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Transformation Properties of the E2a-Pbx1 Chimeric Oncoprotein: Fusion with E2a Is Essential, but the Pbx1 Homeodomain Is Dispensable

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The t(1;19) chromosomal translocation in acute lymphoblastic leukemias creates chimeric E2a-Pbx1 oncoproteins that can act as DNA-binding activators of transcription. A structural analysis of the functional domains of E2a-Pbx1 showed that portions of both E2a and Pbx1 were essential for transformation of NIH 3T3 cells and transcriptional activation of synthetic reporter genes containing *PBX1* consensus binding sites. Hyperexpression of wild-type or experimentally truncated Pbx1 proteins was insufficient for transformation, consistent with their inability to activate transcription. When fused with E2a, the Pbx-related proteins Pbx2 and Pbx3 were also transformation competent, demonstrating that all known members of this highly similar subfamily of homeodomain proteins have latent oncogenic potential. The oncogenic contributions of E2a to the chimeras were localized to transactivation motifs AD1 and AD2, as their mutation significantly impaired transformation. Either the homeodomain or Pbx1 amino acids flanking this region could mediate transformation when fused to E2a. However, the homeodomain was not essential for transformation, since a mutant E2a-Pbx1 protein (E2a-Pbx$^{\Delta HD}$) lacking the homeodomain efficiently transformed fibroblasts and induced malignant lymphomas in transgenic mice. Thus, transformation mediated by the chimeric oncoprotein E2a-Pbx1 is absolutely dependent on motifs acquired from E2a but the Pbx1 homeodomain is optional. The latter finding suggests that E2a-Pbx1 may interact with cellular proteins that assist or mediate alterations in gene expression responsible for oncogenesis even in the absence of homeodomain-DNA interactions.

Homeodomain proteins constitute a specific class of DNA-binding transcription factors that are involved in multiple gene regulatory and developmental decisions (43). Although perturbations in the expression of homeodomain proteins are generally associated with morphologic defects, recent studies demonstrate that their deregulated expression may also contribute to neoplastic transformation. This was initially demonstrated by studies of the homeobox gene *Hox2.4*, which is ectopically expressed by integration of an adjacent intracisternal A particle in the myeloid leukemia cell line WEHI3 (6, 25). Experimentally, *Hox2.4* transforms rodent fibroblasts and in conjunction with interleukin-3 induces myeloid leukemias in mice (1, 39). *Hox* genes are also implicated in the pathogenesis of a subset of human leukemias by virtue of their locations at chromosome translocation breakpoints, as evidenced by the ectopic expression of *Hox11* following its juxtaposition with a T-cell receptor gene in t(10;14)-bearing leukemias (10, 16, 30). A normal role for a subset of *Hox* genes in the function and lineage determination of hematopoietic cells is suggested by characteristic patterns of expression (26, 27), whereas forced expression of several but not all *Hox* genes has been shown experimentally to induce transformation of NIH 3T3 fibroblasts (33).

These and other studies suggest that oncogenic activation of *Hox* genes may involve a simple gain-of-function mechanism involving misdirected or ectopic expression (8). However, this mechanism may not apply to all *Hox* genes with oncogenic potential. We and others have shown that a divergent member of the homeodomain family, Pbx1, undergoes protein fusion as a result of t(1;19) chromosomal translocations in acute leukemias (23, 38). This translocation brings together the *E2A* and *PBX1* genes, resulting in synthesis of chimeric proteins composed of amino terminal portions of E2a containing transcriptional activation motifs and carboxy-terminal portions of Pbx1 containing its homeodomain. Pbx1 is distantly related to yeast MATa1 and MATα2 (38), and *PBX*-related genes have been isolated from humans, *Caenorhabditis elegans*, and *Drosophila melanogaster* (7, 12, 34, 41). In *D. melanogaster*, a homolog of Pbx is the product of the *extradenticle* (*exd*) gene, whose mutations suggest a unique role in segmental identity by functioning in parallel with the homeotic selector proteins (41).

Although Pbx1 undergoes protein fusion following chromosomal translocations in acute leukemias, the structural requirements for oncogenic activation of Pbx1 have not been defined. The *PBX1* gene is not transcriptionally active in lymphoid cells (34, 38), raising the possibility that fusion with E2A simply redirects expression of the Pbx1 protein to cells in which it is not normally found. Alternatively, fusion with E2a may constitute an essential event for unmasking the latent transforming capability of Pbx1 which would otherwise not be evident regardless of the cellular context in which it was expressed. Indeed, the translocated, chimeric form of Pbx1 has been shown to transform several cell types, including fibroblast, myeloid, and lymphoid cells (9, 21, 22), in spite of its restricted association with a specific subtype of human leukemia.

In this study, we investigated the structural components of chimeric Pbx proteins required for transcriptional activation and transformation. Our results demonstrate that unlike other Hox proteins, the Pbx subfamily of homeodomain proteins lacks inherent transforming potential in the absence of fusion

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TABLE 1. Focus-forming capabilities of wild-type and chimeric Pbx proteins in NIH 3T3 cells

Retroviral construct	Focus formation[a]
cos-MSV E2a-Pbx1a	507 (227)
cos-MSV E2a-Pbx1b	436 (286)
cos-MSV E2a-Pbx2	397 (165)
cos-MSV E2a-Pbx3a	200 (20)
cos-MSV E2a-Pbx3b	367 (220)
cos-MSV Pbx1a	0
cos-MSV Pbx1b	0
cos-MSV Pbx2	0
cos-MSV Pbx3a	0
cos-MSV Pbx3b	0
cos-MSV vector	0.4 (0.7)

[a] Values indicate number of foci observed per 10^4 G418-resistant colonies and are the averages resulting from two to six experiments examining serial 10-fold dilutions of each viral stock (standard deviations in parentheses).

with the heterologous E2a protein. Oncogenic conversion of Pbx1 occurred in parallel with acquisition of transcriptional activity and required the transcriptional activation domains of E2a. Unexpectedly, the Pbx1 homeodomain was not essential for transformation, suggesting that E2a-Pbx1 does not need to directly bind DNA through its homeodomain to transform fibroblasts or lymphoid cells. These findings are consistent with a model in which E2a-Pbx1 may interact with cellular proteins that assist or mediate its effects on gene expression responsible for oncogenesis.

MATERIALS AND METHODS

Expression constructs and mutagenesis. Deleted forms of *PBX1* and *E2A-PBX1a* cDNAs (34, 38) were constructed by using PCR and standard cloning techniques. Constructs encoding E2a fused with Pbx2 or Pbx3 protein were created by overlap extension PCR so that the sites of fusion were identical to that in E2a-Pbx1. All DNA fragments generated by PCR were completely sequenced to rule out mutations introduced by the amplification procedures. Intact deletion derivatives were assembled in the pSP64 or pSP65 vector (Promega, Madison, Wis.) and subjected to in vitro transcription and translation to assess the integrity of the encoded proteins prior to subcloning into the cos-MSV-tk-Neo plasmid vector. Amino acid numbering corresponds to published sequences (34, 38).

Retroviral stocks. The various deleted forms of *E2A-PBX1* and *PBX* cDNAs were cloned into the *Eco*RI site of the cos-MSV-tk-Neo plasmid vector. Retroviral stocks were generated by transient transfection of COS7 cells essentially as described elsewhere (36, 37). COS7 cells were cotransfected with various MSV-tk-Neo constructs and ecotropic packaging plasmids. Culture supernatants containing viral stocks were collected from day 3 to 5 after transfection, centrifuged, and filtered.

Focus-forming assays. Contact-inhibited NIH 3T3 cells were obtained from the American Type Culture Collection. Serial dilutions of COS cell supernatants containing retroviruses were layered onto monolayers of low-passage-number NIH 3T3 cells and incubated for 3 h in the presence of 6 μg of Polybrene per ml. Fresh medium was exchanged, and after 1 more day, G418 was added to 500 μg/ml. Media were changed every 3 to 4 days for approximately 3 to 4 weeks, at which time the cells were fixed with paraformaldehyde and stained with crystal violet. The total number of G418-resistant colonies was determined on plates in which the viral dilution yielded individual colonies. Foci were scored on more confluent plates, and their numbers were expressed relative to the extrapolated total number of G418-resistant colonies. For comparative analyses of mutant E2a-Pbx1a proteins, the number of foci per 10^4 G418-resistant colony was divided by the number observed for *E2A-PBX1a* analyzed in parallel and expressed as a percentage. Focus-forming assays were performed under selection to maximize sensitivity given the variable focus-forming capabilities of E2a-Pbx1 under nonselective conditions (22, 35). Performance of focus assays under conditions that selected against nontransfected cells also allowed a more sensitive assessment of the relative focus-forming abilities of wild-type versus fusion Pbx proteins. Focus assays performed under nonselective conditions involved plating 10^3 and 10^4 G418-resistant cells on a monolayer of 10^5 NIH 3T3 cells. Cultures were grown until the appearance of macroscopic foci (2 to 3 weeks), at which time cells were fixed and stained and foci were counted as described above. The anchorage independence and tumorigenicity of *E2A-PBX1*-transformed fibroblasts were not addressed in these studies but have been characterized previously (22).

Transient transfections and transactivation assays. Transcriptional properties were assessed on *PBX* reporter constructs as described previously (28). For lymphoid transfections, the *PBX* reporter contained 10 concatamerized *PBX* consensus binding sites (28) cloned immediately 5′ to a minimal herpes simplex thymidine kinase gene promoter upstream of a chloramphenicol acetyltransferase (CAT) reporter gene (31). NIH 3T3 transfections were done with a reporter plasmid incorporating four *PBX* consensus binding sites linked to a liver/bone/kidney alkaline phosphatase gene promoter (47) upstream of a CAT coding sequence. Constructs for transient expression of Pbx, E2a-Pbx, or various deleted forms of these proteins consisted of the respective cDNA inserts cloned downstream of a cytomegalovirus promoter in pCMV1 (28).

Lymphoid cells were transfected as previously described (13, 28). REH cells in log-phase growth were washed once in serum-free RPMI, and 3×10^6 cells were resuspended in 0.8 ml of RPMI containing 0.38 μg of each expression and reporter plasmid per ml, 0.25 μg of pSVtk-luciferase plasmid per ml, and 5 μg of DEAE-dextran per ml. The mixture was pulsed at room temperature in a 4-mm electroporation cuvette, using a Bio-Rad Gene Pulser set at 300 V and 960 μF. Following electroporation, the cells were transferred to 4 ml of complete medium and incubated for 24 h at 37°C in a 5% CO_2 environment. Cells were lysed in 50 μl of Tris (250 mM, pH 7.8) for luciferase and CAT assays. CAT assays were carried out by using 5 to 20 μl of cell lysate as previously described with an incubation time of 3 h (14). Results were quantitated on a PhosphorImager (Molecular Dynamics). Luciferase assays were performed by adding 20 μl of cell lysate to 200 μl of assay buffer (25 mM KPO_4 [pH 7.8], 15 mM $MgSO_4$, 4 mM EGTA, 1 mM dithiothreitol, 2 mM ATP) immediately before quantitation of light emission with a luminometer (Analytical Luminescence model 2010). NIH 3T3 fibroblasts were transfected by using calcium phosphate-DNA precipitates as described previously (28).

Immunoblotting. Single-cell suspensions from stably infected NIH 3T3 cells, transiently transfected COS7 cells, or mouse tissues were lysed in radioimmunoprecipitation assay buffer (1% Triton X-100, 0.1% sodium dodecyl sulfate [SDS], 0.1% deoxycholate, 150 mM NaCl, 5 mM EDTA) containing protease inhibitors. Proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) (11.5% gel) and immobi-

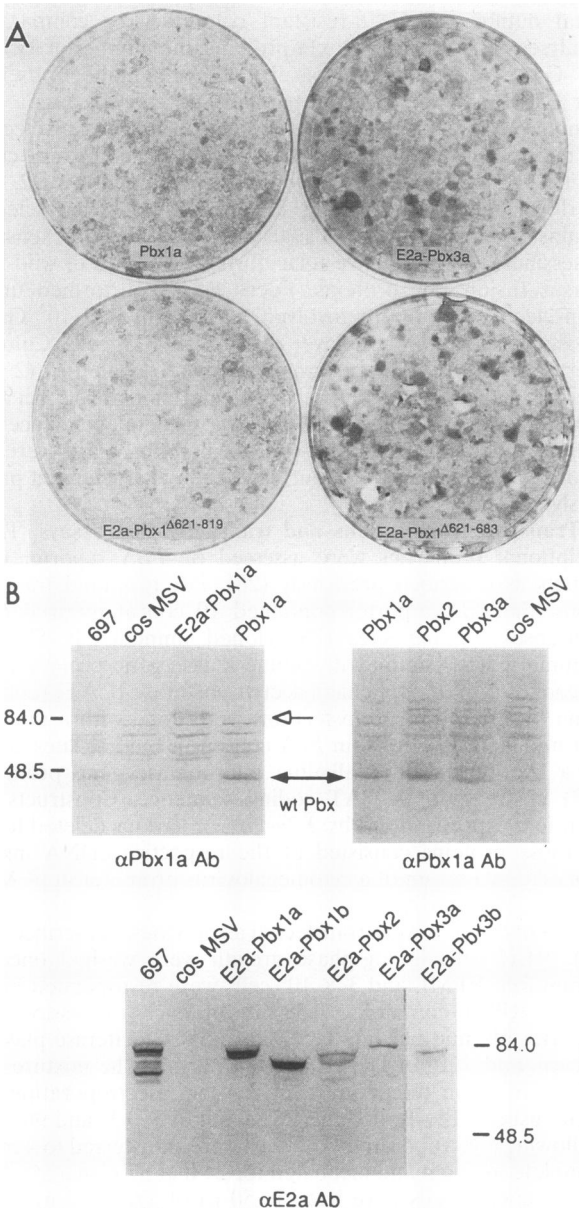

FIG. 1. Fusion with E2a activates the transforming properties of Pbx proteins. (A) Representative results of focus-forming assays. NIH 3T3 cells were infected with recombinant retroviruses expressing wild-type or chimeric Pbx proteins as indicated. Dark-staining foci correspond to transformed cells that have overgrown nontransformed cells. (B) Expression of wild-type and chimeric Pbx proteins in transfected fibroblasts. Protein extracts from NIH 3T3 cells stably transfected with various Pbx expression constructs were analyzed by Western blotting using an anti-Pbx1a antiserum (34) or an anti-E2a monoclonal antibody (Ab) (19) as indicated below the panels. Transfected constructs are indicated above the lanes. The t(1;19)-bearing cell line 697 served as a positive control, and fibroblasts infected with the retroviral vector lacking an insert cDNA (cos-MSV) served as a negative control. The open arrow indicates migration of E2a-Pbx1a; the double-headed arrow marks migration of wild-type (wt) Pbx proteins detected with the anti-Pbx1a antiserum. Sizes are indicated in kilodaltons.

lized on nitrocellulose filters by semidry electrophoretic transfer. E2a-Pbx chimeric proteins were detected with an E2a-specific monoclonal antibody (19). Pbx proteins were detected with a Pbx1a-specific antiserum, using conditions reported previously (34). This serum recognizes an epitope at the conserved carboxy terminus of PBX1a and thus reacts with Pbx2 and Pbx3a but not Pbx1b or Pbx3b.

Construction and characterization of transgenic mice. A cDNA insert coding for E2a-Pbx$^{\Delta 621\text{-}683}$ was used to replace the *E2A-PBX1a* insert of the transgene construct reported previously (9). This placed the *E2A-PBX*$^{\Delta 621\text{-}683}$ cDNA downstream of the immunoglobulin heavy-chain (IgH) enhancer/V_H promoter (5) and upstream of the simian virus 40 small intron and polyadenylation sequences. The entire E_μ/V_H*E2A-PBX*$^{\Delta HD}$ insert was released from the vector backbone, purified, and used for injection of pronuclei of FVB/N fertilized eggs, using standard methods (18). Transgenic progeny were identified by Southern blotting of *Eco*RI-digested tail biopsy DNA, using an *E2A-PBX1* probe. Transgene-positive lines were propagated by mating to FVB homozygous mice. Six founder animals were obtained, three of which (F8, A4, and B1F) died of malignant lymphomas before 5 months of age. Three lines showed germ line transmission of the transgene. Progeny from two of the lines (designated DF8 and DA4) showed alterations in the phenotype profiles of T-lineage cells and were selected for further analyses.

Freshly isolated cells from thymuses and tumors were stained with fluorochrome-tagged antibodies for four-color analysis, and the fluorescence was analyzed with a dual-laser FACSIV apparatus as described previously (44). Sources, specificities, and fluorochrome modifications of monoclonal antibodies have been described in detail previously (9). Tissues for light microscopy were fixed in 10% buffered formalin and processed for paraffin embedding. Sections were stained with hemotoxylin and eosin by using standard procedures.

Thymuses from transgenic animals were removed by blunt dissection, and total cell counts were determined on single-cell suspensions. Viable cells were determined by trypan blue exclusion (9). A mean age of 122 days for tumor onset was determined on a cohort of 17 animals from the DF8 founder line of Tg.*E2A-PBX*$^{\Delta HD}$ mice; line DA4 showed more rapid tumor onset (mean of 79 days, $n = 6$). All transgene-positive animals that died of malignant lymphomas did so by 160 days of age. Morphology, phenotype, clonality, and transplantability were assessed on at least four Tg.*E2A-PBX*$^{\Delta HD}$ tumors, using methods described previously for analysis of Tg.*E2A-PBX1* mice (9).

PCR analysis of mouse genomic DNA. Genomic DNA from tail biopsies of transgenic mice was analyzed by 30 cycles of PCR using primers homologous to sequences within or immediately flanking the human Pbx1 homeodomain. DNA from Tg.*E2A-PBX1a* but not Tg.*E2A-PBX*$^{\Delta HD}$ mice resulted in a band of approximately 200 bp, indicating that the Tg.*E2A-PBX*$^{\Delta HD}$ animals did not contain human DNA with an intact Pbx1 homeodomain in their genomes. DNA encoding the endogenous mouse Pbx1 homeodomain was not amplified by this procedure.

RESULTS

Oncogenic transformation by Pbx1 requires fusion with E2a. The structural requirements for oncogenic conversion of Pbx homeodomain proteins were assessed in NIH 3T3 cells following retroviral gene transfer. Consistent with previous studies (22), E2a-Pbx1b induced morphological transformation and loss of contact inhibition in NIH 3T3 cells (Table 1).

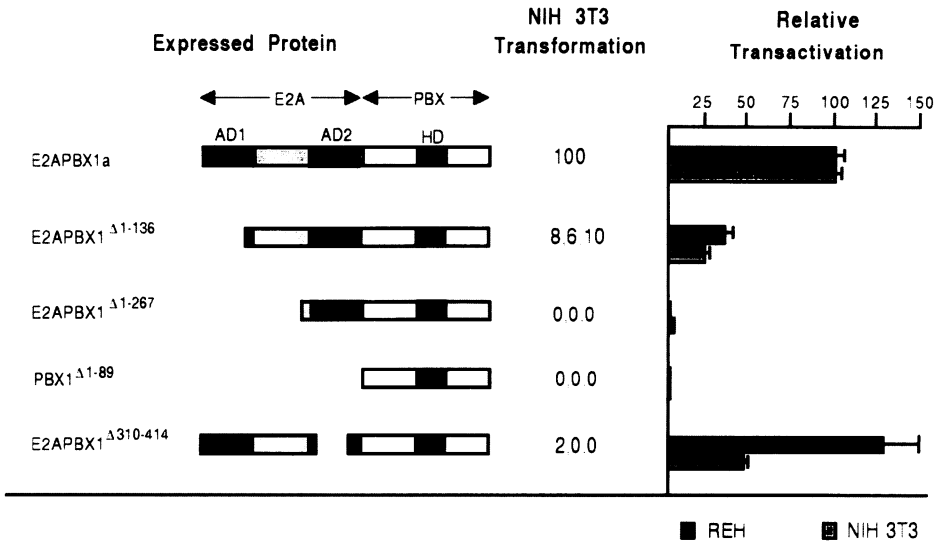

FIG. 2. Transcriptional and transforming activities of mutant E2a-Pbx1 proteins in tissue culture cells. Deletion derivatives of E2a-Pbx1 are shown schematically on the left. E2a- and Pbx-derived amino acids are shown at the top. The Pbx homeodomain is indicated as HD; AD1 and AD2 refer to transcriptional activation domains described elsewhere (3). Transactivation properties were assessed in precursor B (REH) cells and NIH 3T3 fibroblasts, using Pbx-dependent reporter plasmids PBX_{10}-TK-CAT and PBX_4-AP-CAT (28), respectively. Transactivation is shown as percentage of that observed for E2a-Pbx1a. All determinations are the mean values from two to three experiments performed in duplicate, and transfection efficiency was monitored by using an internal control Rous sarcoma virus-luciferase plasmid. NIH 3T3 cell focus-forming determinations are shown for three separately performed experiments. Data are expressed as a percentage relative to the number of foci per G418-resistant colony observed for E2a-Pbx1a.

Under these sensitive assay conditions, E2a-Pbx1a (which differs from E2a-Pbx1b in its carboxy-terminal portion as a result of alternative splicing of a single exon) also induced foci with an efficiency comparable to that of E2a-Pbx1b (Table 1). The latter contrasts with previous observations (22) and may reflect differences in levels of expression from the vectors used or differences in the NIH 3T3 cells. The anchorage independence and tumorigenicity of E2a-Pbx1-expressing cells have been reported previously (22) and were not further studied here.

To determine whether wild-type Pbx1 proteins might possess intrinsic oncogenic capabilities in the absence of protein fusion with E2a, they were hyperexpressed under identical conditions in NIH 3T3 cells. Neither form of the wild-type Pbx proteins (Pbx1a and Pbx1b) resulted in loss of contact inhibition or morphological transformation (Table 1) in spite of the fact that they were expressed at levels comparable to those of the chimeric proteins (Fig. 1). Growth rates and saturation densities were indistinguishable between Pbx1-expressing cells and NIH 3T3 or controls transfected with vector alone (35). Since Pbx required protein fusion with the heterologous E2a protein, its properties differed from those of all previously studied oncogenic homeodomain proteins whose transforming properties were evident in NIH 3T3 cells following simple overexpression. The essential contribution of E2a for activation of Pbx1 was not evident from this initial analysis, prompting additional studies to further define minimal functional domains contributed by both E2a and Pbx proteins.

Pbx2 and Pbx3 can be converted to oncoproteins by fusion with E2a. The Pbx1 proteins are extensively similar both within and outside their homeodomains with two other mammalian proteins named Pbx2 and Pbx3 (34). To establish whether the extensive similarities in primary sequence conferred similarities in function, the oncogenic potentials of Pbx2 and Pbx3 were tested in NIH 3T3 cells either in their wild-type conformations or as experimental fusions with the identical portion of E2a (amino acids 1 to 477) fused to Pbx1 following t(1;19) in acute leukemias. Each of the chimeric proteins E2a-Pbx2 and E2a-Pbx3a and a splice variant E2a-Pbx3b displayed focus-forming properties in NIH 3T3 fibroblasts comparable to those of Pbx1 chimeras (Table 1 and Fig. 1). In contrast, none of the wild-type proteins (Pbx2, Pbx3a, or Pbx3b) resulted in transformation of NIH 3T3 cells. These results demonstrated that all three of the highly related Pbx homeodomain proteins (and their splice variants) possessed latent transforming properties that were similar in potency and dependence on fusion with E2a.

E2a-Pbx1 contains modular DNA-binding and transcriptional activation domains that define its oncogenic potential. To further characterize the functional domains of E2a-Pbx1,

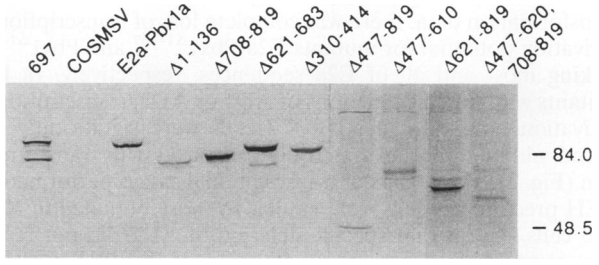

FIG. 3. Expression of E2a-Pbx1 proteins in transfected fibroblasts. Protein extracts from NIH 3T3 cells stably transfected with various E2a-Pbx1 expression constructs were analyzed by Western blotting using an anti-E2a monoclonal antibody (19). Transfected constructs are indicated above the lanes. The t(1;19)-bearing cell line 697 served as a positive control, and fibroblasts infected with the retroviral vector lacking an insert cDNA (cos-MSV) served as a negative control. Sizes are indicated in kilodaltons.

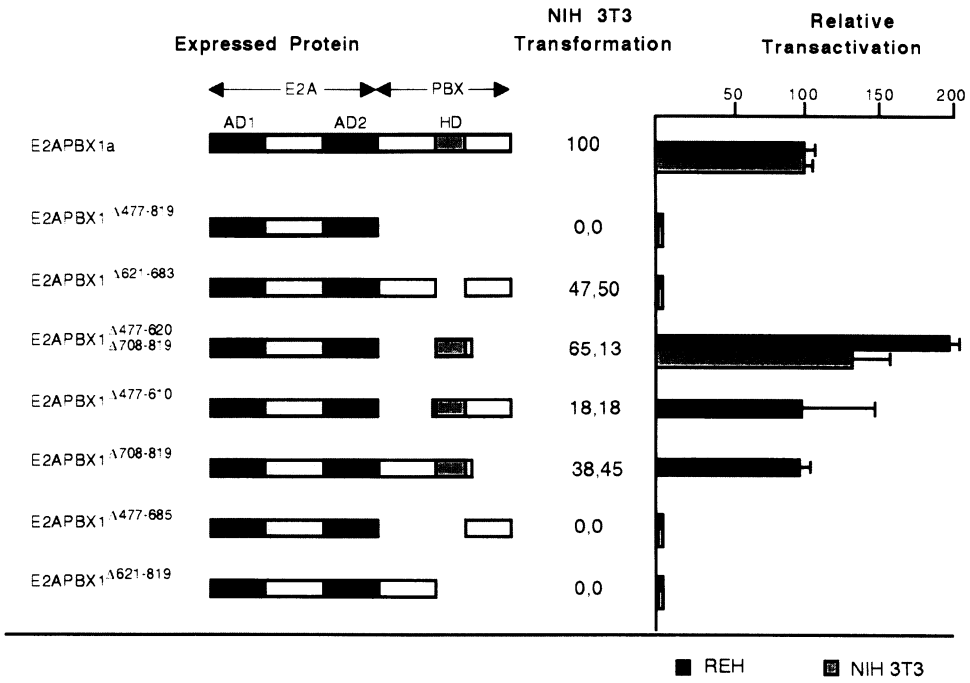

FIG. 4. Contributions of Pbx1 to the transcriptional and transforming properties of E2a-Pbx1. Transcriptional and focus-forming assays were performed as described in legend to Fig. 2. Transactivation is shown as percentage of that observed for E2a-Pbx1a. NIH 3T3 cell focus-forming data for two independently performed experiments are expressed as a percentage relative to the number of foci per G418-resistant colony observed for E2a-Pbx1a.

we generated a series of deletion mutants for subsequent expression in cultured cells to compare their relative transforming and transcriptional properties. Transcriptional capabilities of the deleted proteins were measured in lymphoid (REH) or fibroblast (NIH 3T3) cell lines in cotransfections using the PBX_{10}-tk-CAT or PBX_4-AP-CAT reporter plasmid, respectively, that responds to chimeric but not wild-type Pbx proteins (28). Specific domains of E2a that mediate transcriptional activation in certain cell types have been reported in previous studies (3, 17, 40). Two of the E2a mutants (E2a-$Pbx1^{\Delta 1\text{-}136}$ and E2a-$Pbx1^{\Delta 310\text{-}414}$) resulted in deletion of the previously mapped activation domains AD1 and AD2, respectively. Other mutants removed most (E2a-$Pbx1^{\Delta 1\text{-}267}$) or all ($Pbx1^{\Delta 1\text{-}89}$) of the E2a sequences present in E2a-Pbx1 (Fig. 2).

When assayed for their focus-forming properties, these mutant proteins showed either complete or nearly complete loss of transforming potential (Fig. 2). Concordant with the transformation data, there was complete loss of transcriptional activation potential for mutants E2a-$Pbx1^{\Delta 1\text{-}267}$ and $Pbx1^{\Delta 1\text{-}89}$, lacking most and all of E2a sequences, respectively. In the mutants with specific deletions of AD1 or AD2, transcriptional activation potentials in NIH 3T3 cells were significantly reduced, although not as drastically compared with transformation (Fig. 3). The results of transcriptional assays performed in REH precursor B cells were similar to those obtained in NIH 3T3 cells, except that specific deletion of AD2 did not result in a comparable decrease in activation of the *PBX* reporter gene in lymphoid cells. This finding is consistent with earlier observations that the transcriptional activation potential of AD2 varies in different cell types (3). The differences in transcriptional and transforming properties observed for E2a-$Pbx1^{\Delta 1\text{-}267}$ versus E2a-$Pbx1^{\Delta 1\text{-}136}$ likely reflect a more complete deletion of AD1 in E2a-$Pbx1^{\Delta 1\text{-}267}$, as earlier studies have not detected transcriptional activation potential in the proline-rich region between AD1 and AD2 (3, 40). Western blot (immunoblot) analyses showed that proteins with the predicted molecular weights were expressed in stably transfected NIH 3T3 cells (Fig. 3) or transiently transfected COS7 cells (35) at levels comparable to those of constructs with focus-forming properties and were capable of localizing to the nucleus (29). These observations suggested that both activation domains of E2a needed to remain intact for efficient transformation of NIH 3T3 cells. The inability of certain E2a-Pbx1 or Pbx1 mutant proteins to transform cells demonstrated that the oncogenic potential of Pbx1 was not simply induced by removal of the amino-terminal region that is deleted from Pbx1 following fusion with E2a but, rather, suggests that the intact chimera undergoes a gain-of-function transition by acquiring functional motifs from E2a.

Another set of E2a-Pbx1 mutants was constructed to assess the contributions of Pbx1 sequences to the transcriptional and transforming capabilities of the chimeric proteins. Not surprisingly, the homeodomain of Pbx1 proved to be essential for activated transcription of the PBX reporter construct, as its deletion from the chimera abolished CAT activity (Fig. 4, construct $E2APBX^{\Delta 621\text{-}683}$). Furthermore, all mutants with an intact homeodomain, including one with only 87 Pbx amino acids fused to E2a (construct $E2APBX1^{\Delta 477\text{-}620,708\text{-}819}$), displayed robust activator activity. Taken together, these studies demonstrated that E2a-Pbx1 contains modular DNA-binding and transcriptional activation domains contributed separately by the heterologous proteins fused together by the t(1;19).

E2a-Pbx1a displays homeodomain-independent transformation. The constructs containing large deletions of Pbx amino acids upstream and/or downstream of the homeodomain were not significantly impaired in transformation (constructs $E2A\text{-}PBX^{\Delta 477\text{-}620,708\text{-}819}$, $E2A\text{-}PBX^{\Delta 477\text{-}610}$, and $E2A\text{-}PBX^{\Delta 708\text{-}819}$), findings that paralleled results obtained for

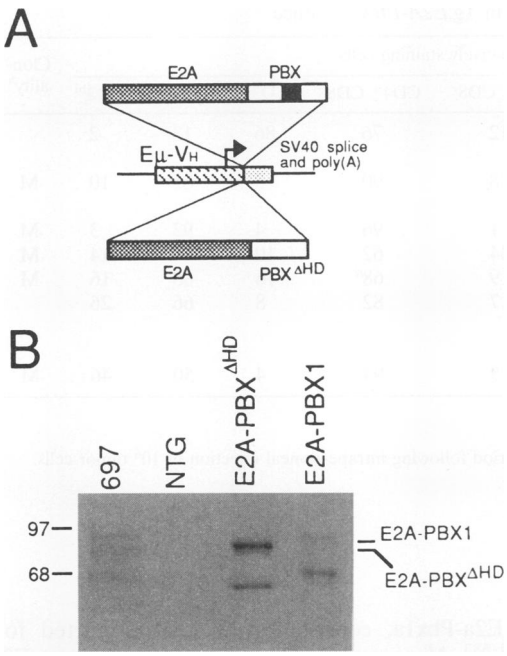

FIG. 5. Structure and expression of the *E2A-PBX*$^{\Delta HD}$ transgene construct. (A) Schematic comparison of the transgene constructs used for Tg.*E2A-PBX* and Tg.*E2A-PBX*$^{\Delta HD}$ mice. SV40, simian virus 40. (B) Western blot analysis of thymocytes from transgenic mice. Chimeric Pbx proteins were detected with an anti-E2a monoclonal antibody which does not significantly cross-react with mouse E2a proteins (19). Lower bands reacting with the anti-E2a antibody in both lines of transgenic mice are presumed to result from differential splicing. The t(1;19)-bearing cell line 697 served as a positive control. NTG, nontransgenic control. Sizes are indicated in kilodaltons.

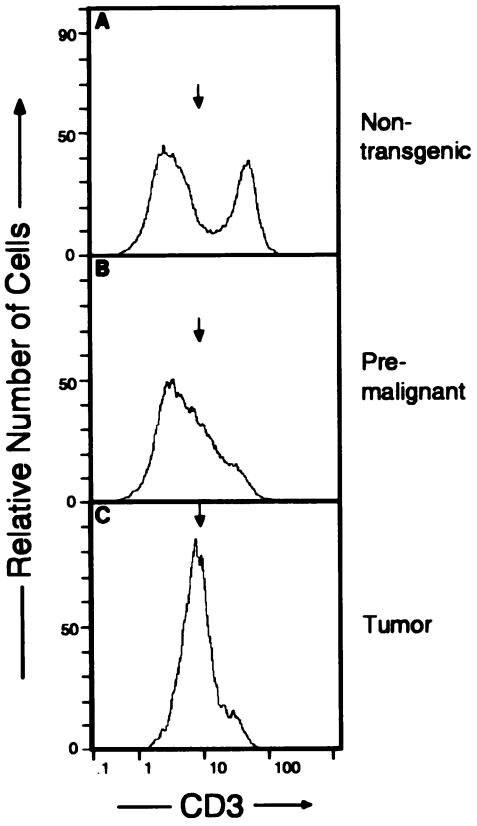

FIG. 7. Homeodomain-independent induction of lymphoid malignancies by E2a-Pbx1 in transgenic mice. FACS analyses are shown for thymocytes from control and Tg.*E2A-PBX*$^{\Delta HD}$ transgenic mice. Relative numbers of thymocytes expressing different levels of CD3 are shown for 4-week thymus from a nontransgenic animal (A) and a transgenic animal (B) and for an *E2A-PBX*$^{\Delta HD}$-induced malignant lymphoma (C). Arrows serve as internal reference standards for CD3 expression.

transcriptional activation by these constructs. Unexpectedly, the Pbx1 homeodomain was not required for transformation, as the construct expressing E2a-Pbx$^{\Delta 621\text{-}683}$ retained significant focus-forming ability in spite of the fact that it could not activate the PBX-specific reporter gene (Fig. 4), whereas a truncated fragment containing only the E2a portion of the chimera (construct E2A-PBX1$^{\Delta 477\text{-}819}$) showed no transforming activity or transcriptional activation. Analysis by Western blotting confirmed that each of these transformants expressed proteins of the predicted molecular weights (Fig. 3) that localized to the nucleus, as assessed by cell staining with an anti-E2a or anti-Pbx antibody (29). Attempts to further define the minimal region of Pbx necessary for homeodomain-independent transformation by deletion mutagenesis resulted in complete loss of focus-forming ability (constructs E2A-PBX$^{\Delta 477\text{-}685}$ and E2A-PBX$^{\Delta 621\text{-}819}$). Thus, transformation in this system was not a consequence of simple overexpression of a truncated E2a protein but, rather, required specific portions of Pbx fused to E2a. The data demonstrated that a minimal portion of Pbx containing its homeodomain was sufficient in this regard although the homeodomain was not essential, as its deletion out of the Pbx backbone of E2a-Pbx1 did not abrogate focus-forming ability.

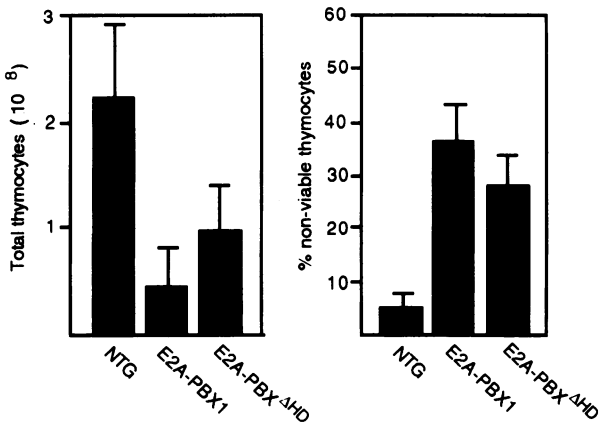

FIG. 6. Thymocyte numbers and viability in Tg.*E2A-PBX*$^{\Delta HD}$ mice. Thymuses from transgenic and nontransgenic (NTG) animals were removed by blunt dissection, and total cell counts were determined on single-cell suspensions. Viable cells were determined by trypan blue exclusion. Data shown were obtained from a minimum of three thymuses in each category.

E2a-Pbx1$^{\Delta HD}$ induces malignant lymphomas in transgenic mice. To further characterize the homeodomain-independent oncogenic properties of E2a-Pbx1, lymphoid transformation was assessed in transgenic mice. In earlier studies, we observed impaired differentiation of lymphoid cells and development of malignant lymphomas in transgenic mice expressing full-length E2a-Pbx1 chimeric proteins (9). Construct Tg.*E2A-PBX*$^{\Delta HD}$,

TABLE 2. Characteristics of tumors arising in Tg.*E2A-PBX*$^{\Delta HD}$ mice

Mouse	Age[a]	Tumor site(s)	% Positively staining cells							Clonality[b]	Transfer[c]
			CD4$^-$ CD8$^-$	CD4$^+$ CD8$^-$	CD4$^-$ CD8$^+$	CD4$^+$ CD8$^+$	CD3^{lo}	CD3^{med}	CD3^{hi}		
F8 (founder)	115	Spleen, thymus	12	1	12	76	86	13	2		+
DF8MB3	68	Spleen, thymus									
M2M	105	Spleen, lung	2	1	8	90	5	85	10	M	+
M8F[d]	158	Spleen, liver									
A4 (founder)	106	Kidney	1	3	1	96	4	93	3	M	
K2M	68	Spleen, lung, kidney	4	1	34	62	10	76	14	M	−
K6M	57	Spleen, lung	2	10	19	68[d]	16	68	16	M	+
L1F	70	Spleen, lung, thymus	1	1	17	82	8	66	26		
DA4MA1[e]	93	Spleen, lung, kidney									
DA4MB4	77	Spleen									
B1F (founder)	133	Spleen, thymus	1	1	5	94	4	50	46	M	+

[a] Age in days at time of death.
[b] M, monoclonal.
[c] Presence (+) or absence (−) of tumors in syngeneic hosts over a 4-month observation period following intraperitoneal injection of 10^6 tumor cells.
[d] Mean fluorescence intensity for CD8 corresponded to medium levels of expression.
[e] Mouse found dead with typical tumor by gross appearance but no viable cells for analysis.

containing immunoglobulin regulatory elements (Igμ enhancer fused to an IgH V-gene promoter) identical to those employed for our previous studies, was used to introduce the *E2A-PBX*$^{\Delta 621\text{-}683}$ cDNA into the germ line of FVB inbred mice (Fig. 5A). From six founder animals three lines were obtained, two of which (DF8 and DA4) showed lymphoid changes associated with transgene expression and were selected for further analyses.

Transgenic mice at 2 months of age showed lymphoid-specific expression of E2a-Pbx$^{\Delta HD}$, as demonstrated by Western blot analysis (Fig. 5B). Similar to Tg.*E2A-PBX1* mice, the Tg.*E2A-PBX*$^{\Delta HD}$ mice showed highest levels of transgene expression in the thymus. The immunoreactive protein had a migration in SDS-PAGE that was slightly different from that of intact E2a-Pbx1a, consistent with that predicted for E2a-Pbx$^{\Delta 621\text{-}683}$. No protein with a migration of intact E2a-Pbx1 was detected in the Tg.*E2A-PBX*$^{\Delta HD}$ mice by this sensitive analysis, nor was there evidence of DNA encoding the human Pbx1 homeodomain in these animals by PCR (35).

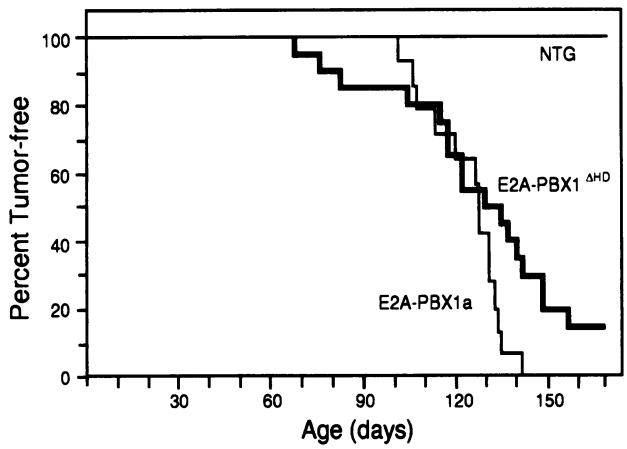

FIG. 8. Development of malignant lymphomas in Tg.*E2A-PBX*$^{\Delta HD}$ mice compared with that in Tg.*E2A-PBX1a* mice and nontransgenic littermates. Data represent the percentages of animals alive and free of tumor over time. For Tg.*E2A-PBX*$^{\Delta HD}$ mice (n = 20), the data derive from a single founder line (DF8) in which 85% of animals developed fatal lymphomas by 160 days (mean, 122 days). Malignant lymphoma was confirmed by autopsy or histologic examination of involved tissues. Results for Tg.*E2A-PBX1a* mice (n = 14; mean age, 130 days) have been reported previously (9). For nontransgenic (NTG) littermate controls, n = 20.

Tg.*E2A-PBX*$^{\Delta HD}$ mice showed alterations of thymocyte differentiation similar to those observed in Tg.*E2A-PBX* mice (9). Thymus sizes were less than normal, and total numbers of viable thymocytes from 5- to 9-week-old Tg.*E2A-PBX*$^{\Delta HD}$ mice were less than half the level seen in nontransgenic FVB/N mice (Fig. 6). Similarly, increased levels of thymocyte death were evidenced by elevated percentages of nonviable cells in transgenic thymuses (28% versus 5% in controls) comparable to levels seen in Tg.*E2A-PBX1* mice and consistent with an increased propensity to undergo apoptosis as reported earlier (9). The relative ratios of specific thymocyte subpopulations identified on the basis of their surface antigen expression profiles were altered similarly in Tg.*E2A-PBX1* and Tg.*E2A-PBX*$^{\Delta HD}$ mice. Animals from both lines showed a markedly increased proportion of cells with a transitional phenotype characterized by coexpression of high levels of CD4 and CD8 and intermediate levels of CD3 (Fig. 7) and reduced numbers of mature T cells in the blood and spleen (data not shown).

Tg.*E2A-PBX*$^{\Delta HD}$ mice also developed lymphoid malignancies with pathologic and phenotypic features similar to those observed in Tg.*E2A-PBX1* transgenic mice. Of a cohort of 20 transgenic animals from the DF8 founder line of Tg.*E2A-PBX*$^{\Delta HD}$ mice, 85% succumbed to malignant lymphomas by 5 months of age (Fig. 8). The mean age of tumor-induced mortality for this line of Tg.*E2A-PBX*$^{\Delta HD}$ mice is virtually identical to that of the previously analyzed Tg.*E2A-PBX1a* mice (122 versus 130 days; Fig. 8). Age of lymphoma onset was somewhat more variable for individual Tg.*E2A-PBX*$^{\Delta HD}$, mice and tumors more frequently involved the spleen, lymph nodes, and extranodal sites such as the kidney (Table 2). The other Tg.*E2A-PBX*$^{\Delta HD}$ founder line (DA4) with T-lineage expression also developed malignant lymphomas at high frequency but with a shorter latency (mean, 79 days; data not shown). All transgene-positive animals that died of malignant lymphomas did so by 5 months of age. Histologically, tumors were diffuse lymphomas composed of medium to large cells with numerous mitotic figures and were monoclonal by Southern blot analysis (Fig. 9). Fluorescence-activated cell sorting (FACS) analyses

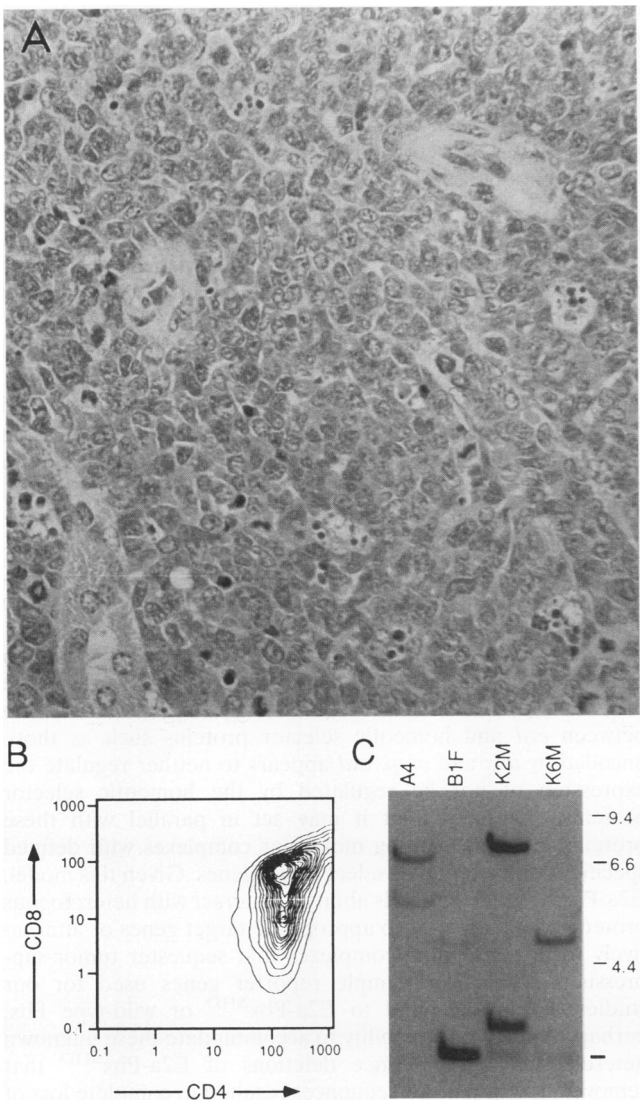

FIG. 9. Histologic, phenotypic, and molecular features of tumors arising in Tg.*E2A-PBX*$^{\Delta HD}$ mice. (A) Histological appearance of malignant lymphoma infiltrating the kidney of an 8-week-old Tg.*E2A-PBX*$^{\Delta HD}$ mouse. Tissue section was stained with hematoxylin and eosin. Magnification, ×308. (B) FACS analysis for CD4 and CD8 expression by tumor cells from a Tg.*E2A-PBX*$^{\Delta HD}$ mouse. (C) Southern blot of DNA from tumors. Tumor DNA was digested with *Eco*RI and analyzed with a probe specific for the T-cell receptor Jβ2 genes. Designations correspond to those in Table 1. Dash indicates position of germ line band. Sizes are indicated in kilodaltons.

showed that the tumors were composed of cells that coexpressed CD4, CD8, and CD3^{med} (Table 2 and Fig. 9), a surface antigen profile identical to that displayed by the phenotypically abnormal population of thymocytes in premalignant animals (Fig. 7). Tumors could be serially transplanted to syngeneic hosts but not cultured in vitro (Table 2). Thus, by these criteria, E2a-Pbx$^{\Delta HD}$ appeared to have oncogenic properties comparable to those associated with intact E2a-Pbx1, demonstrating that the homeodomain was dispensable for in vivo transformation of lymphoid cells as well as fibroblasts in vitro.

DISCUSSION

Earlier studies have shown that E2a-Pbx1, originally discovered as the fusion protein resulting from t(1;19) in acute leukemias, is capable of inducing oncogenic changes in several different cell types, including fibroblasts, myeloid cells, and lymphocytes (9, 21, 22). However, these studies did not address the oncogenic potential of wild-type Pbx proteins or the structural components that establish and define the transformation properties of chimeric E2a-Pbx1 proteins. The current study demonstrates that none of the known Pbx-like proteins (Pbx1, Pbx2, and Pbx3) comprising a small subfamily of atypical homeodomain proteins contains intrinsic oncogenic capability when hyperexpressed as wild-type proteins in rodent fibroblasts. However, all three Pbx family members were converted to oncogenic forms following fusion with the heterologous basic helix-loop-helix protein E2a, demonstrating their latent oncogenic potentials. Furthermore, the portions of E2a required for oncogenic conversion of Pbx1 coincided with its known transcriptional activation domains. The DNA-binding homeodomain was the minimal portion of Pbx1 sufficient for transformation. Thus, these studies show that E2a-Pbx1 functions as a chimeric oncoprotein which contains modular DNA-binding and transcriptional activation domains separately contributed by each of the constituent proteins giving rise to the chimera.

The required fusion with E2a for oncogenic activation of Pbx proteins distinguishes them from a growing number of homeodomain proteins that possess intrinsic oncogenic capabilities following simple overexpression. Several of the clustered *Hox* genes related to *Drosophila Hom* genes display such properties in rodent fibroblasts. These include *Hoxa-7*, *Hoxa-5*, *Hoxb-7*, and *Hoxc-8*, belonging to the *Antp* and *Ubx* subfamilies in several different clusters or paralog groups (33). Other more divergent *Hox* genes, such as *Hoxa-1*, *Evx-1*, and *Cdx-1*, display similar experimental properties (33), but only *Hoxb-8* (otherwise known as *Hox2.4*) and *Hox11* (highly similar to *Hoxa-1*) are implicated in naturally arising malignancies. *Hox2.4* expression was deregulated by adjacent proviral insertion in the WEHI3 myeloid leukemia cell line (6, 25). *Hox11* was discovered by virtue of its translocation into the T-cell receptor locus, resulting in its deregulated expression in 5 to 10% of acute T-lineage leukemias (10, 16, 30). Although Pax3 is involved by protein fusion following its translocation in alveolar rhabdomyosarcomas, it contains a paired DNA-binding domain in addition to its homeodomain and experimentally does not require protein fusion for oncogenic activity (4, 32, 45).

We used a fibroblast focus-forming assay to study the structural requirements for oncogenic activation of Pbx proteins. These assays were performed under selective conditions to increase their sensitivity by eliminating nontransfected cells, thereby maximizing our capability for detecting any oncogenic potential associated with wild-type Pbx proteins. Furthermore, earlier studies indicated that E2a-Pbx1a possessed relatively weak focus-forming properties in fibroblasts even under selective conditions (22). In our studies, both forms of E2a-Pbx1, resulting from differential splicing of their carboxy-terminal portions, were capable of inducing foci, perhaps reflecting higher levels of expression than those obtained in previous studies (22) or differences in the NIH 3T3 cells. The experimental conditions used in our studies reproducibly measured differences in the focus-forming properties of various mutant E2a-Pbx proteins, but a more comprehensive assessment of the oncogenic properties of these proteins in fibroblasts was not conducted. Our subsequent studies focused instead on the lymphoid-transforming properties of select mutants in trans-

genic mice, a more relevant test of their oncogenicity given that lymphoid precursors constitute the target cell population in human leukemias. Nevertheless, further studies in fibroblasts are warranted to characterize potential differences in their tumorigenic potential and ability to induce foci under nonselective conditions.

The oncogenic incompetence of wild-type Pbx proteins likely reflects their lack of transcriptional activator potential, as demonstrated on reporter genes containing two different minimal promoters. Oncogenic conversion of Pbx1 occurred in parallel with acquisition of transcriptional competence in these assays. This correlation strongly supports a model for the oncogenic role of E2a-Pbx1 that is mediated by transcriptional deregulation of specific subordinate genes. However, the transcriptional assays performed here did not completely reflect the oncogenic activity of E2a-Pbx1, since transformation and transactivation did not correlate for all constructs. This finding suggests that the transcriptional roles for chimeric and wild-type Pbx proteins may require interactions with heterologous proteins, perhaps providing a molecular explanation for the observed homeodomain-independent transformation as discussed below. Our studies of the transcriptional properties of E2a-Pbx1 confirm earlier observations that the activation potential of E2a is contained in two distinct domains named AD1 and AD2 (3, 17, 40). These domains vary in their potencies in different cell types (3), consistent with our observations that E2a-Pbx1-mediated activation is significantly reduced in fibroblasts but essentially unaffected in lymphoid cells by deletion of AD2. These results predict that AD2 may not be essential for induction of lymphoid malignancies, in contrast to fibroblasts, in which case deletion of AD2 drastically impaired focus-forming potential. It would be of interest to test an AD2 deletion mutant for oncogenic potential in a lymphoid transformation assay.

Although a minimal portion of Pbx1 containing its homeodomain and flanking amino acids was sufficient for transcriptional activation and transformation when fused to E2a, paradoxically the homeodomain was not absolutely required for induction of foci in NIH 3T3 cells and malignant lymphomas in transgenic mice. These observations suggest that in the absence of homeodomain-DNA interactions, a compensatory mechanism allows E2a-Pbx to function as a transcriptional protein. This could occur by several different potential mechanisms. For example, Pbx may contain an accessory DNA-binding domain outside the homeodomain. This appears to be the case for two of the Pax proteins (Pax3 and Pax6) which contain homeodomains in addition to DNA-binding motifs known as paired domains (32). Not only are Pax3 and Pax6 transforming, but so are Pax1, Pax2, and Pax8, which lack or contain only rudimentary homeodomains (32). Thus, in Pax proteins, the paired domain is the dominant DNA-binding motif, as evidenced by point mutations that impair both DNA binding and transformation. In contrast, a secondary DNA-binding motif is not apparent in any of the Pbx/Exd family members, and binding site selection experiments with the isolated homeodomain (28) or a larger portion (46) of Pbx resulted in the same high-affinity binding sequence. Furthermore, unlike the Pax proteins, the homeodomain of Pbx is both sufficient and dispensible for transformation.

A more likely possibility is that the observed homeodomain-independent transformation is initiated by protein-protein interactions alone. Such interactions may be analogous to those proposed for the homeodomain-independent activity of *fushi tarazu* (*ftz*) that result in deletion of odd-numbered parasegments (the so-called anti-*ftz* phenotype) in *Drosophila* blastoderm embryos (11). Deletion of the homeodomain also does not impair synergistic activation of an *engrailed*-derived promoter by *ftz* and the PRD homeodomain protein (2). These and other data (42) indicate that the functional specificity of homeodomain proteins is determined by the combined effects of various individual interactions and not a single high-affinity protein-DNA interaction. However, although the *ftz* homeodomain was not necessary to generate the anti-*ftz* phenotype, it is required to rescue the *ftz* null phenotype (11). Thus, in contrast to E2a-Pbx1-mediated transformation, full biological activity of *ftz* requires an intact homeodomain. The role of the homeodomain in mediating biological functions of wild-type Pbx proteins cannot be assessed at this time.

Protein-protein interactions have been shown to be essential for the biological properties of several homeodomain proteins. The most extensively characterized are yeast MAT**a**1 and MATα2, which regulate mating-type-specific gene expression in yeast cells through combinatorial interactions which serve to direct their binding to specific DNA recognition elements (20). More recently, the pHOX protein was shown to regulate the DNA-binding activity of serum response factor (15), suggesting that Hox proteins, through their heterotypic interactions, may help define tissue-specific transcriptional responses to generic intracellular signalling events.

Identification of the product of the *Drosophila exd* gene as a Pbx homolog has suggested a model for the activity of Pbx-like proteins (41). Genetic evidence strongly suggests interaction between *exd* and homeotic selector proteins such as those encoded by *ubx* and *antp*. *exd* appears to neither regulate the expression of nor be regulated by the homeotic selector proteins, suggesting that it may act in parallel with these proteins, perhaps forming molecular complexes with defined specificity and affinity for select target genes. Given this model, E2a-Pbx$^{\Delta HD}$ may retain its ability to interact with heterologous proteins which direct it to appropriate target genes or alternatively form nonbinding complexes that sequester tumor-suppressing factors. The simple reporter genes used for our studies did not respond to E2a-Pbx$^{\Delta HD}$ or wild-type Pbx, perhaps reflecting an inability to accommodate these unknown heterologous factors. Since deletions of E2a-Pbx$^{\Delta HD}$ that removed additional Pbx sequences resulted in complete loss of transforming ability, homeodomain-independent activity of the chimera may result from relatively weak heterotypic interactions requiring two or more specific regions of Pbx. A more refined mutational analysis will be required to further map the sites on Pbx that mediate this effect. However, homeodomain-independent transformation by E2a-Pbx1 should serve as a useful in vitro assay to further characterize the biochemical properties of homeodomain proteins and allow a functional test of the hypothesis that the normal and perhaps oncogenic roles of Pbx/Exd proteins involve interactions with heterologous proteins of the homeodomain family.

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