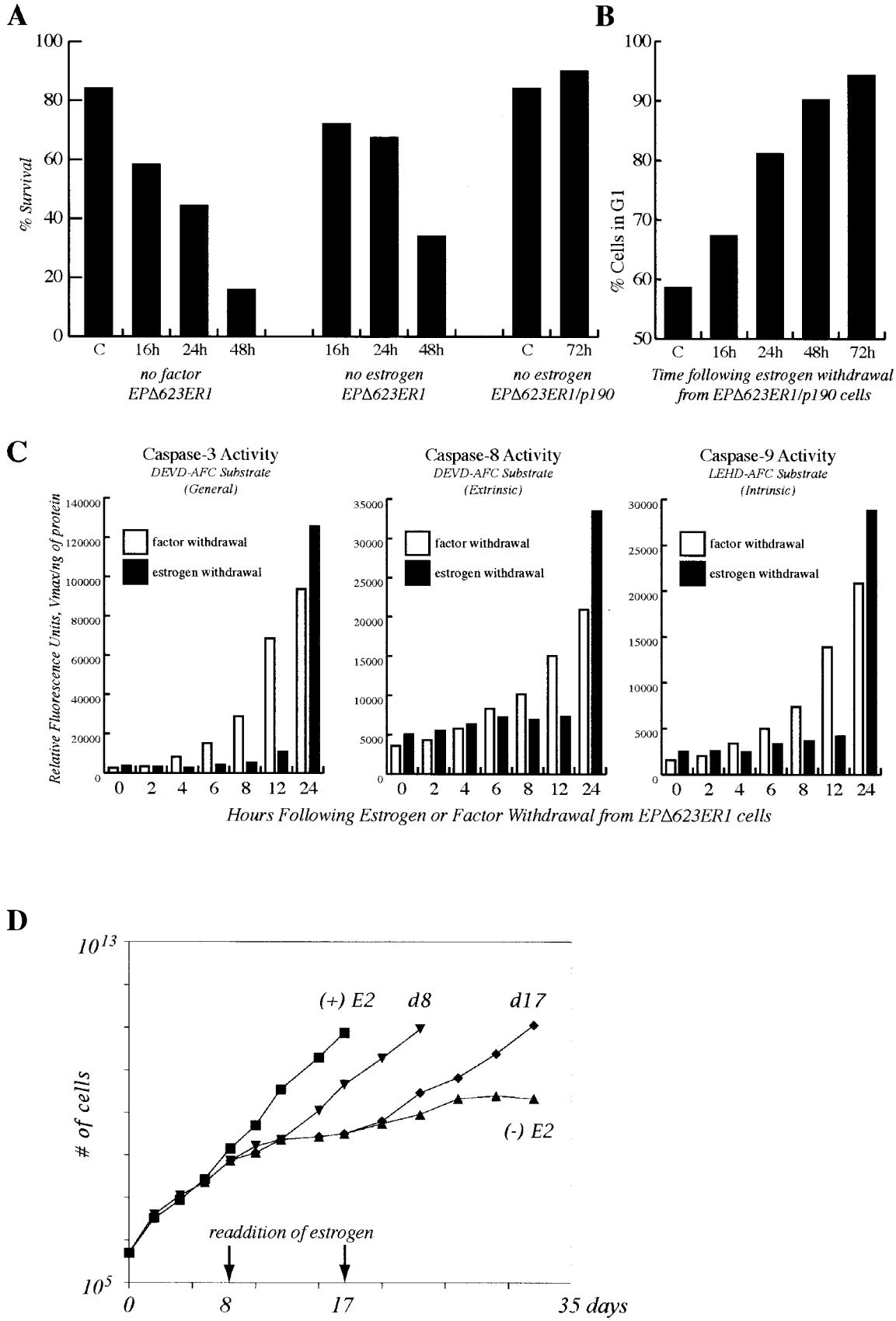
FIG. 2. Northern analysis demonstrates a pattern of early T/progenitor cell gene expression in the E2a/Pbx1-immortalized cell lines. Cells were cultured in the presence or absence of estrogen as indicated for 72 h. Controls are represented by uninfected cells cultured in SCF and IL-7 (lane 15), and BL3 Scid.adh, EML.C1, and NIH 3T3 cells (lanes 11 to 14). ECoM-G cell RNA (cultured in the presence and absence of estrogen for 48 h) was used as a control for myeloid gene expression (lanes 16 and 17).

E2a/Pbx1 stimulates the cell cycle in SCF-dependent thymocytes. The conditional protein E2a/Pbx1Δ623ER was used to characterize the transforming functions of E2a/Pbx1 in pro-T1/pro-T2 thymocytes. E2a/Pbx1Δ623ER is an estrogen receptor fusion of E2a/Pbx1 whose ability to activate transcription, transform NIH 3T3 cells, and block myeloid differentiation is tightly regulated by estradiol (40). Marrow infected with E2a/

TABLE 1. Characteristics of leukemias induced by populations and clones of E2a/Pbx1-immortalized pro-T1/2 thymocytes

Mouse	Peripheral blood cell analysis[a]					Hematopoietic tissue mass (mg)				Ascites fluid vol (ml)	Differential count (%) in hematopoietic tissues											
	WBC (10^3/μl)	G (%)	L (%)	Platelets (10^3/μl)	Hemat (%)	Spleen	Thymus	Node	Mass		Marrow			Spleen			Thymus			Node		
											B[b]	L	G	B	L	G	B	L	G	B	L	G
Controls (avg values for 4 mice)	2.8	25	67	817	45	106	29	<1			31	30	39	2	96	2	0	100	0			
Mice with leukemias induced by E2a-Pbx1-immortalized thymocyte populations																						
1.1	2.5	67	31	103	26	49	3.1	NC[d]	1,092		49	14	37	2	98	0	11	87	2			
1.2			ND[c]			NC	NC	NC		4.0												
1.3			ND			858	5.0	NC														
2.3			ND			NC	NC	NC			ND	ND	ND	20	80	0	ND	ND	ND			
3.1	4.4	31	66	75	38	255	5.0	NC	1,340		84	4	12	64	34	2	3	96	1			
3.2	8.6	21	73	74	35	1,429	11	NC			77	12	11	87	10	3	0	100	0	3	97	0
3.3	2.4	36	63	192	42	59	4	NC			ND	ND	ND	ND	ND	ND	ND	ND	ND			
Mice with leukemias induced by E2a-Pbx1 clone 4 (EPS4)																						
1	17	88	10	271	19	732	20	16			47	36	17	35	57	8	30	64	6	49	50	1
2	60	6	89	259	18	1,091	42	13			74	20	6	63	36	1	38	60	2	73	27	0
3	22	19	73	534	23	1,165	79	5			64	21	15	46	52	2	68	31	1	11	88	1
Mice with leukemias induced by E2a-Pbx1 clone 4 expressing p190bcr-abl (EPS4-p190)																						
1	10	82	9	734	36	417	31	1			25	2	73	31	4	65	61	30	9			
2	21	85	4	698	36	580	52	2			38	1	61	26	2	72	47	48	5			
3	5	84	11	642	39	429	78	2			43	2	55	33	12	55	60	24	16			
4	105	ND	ND	ND	ND	639	54	17			50	5	45	30	18	52	73	22	5	51	37	12
5	128	91	3	862	28	990	7	NC			39	3	58	45	14	41	7	88	5			
6	124	90	5	960	32	822	26	7			54	2	44	50	10	40	56	32	12	29	57	14

[a] WBC, white blood cells; G, granulocytes; L, lymphocytes; Hemat, hematocrit.
[b] B, blasts.
[c] ND, not determined
[d] NC, no significant change.



Pbx1Δ623ER retrovirus produced similar pro-T-cell outgrowths in the presence but not in the absence of estradiol, reemphasizing the requirement for E2a/Pbx1-mediated transactivation (Fig. 1C). The cells expressed E2a/Pbx1Δ623ER at levels comparable to that of wild-type E2a/Pbx1 (Fig. 1B, lanes 6 and 7), required SCF for survival, and exhibited very similar cell surface staining and gene expression profiles.

Fifteen clones were generated from five distinct populations. Estrogen was removed from the culture medium to determine how E2a/Pbx1 impacted cell proliferation, apoptosis, and genetic events indicative of pro-T-cell differentiation. Removal of estrogen had little or no effect on the expression of cell surface antigens (Table 1) or pro-T-cell genes (Fig. 2, lanes 7 to 10). In contrast, dramatic changes in either apoptosis or cell division were observed in all clones. A minority underwent apoptosis within 48 h, exhibiting annexin V staining (Fig. 3A) and activation of caspases-3, -8, and -9 within 24 h following estrogen withdrawal, as evidenced by the cleavage of caspase-specific fluorescent substrates (Fig. 3C). Caspase activation produced by E2a/Pbx1 inactivation lagged behind that induced by factor withdrawal, consistent with a mechanism involving E2a/Pbx1-induced gene transcription.

Rapid apoptosis upon E2a/Pbx1Δ623ER inactivation was blocked by coexpression of $p190^{Bcr/Abl}$, resulting instead in cellular quiescence (Fig. 3B). Thus, Bcr/Abl required a cooperative E2a/Pbx1 function to stimulate thymocyte cell division. This is particularly relevant considering that coexpression of E2a/Pbx1 and Bcr/Abl has been observed in human pre-B-cell acute lymphoblastic leukemia (13). Most E2a/Pbx1 thymocyte clones did not undergo apoptosis, but rather slowed or stopped proliferating, and resumed proliferation upon readdition of estradiol (Fig. 3D). Thus, E2a/Pbx1 stimulates pro-T thymocyte cell division in cooperation with SCF or with Bcr/Abl.

Failure to express TCF-1 and Notch and enforced transcription of the TLE1 corepressor gene are possible mechanisms that could alter the proliferation and differentiation of E2a/Pbx1 pro-T cells. E2a/Pbx1 pro-T cells expressed little to no T-cell-specific transcription factor (TCF-1) or Notch1 (Fig. 2), despite the fact that both of these genes are required for T-cell progenitor differentiation in vivo and would be expected to be expressed in E2a/Pbx1-immortalized pro-T-cell lines similar to their strong expression in BL3 and Scid.adh control cell lines. In one conditional line, inactivation of E2a/Pbx1Δ623ER resulted in strong upregulation of TCF-1, suggesting a possible active mechanism of differentiation arrest.

TLE1 was expressed by each pro-T1 cell clone and downregulated within 72 h following estrogen withdrawal from those thymocytes expressing the conditional E2a/Pbx1Δ623ER. TLE1 encodes a transcriptional corepressor that binds activators such as AML1 and TCF1 (47), converting them into context-specific repressors. TLE1 is also expressed in myeloid progenitors blocked in differentiation by E2a/Pbx1 and downregulated following E2a/Pbx1Δ623ER inactivation (39a). By repressor conversion and dominant-negative mechanisms, the myeloid oncoproteins AML1-Eto, and the lymphoid oncoprotein Tel-AML1 also prevent AML1 function. Therefore, overproduction of TLE1 may represent a third mechanism for inhibiting AML1 in human progenitor leukemia.

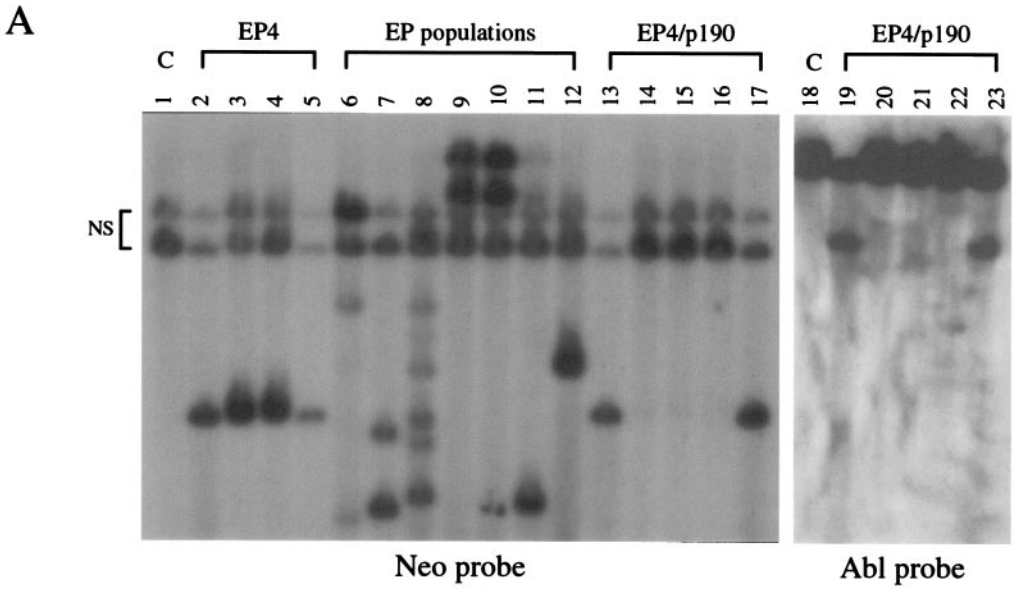
E2a/Pbx1-immortalized pro-T cells cause acute myeloid leukemia. Twenty one days postinfection, three polyclonal pro-T thymocyte populations generated by infection of primary marrow with E2a/Pbx1 retrovirus (100% $CD44^+$, 10% $Mac1^{lo}$, 35% $CD25^+$, 45% $Thy1.2^+$) were each injected into three sublethally irradiated mice. Polyclonality was demonstrated by enumerating proviral integrations in DNA (Fig. 4A, lanes 6 to 8). Early-passage, polyclonal cells were used for leukemogenesis assays rather than cloned cell lines in order to avoid the possibility that mutations arising during cell cloning might accelerate leukemogenesis in vivo or that a representative result might not be evidenced by analyzing a limited number of clones. The three populations of pro-T cells yielded E2a/Pbx1-expressing leukemias after a mean latency of 230, 170, and 85 days, respectively (Fig. 4B). Leukemias were clonal (Fig. 4A, lanes 4 to 7A) and expressed E2a/Pbx1 (Fig. 4D, lanes 5 to 14 versus 2 to 4).

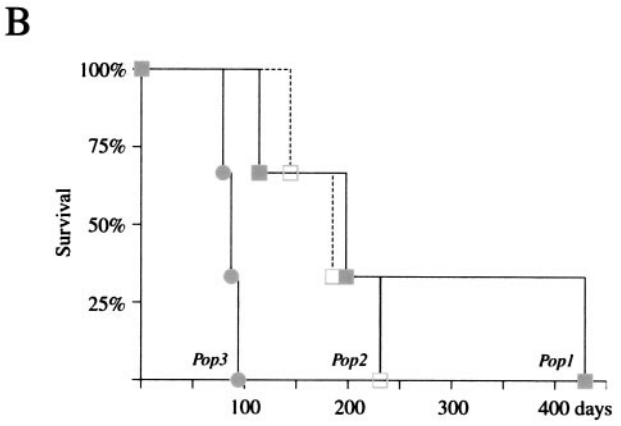
Surprisingly, E2a/Pbx1-containing leukemias were composed of myeloid progenitors, based on morphology (Fig. 5 and quantitated in Table 1) and cell surface antigen expression ($Mac1^+$, $CD25^-$, $CD19^-$; Table 2) that were located principally in marrow and spleen (Table 1). Thymuses were acellular (Table 1) and inguinal lymph nodes were not enlarged. Three mice exhibited extramedullary disease in the form of myeloblastic ascites or large granulocytic sarcomas comprised exclusively of myeloblasts containing the leukemia clone as that which populated other hematopoietic tissues (Table 1, Fig. 4B, lanes 9 and 10). Unlike parental pro-T cells, the leukemic myeloblasts derived from the pro-T cells proliferated in granulocyte-macrophage colony-stimulating factor, consistent with their myeloid character. Two mice developed T-cell leukemias that did not express E2a/Pbx1 and did not evidence E2a/Pbx1 retroviral integration sites (mice 1 and 2 from E2a/Pbx1-immortalized population 2).

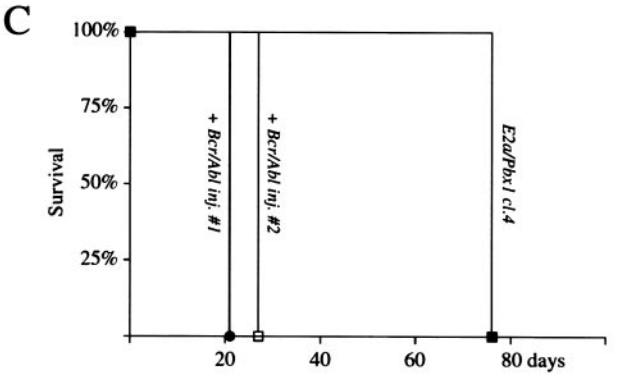
Collectively, these results suggested that the E2a/Pbx1 pro-T cells were not irreversibly committed to T-cell differentiation. However, the possibility remained that a small subset of myeloid progenitors contained in the polyclonal pro-T-cell population existed in the polyclonal populations, and expanded in vivo to caused acute myeloid leukemia. Therefore, pro-T thymocyte clones were subsequently evaluated for disease induction, as described below.

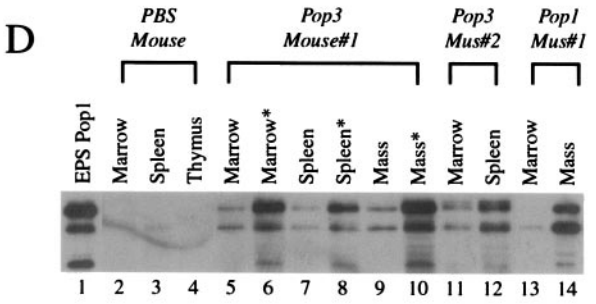
Pro-T-cell clone immortalized by E2a/Pbx1 causes pro-T acute lymphoblastic leukemia but induces acute myeloid leu-

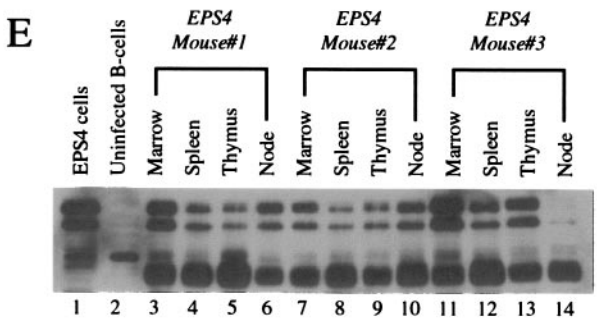
FIG. 3. Inactivation of E2a/Pbx1 results in apoptosis or quiescence of pro-T thymocytes. (A) Apoptosis following factor withdrawal or estrogen withdrawal from pro-T thymocyte clone EPΔ623ER1 is prevented by $p190^{Bcr/Abl}$ (derivative clone designated EPΔ623ER1/p190). Cell survival was assayed by annexin V and propidium iodide staining. (B) E2a/Pbx1 inactivation in EPΔ623ER1/p190 thymocytes results in G_1 arrest. (C) The kinetics of caspase activation induced by E2a/Pbx1 inactivation (estrogen withdrawal) differ from those following factor withdrawal in the pro-T clone EPΔ623ER1. (D) Pro-T1 cell populations expressing conditional EPΔ623ER slow proliferation dramatically (>10-fold) upon removal of estrogen (EPΔ623ER inactivation) and can resume proliferation upon readdition of estrogen and reactivation of E2a/Pbx1.

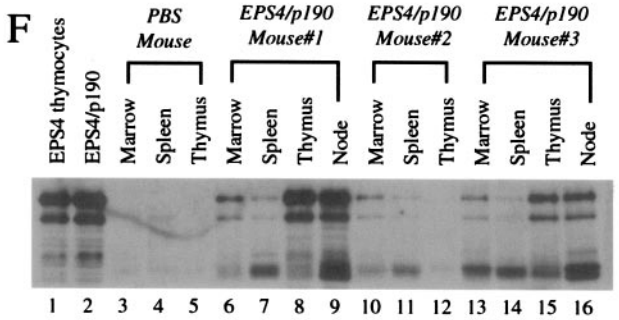












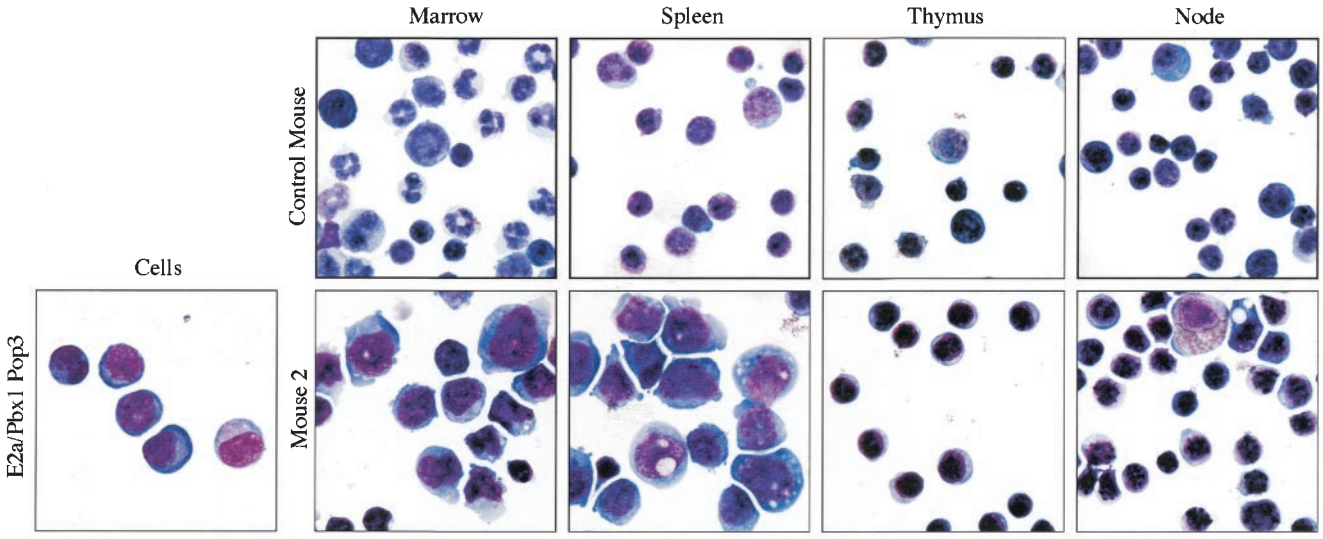


FIG. 5. Wright-Giemsa staining on cytocentrifuged preparations of cells derived from the hematopoietic tissues of a representative leukemic mouse (mouse 2) infused with a population of E2a/Pbx1-immortalized pro-T1/2 thymocytes (population 3). Cell morphologies are compared with those derived from control mouse tissues. As stated in the Results, the leukemic cells from mice infused with these cell populations were principally myeloid.

kemia when converted to factor independence by Bcr/Abl. The EP4 clone of E2a/Pbx1 pro-T cells (100% $CD44^+$, 10% $CD25^+$, CD19-, Mac1-) produced leukemia by day 77 (Fig. 4C) that was comprised of the same pro-T-cell phenotype (Table 2) and that contained the same proviral integration as parental EP4 progenitors (Fig. 4A, lanes 2 to 5). Leukemic progenitors infiltrated the thymus, inguinal lymph nodes, and spleen (Fig. 6), resulting in a 2-, 5-, and 10-fold increase in the mass of these tissues. No granulocytic sarcomas arose. In contrast, factor-independent EP4 pro-T1 cells expressing Bcr/Abl (46) induced leukemia within 25 days (Fig. 4C) that was comprised of pro-T1 cells, myeloid leukemia cells, and a reactive granulocytosis (Fig. 6). The thymus was enlarged twofold, and leukemic cells in thymus and nodes were identical to the parental $CD25^+$ pro-T1 cells (Table 2), representing an expanding pro-T-cell leukemia. The sixfold enlarged spleen, by contrast, contained 80% myeloid cells in a 2:1 ratio of progenitors to neutrophils, yet despite its sixfold enlargement, spleen DNA contained extremely low levels of the retroviral integration found in parental EP4 progenitors (Fig. 6, lanes 14 to 16 versus lane 13), demonstrating that most of the cells arose from a normal reactive granulocytosis.

Such a reactive granulocytosis is likely induced by secretion of IL-3 and granulocyte-macrophage colony-stimulating factor from leukemic blasts, as has been reported in leukemia induced by v-Abl or Bcr/Abl (33, 37, 49). When cultured, approximately 10% of the progenitors found in the spleen proliferated as myeloid blasts marked by the original proviral integration (Fig. 6, lane 17 versus 13). These cells had lost surface CD25, gained surface Mac1 (Fig. 7A), expressed the myeloid genes neutrophil elastase and myeloperoxidase, and lost expression of the T-cell progenitor gene GATA-3 (Fig. 7B). These cells therefore represented a developing myeloid leukemia derived from the same parental clone. Myeloid leukemia was not produced by the activity of Bcr/Abl alone, as pro-T1 cells generated by estrogen-dependent EPΔ623ER and rendered factor-independent by expression of Bcr/Abl yielded no disease following injection in mice (followed to 166 days).

FIG. 4. E2a/Pbx1 causes clonal leukemias. (A) Southern analysis of genomic DNA isolated from cell lines and leukemic mouse tissues. Retroviral integrations were identified by probing for *neo* sequences in the MSCVneo E2a/Pbx1 provirus or *Abl* sequences in the MSCVpuro Bcr/Abl provirus. Two nonspecific bands on the first blot are labeled NS. DNA samples were derived from the following sources. Lane 1, control spleen. Lane 2, E2a/Pbx1 clone 4 cultured pro-T1 cells. Lanes 3 to 5, spleen from three different leukemic mice injected with E2a/Pbx1 clone 4. Lanes 6 to 8, polyclonal populations 1, 2, and 3, comprised of pro-T1/2 thymocytes derived by infection of progenitors with E2a/Pbx1 retrovirus followed by culturing in SCF and IL-7 for 14 days. Lanes 9 and 10, spleen and granulocytic sarcoma derived from a single mouse injected with ProT1/2 cell population 3. Lane 11, spleen from a second leukemic mouse injected with pro-T1/2 cell population 3. Lane 12, granulocytic sarcoma from a leukemic mouse injected with pro-T1/2 cell population 1. Lane 13, factor-independent E2a/Pbx1 clone 4 pro-T1 cells expressing $p190^{Bcr/Abl}$ retrovirus. Lanes 14 to 16, spleen from a three different leukemic mice (mice 1 to 3) injected with E2a/Pbx1 clone 4 expressing Bcr/Abl. Lane 17, progenitors cultured from leukemic spleen analyzed in lane 16. Lanes 18 to 23, analysis of DNA'=s from lanes 1 and 13 to 17 with a probe for Abl. (B) Kaplan-Meier survival curves for sublethally irradiated mice infused with E2a/Pbx1-immortalized pro-T1/2 cell populations 1, 2, and 3. Three mice were infused with each population. (C) Kaplan-Meier survival curves illustrating the effect of $p190^{Bcr/Abl}$ on expression of sublethally irradiated mice infused with E2a/Pbx1 clone 4 pro-T1 thymocytes. Solid lines indicate mice that died from a myeloid disease expressing E2a/Pbx1, while dashed lines indicate mice that died from an immature T-cell disease that did not express E2a/Pbx1. (D to F) Quantitation of E2a/Pbx1 abundance by anti-E2a immunoblot analysis in pro-T1/2 cell populations and clones immortalized by E2a/Pbx1 and in tissues from leukemic mice infused with these cell lines. The identities of the clones and sources of tissue are indicated.

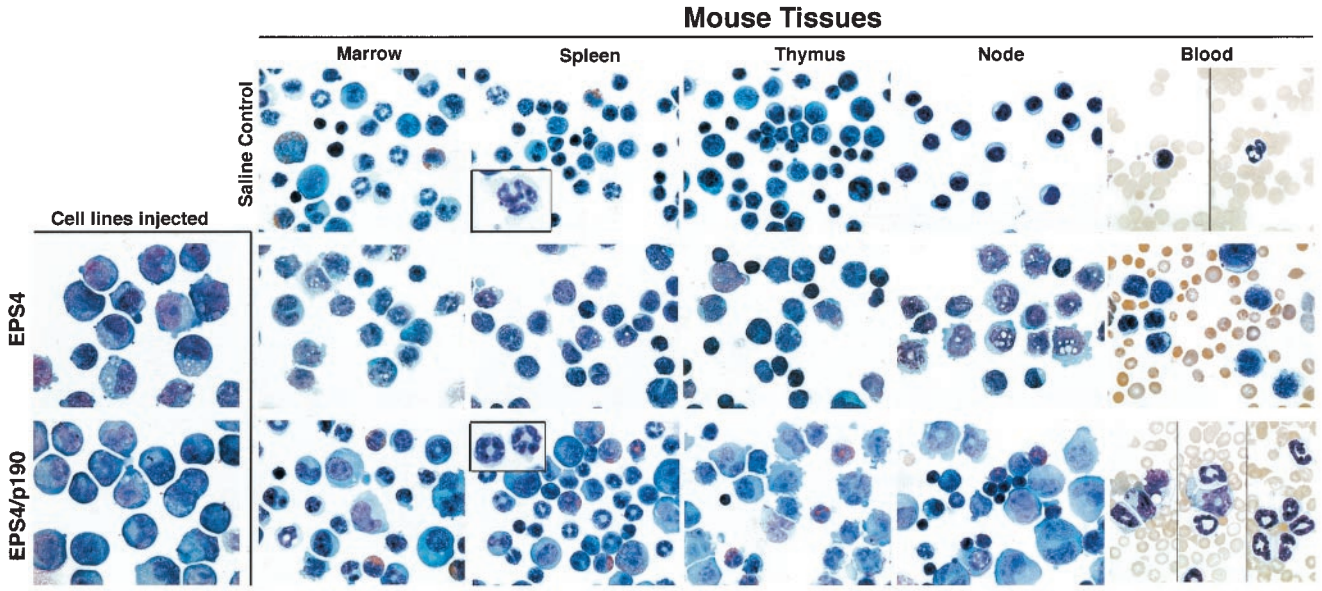


FIG. 6. Wright-Giemsa staining on cytocentrifuged preparations of cells derived from the hematopoietic tissues of mice infused with E2a/Pbx1 clone 4 and E2a/Pbx1 clone 4 expressing $p190^{Bcr/Abl}$ and compared with those from a control mouse. Leukemias initiated by E2a/Pbx1 clone 4 were striking for the presence of many immature pro-T-cell blasts, while its derivative clone that expressed $p190^{Bcr/Abl}$ induced a combination of acute myeloid leukemia and pro-T-cell acute lymphoblastic leukemia accompanied by a reactive granulocytosis.

Supporting the notion that Bcr/Abl can cause the pro-T thymocyte to exhibit myeloid differentiation, we also found that pro-T-cell clones coexpressing EPΔ623 plus Bcr/Abl also developed acute myeloid leukemia with accompanying granulocytic sarcomas (data not shown). The ability of Bcr/Abl to promote myeloid differentiation of a pro-T thymocyte is particularly interesting in light of the fact that enforced IL-2Rα signaling can redirect the differentiation of uncommitted T-cell progenitors along the myeloid lineage (26, 27) and that expression of Bcr/Abl in EP4 thymocytes induced expression of CD25 (IL-2Rα; Table 2).

DISCUSSION

E2a/Pbx1 immortalizes a pro-T lymphoblast that fails to undergo further differentiation in vivo. Here we describe the first lymphoid target cell for transformation by E2a/Pbx1, a pro-T1/2 thymocyte. Because leukemic progenitors can exhibit abnormal patterns of gene expression, multiple clones of the E2a/Pbx1-immortalized pro-T-cell target were characterized both by immunologic and genetic criteria. In all cases, the pro-T1/2 phenotype was confirmed by both its $CD44^{+}/CD25^{-/lo}$ surface phenotype and by expression of marker

TABLE 2. Flow-cytometric analysis of cell lines used to produce leukemias in mice and also of cells derived from those leukemic mice[a]

Cell line	FACS[b] analysis of cell line:					Leukemic mouse no.	FACS analysis of tissue from leukemic mice injected with the indicated cell line													
							Marrow				Spleen				Thymus				Mass		
	Mac1	CD2S	CD19	Thy1.2	Lin^{-}		Mac1	CD25	CD19	Lin^{-}	Mac1	CD25	CD19	Lin^{-}	Mac1	CD25	CD19	Lin^{-}	Mac1	CD25	CD19
EPS Pop 1	12	56	0	45	0	1	64	4	20		9	7	24						95	2	2
EPS Pop 2	4	29	0	50	0	1	5	69	0		3	69	5		3	65	2				
EPS Pop 3	12	52	0	38	0	1	87	9	3		62	9	21						85	6	2
						2	84	10	13		80	18	16								
EPS4	0	10	0	0	90	1	17	5	3	75	11	10	13	66	20	12	3	65			
						2	32	1	1	66	19	2	6	73	20	2	3	65			
						3	16	1	1	82	4	1	5	90	7	13	3	77			
EPS4-p190	0	92	0	0	8	1	84	20	1		63	11	5		29	77	0				
						2	70	43	2		66	11	5		25	60	2				
						3	77	19	2		52	8	11		30	72	7				
						4	56	50	2		86	11	5		20	85	1				
						5	65	40	1		79	17	2		10	15	1				
						6	85	44	1		72	10	5		14	2	1				

[a] Values are percent of total.
[b] FACS, Fluorescence-activated cell sorter.

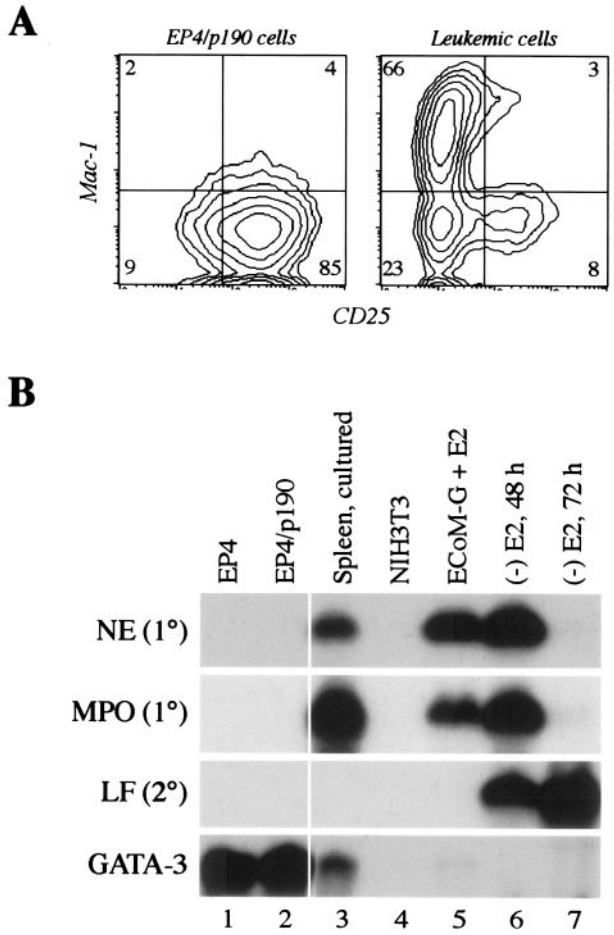


FIG. 7. Expression of $p190^{Bcr/Abl}$ in E2a/Pbx1-immortalized pro-T1 clone 4 (EP4p190) induces acute leukemias that evidence a myeloid progenitor pattern of gene expression. (A) Flow cytometric analysis of EP4p190 thymocytes prior to injection in mice and EP4p190 progenitors cultured from the spleen of a recipient leukemic mouse. Mac-1-positive myeloid cells predominate, while a minority are CD25-positive pro-T1 blasts. (B) Northern analysis demonstrating expression of primary granule genes in the cultured leukemic cells. Note that the low level of GATA-3 probably represents the small proportion of pro-T1 blasts in the culture, which are visualized by flow cytometry analysis below. ECoM-G cell RNA was used as a positive control for myeloid gene expression. ECoM-G cells retain a myeloblast phenotype in the presence of estrogen and differentiated to neutrophils following estrogen withdrawal. NE, neutrophil elastase; MPO, myeloperoxidase; LF, lactoferrin.

genes for T-cell specific progenitors (GATA-3 [42], pro T/B lymphoid progenitors (c-kit (16, 17), SCL/tal1 (19), IL-7Rα (32), Id2 (36), and T and B lymphoid progenitors throughout their differentiation (E2A (3, 14), Ikaros (15), PU.1, c-Myb, and Egr2 (2, 20). In contrast, genes specifically expressed at subsequent pre-T-cell stages, such as Aiolos and Rag-1, were not expressed. Collectively, these data demonstrate that the E2a/Pbx1 target cell is a pro-T1/T2 thymocyte.

The fact that pro-T1 cell leukemias evoked by EP4 pro-T thymocytes did not evidence further differentiation in vivo suggests that E2a/Pbx1 also blocks pro-T-cell differentiation. Failure to express TCF-1 and Notch, and active expression of TLE-1 may underlie this differentiation block. TCF-1 is an HMG-box family of DNA-binding proteins that binds the CD3ε enhancer (45), is expressed in early thymocytes, and is thought to be required for normal production of T-cell progenitors that home to the thymus, as TCF-1 knockout mice have greatly reduced thymus size. Notch1 is critical for T-lineage commitment (35). In the thymus, Notch signaling cooperates with E2a and HEB (41) to activate transcription of genes underlying T-cell differentiation, such as pre-T-cell receptor α (pTa) (12), which forms the pre-T-cell receptor complex with T-cell receptor β. Enforced expression of active Notch1 strongly favors T-lymphoid versus. myeloid lineage commitment (34, 39). Therefore, failure to express Notch could explain why pro-T thymocytes immortalized by E2a/Pbx1 can transdifferentiate to the myeloid lineage under the influence of signaling oncoproteins or host microenvironments.

The leukemias triggered by the EP4 clone of E2a/Pbx1-immortalized pro-T1 cells retained their early pro-T1-cell phenotype ($CD44^+/CD25^-$) similar to those that arise in E2a-deficient mice (3) or in mice coexpressing SCL/Tal1 and LMO2 (29) or SCL/Tal1 and LMO1 (10). In contrast, the T-cell leukemias that arise in transgenic mice expressing E2a/Pbx1 from the Eμ promoter show a later pre-T phenotype characterized by expression of the T-cell receptor (11). In that study, E2a/Pbx1 was proposed to be inactive in the pro-T stage, and to effectively block differentiation in the pre-T stage; however, our results argue that E2a/Pbx1 can arrest T lineage differentiation at a pro-T stage as well, and that pre-T-cell leukemia in this study is likely the result of augmented expression from the Eμ promoter in pre-T cells.

Mitogenic function of E2a/Pbx1 is essential for inducing pro-T-cell outgrowth and is likely essential for human pre-B-cell acute lymphoblastic leukemia. Formerly, we found two Pbx deletion mutants that allowed E2a-Pbx1 to block myeloid differentiation but abrogated its ability to induce fibroblast proliferation—one deleting the cooperativity helix downstream of the homeodomain, and a second deleting sequences between E2a and the Pbx1 autoinhibitory alpha helix, which lies just N-terminal to its homeodomain (5). In our experiments above, neither of these mutants were capable of immortalizing T-cell progenitors, and inactivation of conditional E2a/Pbx1 resulted in a decrease in pro-T-cell proliferation. These data suggest that the same E2a-Pbx1 function that stimulates fibroblast proliferation also stimulates pro-T-cell proliferation.

We propose that E2a/Pbx1 contains two functions, one that induces proliferation and a second that prevents or alters normal differentiation. The ability to induce proliferation is, at least in large part, independent of Pbx1 DNA-binding activity (23, 31), and indeed may be due to the ability of E2a oncoproteins to form dimers that either inhibit or activate a subset of gene transcription through dominant-negative or direct enhancer-binding mechanisms (4). The second activity of E2a fusion oncoproteins would alter differentiation through a fusion partner-dependent mechanism. Both activities would contribute to leukemogenesis, a contention supported by the facts that both activities are required for pro-T1/2 immortalization, and both are exhibited by the two naturally occurring forms of E2a-Pbx1 in human t(1;19) pro-B-cell leukemia. According to this model, E2a-Pbx1 and E2a-Hlf would promote proliferation by a common mechanism but alter differentiation by a distinctive one, which would explain their association with dif-

ferent types of human leukemia. In the case of leukemias produced by E2a proteins expressed from the immunoglobulin promoters, second mutations would provide the intrinsic differentiation blocks of E2a-Pbx1 or E2a-Hlf.

The codependence between E2a/Pbx1 and SCF for pro-T-cell proliferation or between Meis1 and SCF for myeloblast proliferation suggests that hematopoietic Meis1 controls normal progenitor expansion, and that its mechanism may be mimicked by E2a/Pbx1. Our earlier studies found that E2a/Pbx1 could prevent myeloid differentiation and induce myeloblast outgrowth in the presence of the myeloid cytokines granulocyte-macrophage colony-stimulating factor or IL-3. Conditional mutants of E2a/Pbx1 clearly demonstrated that E2a/Pbx1 blocked morphological and genetic differentiation, but could not address whether E2a/Pbx1 played an active role in cell division because terminal differentiation was irreversible. In the mechanism of pro-T-cell immortalization by E2a/Pbx1, either cytokines or the expression of Bcr/Abl are required to prevent apoptosis, yet neither is sufficient to drive cell division in the absence of functional E2a/Pbx1.

The mechanism by which E2a/Pbx1 satisfies a cell division requirement is unknown, and elucidating this mechanism is clearly an important goal. The interdependent roles of E2a/Pbx1 and cytokines and oncoproteins in promoting cell division suggest that normal Pbx proteins and their Meis/Hox cofactors may also cooperate actively with extracellular cytokines to regulate the decision of a hematopoietic progenitor to proliferate. Indeed, the fact that Meis1 is expressed specifically in early hematopoietic progenitors (25, 30), as well as the fact that Meis1 cooperates with SCF or granulocyte colony-stimulating factor to induce progenitor proliferation (6) suggests that Meis1, like E2a/Pbx1, also performs a function required for cell division driven by a subset of cytokines or signaling oncoproteins. As Nup98-HoxA9 enforces Meis1 transcription (7), Nup98-HoxA9 could promote cell division through this same Meis1 mechanism. The fact that Meis1 binds Pbx-Hox complexes on target genes and that E2a/Pbx1 heterodimerizes with Hox proteins on target genes presents the possibility that both E2a/Pbx1 and Meis1 could function by activating transcription of the same target gene(s) that cooperates with SCF signaling to stimulate cell division. Such a mechanism could be relevant in hematopoiesis in a model in which a subset of cytokines (e.g., SCF) signal through pathways that impart progenitor viability and in which a switch between proliferation verses differentiation is based on the presence or absence of coexpressed Meis1. In such a model, understanding the mechanisms regulating Meis1 transcription could explain the dynamics of progenitor cell expansion.

Signaling oncoproteins influence lineage commitment. The fact that pro-T thymocyte populations induced by E2a/Pbx1 under cell culture conditions produce acute myeloid leukemia in syngeneic mice, and the fact that expression of Bcr/Abl converts leukemias induced by EP4 pro-T cells from pro-T acute lymphoblastic leukemia to a combination of pro-T acute lymphoblastic leukemia plus acute myeloid leukemia demonstrates that the E2a/Pbx1-immortalized pro-T-cell is not irreversibly committed to the T-cell lineage. Rather, this cell can be induced toward myeloid differentiation by specific cellular environments or signal transducing oncoproteins. Given this behavior, this pro-T-cell target may represent the initial target responsible for the generation of acute myeloid leukemia in mice transplanted with E2a/Pbx1-transduced marrow.

Immunologic sorting has identified specific populations of progenitors capable of myeloid-restricted common myeloid progenitor (1) or lymphoid-restricted common lymphoid progenitor (28) development, yet even these distinctions are not always absolute. The differentiation of the common lymphoid progenitor, for example, can be biased towards the myeloid lineage by exogenous signaling through the IL-2Rα (26, 27). Still other studies identify progenitors that, given the proper conditions, evidence myeloid/T-lymphoid or myeloid/B-lymphoid differentiation (reviewed in reference 24). Lineage specificity is also mediated by changes in transcription factor content. Lineage-specific differentiation is mediated by cooperativity between genes required for lineage development and genes that restrict developmental outcomes to a subset of potential lineages. While factors such as AML1 and SCL/Tal1 are necessary for the development of all hematopoietic lineages, others, such as PU.1 (critical for both myelopoiesis and B-lymphopoiesis) and E2A (critical for both B and T lymphopoiesis) are required for more restricted development, and some, such as Pax5, are required for lineage-restricted development (B-lymphoid). Based on the fact that signaling, as well as transcription factor content, can alter lineage definition, it should not be surprising that signal transduction oncoproteins and transcription factor oncoproteins can alter lineage definition, as we observed in this study.

Combining the concept that E2a/Pbx1 cooperates with cytokines or oncoproteins to promote cell division, with the concept that specific signaling oncoproteins can influence lineage definition of the leukemic cell, it is possible that human pre-B acute lymphoblastic leukemia containing E2a/Pbx1 originates from a non-pre-B progenitor subjected to expansion by E2a/Pbx1, that a second mutation affecting a proliferation pathway is essential for conversion to acute leukemia, and that the properties of this signaling oncoprotein permit or facilitate a pre-B-cell phenotype.

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