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E2a-Pbx1 Induces Aberrant Expression of Tissue-Specific and Developmentally Regulated Genes When Expressed in NIH 3T3 Fibroblasts

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The E2a-Pbx1 oncoprotein contains the transactivation domain of E2a joined to the DNA-binding homeodomain (HD) of Pbx1. In mice, E2a-Pbx1 transforms T lymphoblasts and fibroblasts and blocks myeloblast differentiation. Pbx1 and E2a-Pbx1 bind DNA as heterodimers with other HD proteins whose expression is tissue specific. While the transactivation domain of E2a is required for all forms of transformation, DNA binding by the Pbx1 HD is essential for blocking myeloblast differentiation but dispensable for fibroblast or T-lymphoblast transformation. These properties suggest (i) that E2a-Pbx1 causes cellular transformation by activating gene transcription, (ii) that transcription of E2a-Pbx1 target genes is normally regulated by ubiquitous Pbx proteins and tissue-specific partners, and (iii) that DNA-binding mutants of E2a-Pbx1 activate a subset of all gene targets. To test these predictions, genes induced in NIH 3T3 fibroblasts by E2a-Pbx1 were identified and examined for tissue- and stage-specific expression and their differential abilities to be upregulated by E2a-Pbx1 in NIH 3T3 fibroblasts and myeloblasts and by a DNA-binding mutant of E2a-Pbx1 in NIH 3T3 cells. Of 12 RNAs induced by E2a-Pbx1, 4 encoded known proteins (a J-C region of the immunoglobulin kappa light chain, natriuretic peptide receptor C, mitochondrial fumarase, and the 3′,5′-cyclic nucleotide phosphodiesterase, PDE1A) and 5 encoded new proteins related to angiogenin, ion channels, villin, epidermal growth factor repeat proteins, and the human 2.19 gene product. Expression of many of these genes was tissue specific or developmentally regulated, and most were not expressed in fibroblasts, indicating that E2a-Pbx1 can induce ectopic expression of genes associated with lineage-specific differentiation.

The homeodomain (HD) protein Pbx1 was first identified as the chromosome 1 participant in the t(1;19) translocation, which is found in 20% of pediatric pre-B-cell acute lymphocytic leukemias (ALL) (27, 49). As a consequence of the t(1;19) translocation, the 3′ portion of the PBX1 gene, which encodes most of the Pbx1 protein, including the HD, is fused to the 5′ half of the E2A gene, which encodes two transactivation domains but lacks sequences encoding the helix-loop-helix DNA-binding domain (27, 49). Pbx1 and its highly related homologs, Pbx2 and Pbx3, are broadly expressed during embryogenesis and in adult tissues (42). The first DNA sequence found to bind the Pbx1 HD was TGATTGAT, and E2a-Pbx1 but not Pbx1 activated transcription through this motif (32, 37, 57). E2a-Pbx1 exhibits multiple transforming activities, inducing myeloblastic (25) and T-lymphoblastic leukemias (16) in mice, blocking differentiation of primary myeloblasts in marrow cultures without altering their factor dependence (28), and producing foci in NIH 3T3 fibroblasts (26). In the same assays, Pbx1 has no transforming potential and disruption of the transactivation function of E2a abolishes all forms of transformation by E2a-Pbx1, suggesting that E2a-Pbx1 transforms by activating gene transcription. Interestingly, DNA binding by the E2a-Pbx1 HD is not required for focus formation in NIH 3T3 cells or for induction of T-cell ALL (16, 29), while it remains essential for blocking myeloid differentiation (29), suggesting that E2a-Pbx1 activates the transcription of two distinct subsets of genes, one that mediates growth stimulation and does not require DNA binding by Pbx1 and one that disrupts differentiation by a DNA-binding-dependent mechanism.

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In *Drosophila melanogaster*, expression of homeotic complex (HOM-C) genes, which are localized within the unlinked Antennapedia and Bithorax complexes, establishes anterior-posterior identity of larval structures (41). Genetic evidence suggests that the *Drosophila* homolog of Pbx proteins, Extradenticle, regulates normal differentiation, in part by activating or repressing gene expression in concert with HD proteins encoded by these HOM-C genes (4, 52). For instance, both Exd and Ultrabithorax are required to activate expression of the *decapentaplegic* (*dpp*) gene in parasegments posterior to number 7 (53). Exd heterodimerizes with HD proteins encoded by HOM-C genes on TGATTGAT (25), the element first identified as a Pbx1-binding motif (32, 37, 57). Like Exd, both Pbx1 and E2a-Pbx1 bind DNA poorly as monomers but tightly as dimers with Hox proteins (the eucaryotic homologs of HOM-C proteins [36, 56]) to adjacent half-sites in the motifs, TGATT GAT, TGATTAAT, and TGATTTAT (9, 10, 30, 35). On these motifs, the Pbx protein appears to bind the 5′ TGAT core and the Hox protein binds the 3′ TGAT, TAAT, or TTAT cores (9, 10, 34). In mice, aberrant expression of Hox genes produces homeotic transformations of structures along the anterior-posterior axis, indicating that Hox genes, like their *Drosophila* cognates, play a role in establishing differentiation of anterior-posterior structures (11–14, 17, 23, 24, 33, 38, 39, 45). In the Pbx-Hox-DNA complex, interaction between the Hox and E2a-Pbx1 or the Pbx protein alters the DNA-binding specificity of the Hox protein at position 2 of its 4-bp core, changing it from TAAT to either TGAT or TTAT (10, 35), but maintains inherent Hox DNA-binding specificity at the dinucleotide GG, GA, TG, or TA 3′ to the Hox core (30). In vivo, E2a-Pbx1 and Hox proteins activate transcription synergistically on Pbx-Hox elements containing TAAT and TTAT 3′ Hox cores (10, 35).

Based on the observation that both E2a-Pbx1 and Pbx pro-

teins heterodimerize with Hox proteins, one model to account for the biochemical basis of transformation by E2a-Pbx1 is that E2a-Pbx1 replaces Pbx1 or other Pbx proteins in heterodimer complexes bound to regulatory elements in cellular promoters, inducing persistent transcriptional activation. This hypothesis would restrict the transforming abilities of E2a-Pbx1 to a cell type or to a stage in differentiation that expresses the appropriate heterodimer partner. Alternatively, E2a-Pbx1 alone may be sufficient to activate transcription from a subset of Pbx-Hox motifs containing the repeated TGAT motif (TGATTGAT), because E2a-Pbx1 activates transcription from this element in reporter constructs in all cell lines tested. Both of these transformation mechanisms predict that E2a-Pbx1 will activate transcription of developmentally regulated or tissue-specific genes whose expression is normally regulated by Pbx-Hox heterodimers. To begin to test this hypothesis and to characterize the activities that are collectively responsible for the E2a-Pbx1-transformed phenotype, we have identified genes transcriptionally activated in NIH 3T3 cells transformed by E2a-Pbx1, using differential display and representational difference analysis (RDA) PCR. Of the collection of 12 such genes that were identified, all 12 were detected by RDA PCR and 1 of these 12 was independently identified by differential display PCR. Most of the 12 genes identified as targets of E2a-Pbx1 were normally expressed in a tissue-specific or developmentally regulated manner and were either not transcribed or transcribed at very low levels in normal NIH 3T3 fibroblasts. A subset were activated by DNA-binding mutants of E2a-Pbx1, and one was strongly activated by oncogenes of the Ras and tyrosine protein kinase families. These data suggest that E2a-Pbx1 exhibits at least two distinct mechanisms of transcriptional activation and that it induces ectopic expression of differentiation genes, some of which can be designated oncofetal antigens.

MATERIALS AND METHODS

Cell lines and cell culture. Generation of both populations and agar colonies of NIH 3T3 fibroblasts transformed by E2a-Pbx1 was described previously (26), as were myeloblast cell lines immortalized by E2a-Pbx1 (28). Agar clones transformed by E2a-Pbx1 contained approximately five times the abundance of E2a-Pbx1 as did the populations of transformed cells. NIH 3T3 fibroblasts expressing the E2a-Pbx1 DNA-binding mutant, N682S, and Lck were also described previously (29, 62). NIH 3T3 fibroblasts infected with the retroviral vector pGD and the myeloid cell line 32D were used as negative controls. All NIH 3T3 fibroblasts were cultured in Dulbecco's modified essential medium supplemented with 10% fetal bovine serum. Myeloid cell lines were cultured in RPMI 1640 supplemented with 10% fetal bovine serum and 50 μM 2-mercaptoethanol.

RDA. RDA was used to clone cDNAs contained in populations of stably transformed NIH 3T3 fibroblasts, as originally described by Hubank and Schatz (21) and modified by Braun et al. (7). Briefly, mRNAs were isolated from NIH 3T3 fibroblasts infected with retrovirus containing the Moloney-based vector (pGD) or with virus containing the same vector expressing E2a-Pbx1. A 3-μg sample of mRNA from each cell population was used for RDA analysis. cDNAs were synthesized from the mRNAs and digested with *Dpn*II. Adapters composed of two oligonucleotides, 5′-AGCACTCTCCAGCCTCTCACCGCA-3′ (RBgl 24) and 5′-GATCTGCGGTGA-3′ (RBgl 12), were ligated to the *Dpn*II-digested cDNA. This mixture was amplified by PCR with RBgl 24 oligonucleotides, and the adapters were excised with *Dpn*II. A second pair of adapters, 5′-ACCGAC GTCGACTATCCATGAACA-3′ (JBgl 24) and 5′-GATCTGTTCATG-3′ (JBgl 12), were ligated to the amplified fragments from the E2a-Pbx1-transformed NIH 3T3 cells and hybridized with the RBgl24 amplified cDNA fragments from the NIH 3T3 pGD-infected control (RBgl adapters removed) at a ratio of 1:100 for 24 h. The hybridization mix was used as template for amplification by PCR. A second round of subtraction was performed by removing the JBgl adapters from an aliquot of the first-round PCR product, ligating a third pair of oligonucleotides adapters, 5′-AGGCAACTGTGCTATCCGAG-GGAA-3′ (NBgl 24) and 5′-GATCTTCCCTCG-3′ (NBgl 12), and hybridized with driver amplicons at a ratio of 1:800. The rest of the procedures are approximately the same as in the first-round subtraction and amplification.

Northern blotting analysis. Total RNA was extracted from cultured cells with acidic phenol. A 20-μg portion of RNA was loaded per lane on a 1% agarose gel containing formaldehyde. After electrophoresis, RNA was transferred to a nylon membrane (Magna Graf; MSI) and immobilized by UV cross-linking. ^{32}P-labeled DNA probes were synthesized by extension of random hexamer, using denatured DNA as specified by the manufacturer (Pharmacia Biotech). The QuikHyb (Stratagene) system was used for annealing the radioactive probe to immobilized RNA. Briefly, after prehybridization of the nylon membrane in QuikHyb for 15 min at 68°C, both the radioactive probe and 100 μl of 10-mg/ml sonicated salmon sperm DNA were denatured and added to the bag containing the membrane and prehybridization mix, and hybridization of the probe was allowed to proceed for 1 h at 68°C. The membrane was washed in 0.25× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and subject to autoradiography at −70°C overnight.

Library screening and cloning. A lambda ZAP II mouse spleen cDNA library (Stratagene) was screened with the 313-nucleotide EF-8 probe as specified by the manufacturer. The pBluescript phagmid was purified from positive clones by excision in vivo from the lambda ZAP II vector, using ExAssist helper phage (Stratagene). The full-length EF-8 clone bound the same E2a-Pbx1-induced transcript in transformed NIH 3T3 cells as did EF-8 and was sequenced.

DNA sequence analysis. Double-stranded DNA sequencing was performed with the Sequenase 2.0 DNA sequencing kit from Amersham Life Science, Inc., as specified by the manufacturer. For GC-rich regions that yielded strong stops in all lanes, 7-deaza-dGTP was substituted for dGTP, ΔTaqTM 2.0 polymerase was used, and extension reactions were performed at 70°C (Amersham).

Nucleotide sequence accession numbers. The nucleotide sequence data reported in this paper have been submitted to GenBank under accession numbers U72672 to U72681.

TABLE 1. Identities of target genes activated by E2a-Pbx1 in NIH 3T3 fibroblasts

Gene	Identity
EF-1	Immunoglobulin kappa chain J and C region
EF-2	Natriuretic peptide receptor C
EF-3	Mitochondrial fumarase
EF-4	PDE1A Ca^{2+}/calmodulin-dependent 3′,5′-cyclic nucleotide phosphodiesterase
EF-5	Novel angiogenin family member (angiogenin-3)
EF-6	Novel villin-related protein
EF-7	Protein related to human 2.19 protein (homolog?)
EF-8	Novel protein related to ion channels (RIC)
EF-9	Novel protein containing EGF-like cysteine repeats
EF-10	Unknown
EF-11	Unknown
EF-12	Unknown

RESULTS

Identification of 12 genes transcriptionally activated by E2a-Pbx1 in NIH 3T3 fibroblasts. Both differential-display PCR and RDA PCR were used to identify genes activated by E2a-Pbx1. Differential-display PCR was time-consuming and labor-intensive and, after a 3-month effort, yielded a single differentially expressed transcript; however, RDA PCR was fast, yielding 12 E2a-Pbx1-induced transcripts within 2 weeks. These transcripts are designated EF-1 to EF-12 (E2a-Pbx1 target gene in fibroblasts 1 to 12) and include the one found by differential-display PCR. In only one case were two RDA PCR products identified that were derived from the same transcript, and in this case, one represented an internal fragment of the other. Addition of an excess of the 12 cloned RDA PCR fragments to the RDA reaction mixtures did not result in the amplification of additional cDNA fragments unique to the E2a-Pbx1-transformed cells. The identity or similarity of EF-1 to EF-12 to known cellular genes is indicated in Table 1. Transcriptional activation of each gene was characterized in NIH 3T3 fibroblasts and primary myeloblasts transformed by E2a-Pbx1, in NIH 3T3 fibroblasts transformed by a DNA-binding mutant of E2a-Pbx1, and in a clone of NIH 3T3 cells transformed by an activated version of the Lck tyrosine protein kinase, cloned from a human T-cell leukemia (Fig. 1). Tissue-specific expression (Fig. 2A; Table 2), developmental expression (Fig. 2B; Table 2), and expression in NIH 3T3 fibroblasts

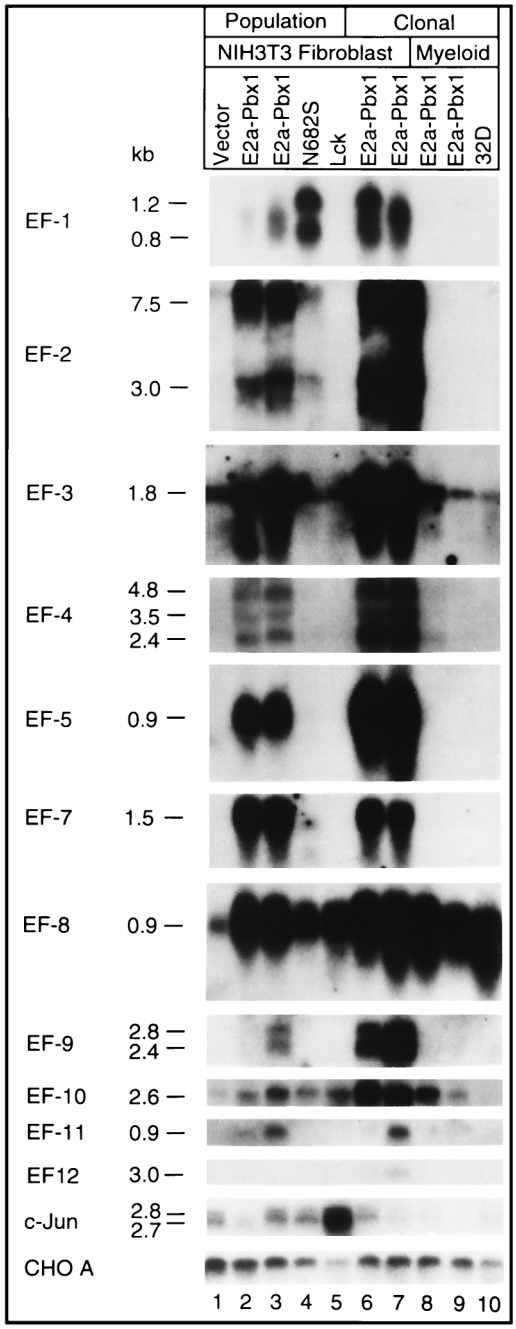

FIG. 1. Expression levels of genes induced by E2a-Pbx1, DNA-binding mutants of E2a-Pbx1, and oncogenic Lck. Northern blot analysis of 12 genes transcriptionally activated by E2a-Pbx1 in NIH 3T3 cells is shown. Lanes: 1, NIH 3T3 cells infected with retroviral vector pGD; 2 and 3, two different populations of NIH 3T3 cells infected by the pGD retrovirus encoding E2a-Pbx1; 4, NIH 3T3 cells transformed by the DNA-binding mutant of E2a-Pbx1 designated EPS N682S; 5, NIH 3T3 fibroblasts transformed by the activated Lck tyrosine protein kinase; 6 and 7, two clones expressing high levels of E2a-Pbx1 in NIH 3T3 cells; 8 and 9, two clones of myeloblasts immortalized by E2a-Pbx1; 10, IL-3-dependent 32D myeloid cells that do not contain E2a-Pbx1. Approximately 20 μg of total RNA was loaded on each lane. Hybridization with a CHO A probe (19) was used as a control for RNA loading. Transcript sizes are marked on the left. Probes were approximately the same specific activity, but exposure times varied as follows: 3.5 h for EF-1, 14 h for EF-2, 33 h for EF-3, 68 h for EF-4, 16 h for EF-5, 22 h for EF-7, 18 h for EF-8, 19 h for EF-9, 18 h for EF-10, 22 h for EF-11, 18 h for EF-12, and 8 h for c-*jun*.

transformed by Ras and other tyrosine protein kinase oncoproteins (Fig. 2C) was also examined.

(i) EF-1. EF-1 contained 388 nucleotides identical to the joining (J5) and constant regions of the immunoglobulin kappa light chain and a portion of the intron preceding J5, and it bound a population of transcripts 0.8 to 1.2 kb in size. These transcripts were not expressed in normal NIH 3T3 cells (Fig. 1, lane 1), were expressed at moderate levels in populations of E2a-Pbx1-transformed NIH 3T3 cells (lanes 2 and 3), and at higher levels in two agar colonies (lanes 6 and 7) of E2a-Pbx1-transformed cells, which contain approximately five times the level of E2a-Pbx1 as does the populations of cells. Cells transformed by the DNA-binding mutant of E2a-Pbx1, designated N682S, also exhibited high expression (lane 4), while two myeloid cell lines immortalized by E2a-Pbx1 failed to express this mRNA (lanes 8 and 9). EF-1 was not induced by oncogenic versions of Lck (lane 5), Ras, Neu, Src, Abl, or Sis (data not shown) and was strongly enriched in the spleen (Table 2). While the presence of this transcript suggested that the recombination machinery might be transcriptionally activated by E2a-Pbx1, neither recombinase-activating gene 1 or 2 (RAG-1, RAG-2) was expressed (data not shown).

(ii) EF-2. EF-2 contained 556 nucleotides encoding a protein 96% identical to rat natriuretic peptide receptor C (NPR-C) and 93 and 92% identical to human and bovine NPR-C, respectively, suggesting that EF-2 encodes murine NPR-C. NPR-C belongs to a family of membrane receptors made up of NPR-A, NPR-B, NPR-C, and NPR-D, which exhibit tissue- and development-specific expression (8, 60). NPF-C is expressed at moderate levels in endothelial cells in the heart and adrenal cortex, at low levels in the glomeruli of the kidney, and at high levels in widely scattered single-cell bodies of the brain (60). In addition to regulating body fluid homeostasis, natriuretic peptides regulate cell growth and differentiation (22, 59). EF-2 transcripts were 3.0 and 7.5 kb, exhibited very low expression in normal NIH 3T3 (Fig. 1, lane 1), and dosage-dependent expression in response to E2a-Pbx1 (compare lanes 2 and 3 with lanes 6 and 7). Expression was severely compromised by eliminating DNA binding by the Pbx1 HD (lane 4) and was not induced by oncogenic versions of Lck (lane 5), Ras, Neu, Src, Abl, or Sis (data not shown) or in myeloblasts transformed by E2a-Pbx1 (lanes 8 and 9). EF-2 transcripts were not detected in any tissue or at any stage in development, most probably because of its highly restricted localization to endothelial cells and single cell bodies within specific organs (8, 60). While NPR-A and NPR-B are ligand-activated receptor guanylyl cyclases, NPR-C lacks G-cyclase activity and is reported to down-regulate cyclic AMP levels and to increase levels of inositol triphosphate, diacylglycerol, and GTPase activity in bovine aortic smooth muscle cells (2, 20). Overexpression in fibroblasts could also activate some of these functions, activating pathways that control proliferation and differentiation.

(iii) EF-3. EF-3 contained 319 nucleotides encoding a protein 97% identical to Rat fumarase and 93% identical to human fumarase, suggesting that EF-3 encodes mouse mitochondrial fumarase, an enzyme that catalyzes the hydration of fumarate to malate in a non-rate-limiting step of the Krebs cycle. Low levels of fumarase mRNA were expressed in NIH 3T3 cells (Fig. 1, lane 1) and in myeloblasts not containing E2a-Pbx1 (lane 10). Expression was stimulated 5-fold in populations of E2a-Pbx1-transformed NIH 3T3 cells (lanes 2 and 3) and 15-fold in the clonal lines expressing higher levels of E2a-Pbx1 (lanes 6 and 7). Induction of fumarase mRNA was severely reduced by eliminating DNA binding by E2a-Pbx1 (lane 4), and expression was not induced by oncogenic versions of Lck (lane 5), Ras, Neu, Src, Abl, or Sis (data not shown) or

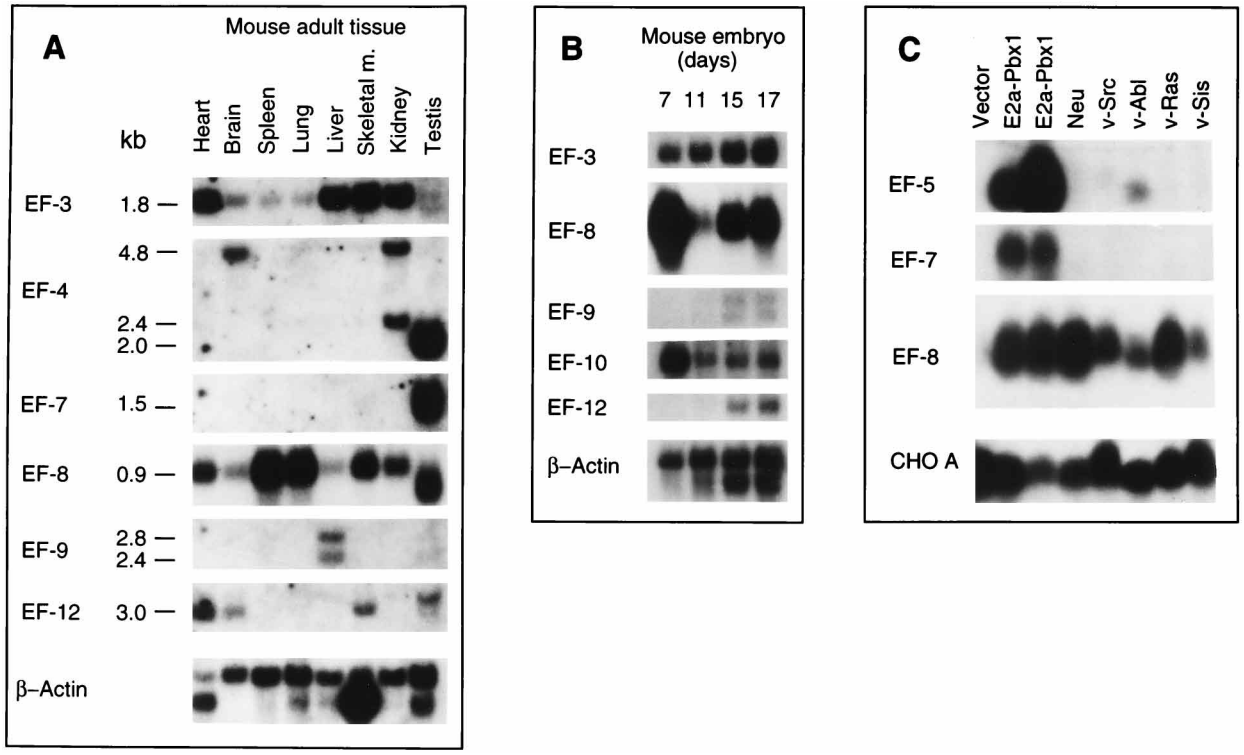

FIG. 2. Expression of E2a-Pbx1 target genes in normal tissues, during development, and in NIH 3T3 cells transformed by Ras and tyrosine kinase oncoproteins. (A) Northern blot analysis of selected target genes in a variety of adult mouse tissues, including heart, brain, spleen, lung, liver, skeletal muscle, kidney, and testis. (B) Expression of selected target genes on days 7, 11, 15, and 17 of mouse embryo development. A 2-μg sample of mRNA extracted from each kind of mouse tissue or embryo was analyzed in each lane. Hybridization with the human β-actin probe as a control is shown at the bottom of panels A and B. (C) Expression of selected target genes in NIH 3T3 cells infected by the pGD retrovirus (lane 1), or by retrovirus containing cDNAs expressing E2a-Pbx1 (lanes 2 and 3), Neu/Her-2 (lane 4), v-Src (lane 5), v-Abl (lane 6), and v-Ras (lane 7). A 20-μg sample of total RNA from each cell line was loaded in each lane. Hybridization with a CHO A probe as a control is shown at the bottom.

in E2a-Pbx1-immortalized myeloblasts (lanes 8 and 9). Normal expression of fumarase is ubiquitous but strongly elevated in the heart, liver, skeletal muscle, and kidney (Fig. 2A; Table 2), suggesting that tissue-specific factors regulate the magnitude of fumarase expression. While up-regulation of fumarase mRNA suggests that its tissue-specific transcription may be regulated, in part, by normal Pbx proteins, it is unlikely that its up-regulation in NIH 3T3 cells alters their growth potential or morphology.

(iv) EF-4. EF-4 contained 263 nucleotides encoding 61 amino acids 100% identical to that of mouse calcium/calmodulin 3′,5′-cyclic nucleotide phosphodiesterase PDE1A. PDE1A transcripts were not expressed in NIH 3T3 cells and exhibited dosage-dependent expression in response to E2a-Pbx1 (Fig. 1, compare lanes 2 and 3 with lanes 6 and 7). PDE1A was weakly activated by the DNA-binding mutant of E2a-Pbx1 (lane 4, more apparent on longer exposures), as well as in myeloblasts immortalized by E2a-Pbx1 (lanes 8 and 9), and was not ex-

TABLE 2. Tissue-specific and developmentally regulated expression[a] of E2a-Pbx1 target genes

Gene	Expression in specific adult tissues								Stage-specific expression at day:			
	Heart	Brain	Spleen	Lung	Liver	Skel. m.[b]	Kidney	Testis	7	11	15	17
EF-1	±	±	++++	++/+++	±	+	±	−	−	−	−	−
EF-2	−	−	−	−	−	−	−	−	−	−	−	−
EF-3	++/+++	+	+	+	++/+++	++/+++	++/+++	±	+	+	+/++	+/++
EF-4	−	+/++	−	−	−	−	+/++	+++	−	−	+	+
EF-5	−	−	−	−	±	−	−	−	±	−	−	−
EF-6	−	−	−	−	−	−	−	−	±	−	−	−
EF-7	−	−	−	−	−	−	−	+++	±	−	−	−
EF-8	++	+	+++	+++	+	++	+/++	++/+++	++++	±	+++	+++
EF-9	−	−	−	−	+/++	−	−	−	−	−	+	+
EF-10	±	−	+	++	+	+/++	±	−	++/+++	±	+	+
EF-11	−	−	−	−	−	−	−	−	−	−	−	−
EF-12	+/++	+	−	−	−	+	−	+	−	−	+	+/++

[a] Levels of expression are indicated on the increasing scale −, ±, +, ++, +++, ++++.
[b] Skeletal muscle.

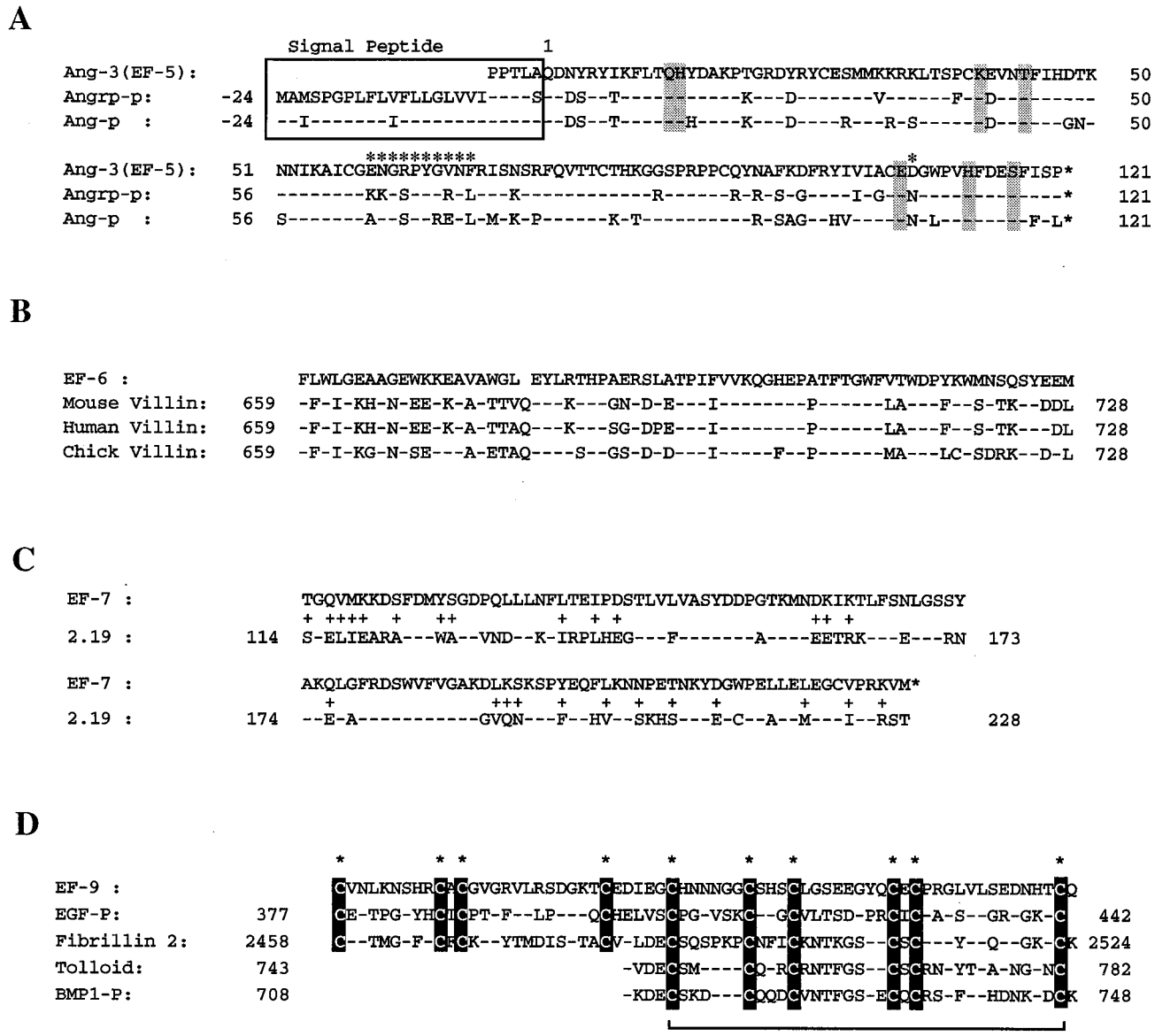

FIG. 3. Protein sequence homology between E2a-Pbx1 target genes and known genes. Dashed lines indicate identical amino acids. (A) Homology among Ang-3 (EF-5), mouse angiogenin-related protein precursor (Angrp-p), and mouse angiogenin precursor (Ang-p). Boxed residues −24 to −1 indicate signal peptides. Residues important for ribonucleolytic activity are shaded, and those in the assumed receptor-binding domain have asterisks overhead. (B) Homology among EF-6 and mouse, human, and chicken villin. (C) Homology between EF-7 and the human 2.19 gene product. + indicates conserved substitutions. (D) Homology among EF-9 and EGF-like cysteine repeats in some other proteins, including mouse EGF precursor (EGF-P), mouse fibrillin 2, the dorsal-ventral patterning protein tolloid of *D. melanogaster*, and the mouse bone morphogenetic protein 1 precursor (BMP1-P). Conserved cysteine residues are shown in highlighted black boxes with asterisks above. The bracket at the bottom indicates one EGF-like cysteine repeat. Asterisks at the end of open reading frames indicate a stop codon.

pressed in NIH 3T3 cells transformed by oncogenic Lck (lane 5), Ras, Neu, Src, Abl, or Sis (data not shown). PDE1A exhibited brain-, kidney-, and testis-specific expression (Fig. 2A; Table 2) and late expression in embryogenesis (Table 2). Members of the PDE family regulate tissue-specific growth and differentiation (3, 15, 63).

(v) EF-5. EF-5 contained 441 nucleotides encoding a third member of the angiogenin gene family (Fig. 3A) 74% identical to mouse angiogenin and 81% identical to mouse angiogenin-related protein. We designated EF-5 angiogenin-3. While all three angiogenin family members retain residues required for ribonucleolytic activity (which are also required for the angiogenic function of angiogenin [Fig. 3A]), each protein diverges at residues in the "receptor-binding" domain (1) (Fig. 3A), and angiogenin-related protein retains ribonucleolytic activity but is not angiogenic. EF-5/Ang-3 was not expressed in NIH 3T3 fibroblasts, exhibited dosage-dependent expression in response to E2a-Pbx1 (Fig. 1, compare lanes 2 and 3 with lanes 6 and 7), and was not induced in NIH 3T3 cells by the DNA-binding mutant of E2a-Pbx1 (lane 4) or by oncogenic Lck (lane 5) Ras, Neu, Src, or Sis (Fig. 2C) or in myeloblasts immortalized by E2a-Pbx1 (Fig. 1, lanes 8 and 9). Very slight up-regulation was observed in Abl-transformed cells. EF-5/Ang-3 was expressed in adult liver and at day 7 in development (Table 2). If angiogenic, EF5/Ang-3 could cause the vascularization of E2a-Pbx1-transformed NIH 3T3 cell tumors in nude mice (26).

(vi) EF-6. EF-6 contained 229 nucleotides encoding a protein 58% identical to residues 659 to 728 of chicken, mouse, and human villin, a calcium-regulated actin-bundling protein found in the microvilli of small intestine and kidney, suggesting that EF-6 is related to villin (Fig. 3B). Detection of EF-6 mRNA required analysis of higher levels of mRNA than those analyzed in Fig. 1, and it was expressed at very low levels in E2a-Pbx1-transformed cells and not in NIH 3T3 cells (data not shown). EF-6 was not expressed detectably in any adult-mouse tissues and at low levels in early embryogenesis (Table 2). If EF-6 encodes a cytoskeletal protein, it could contribute to the fusiform morphology of NIH 3T3 fibroblasts transformed by E2a-Pbx1 (26).

(vii) EF-7. EF-7 contained 465 nucleotides encoding 113 amino acids 55% identical and 78% homologous to the human 2.19 gene product (Fig. 3C), a protein of unknown function. EF-7 was not expressed in NIH 3T3 fibroblasts and was induced at comparable levels in both populations and clones of NIH 3T3 cells transformed by E2a-Pbx1 (Fig. 1, compare lanes 2 and 3 with lanes 6 and 7) but was not induced by the DNA-binding mutant of E2a-Pbx1 (lane 4), in myeloblasts transformed by E2a-Pbx1 (lanes 8 and 9), or in NIH 3T3 cells transformed by oncogenic Lck (lane 5) Ras, Neu, Src, Abl, or Sis (Fig. 2C). Normal EF-7 expression was testis specific (Fig. 2A; Table 2) and early embryonic (Table 2). Because RNA from 17-day embryos does not contain detectable RNA transcripts, EF-7 either must be expressed later in testicular development or may not comprise a large enough fraction of day 17 total mRNA to be detected by this analysis.

(viii) EF-8. EF-8 contained 313 nucleotides encoding the C-terminal 90 amino acids of a protein related to ion channels, including the γ subunit of the Na^+,K^+-ATPase, the Mat-8 chloride channel, and canine phospholemman (PLM), the major plasma membrane substrate for cAMP-dependent protein kinase and protein kinase C (Fig. 4C). Homology was limited to 38 amino acids encompassing the transmembrane channel, and sequence homology within the other members of this family is also confined to this region. EF-8 was most homologous to MAT-8 (21 of 38 identities), a gene up-regulated in epithelial cells by oncogenic Ras and Neu (46) and expressed in human breast tumors (47). While the γ subunit of the Na^+,K^+-ATPase is involved in transfer of sodium and potassium, both Mat-8 and PLM induce chloride conductance in *Xenopus* oocytes (44, 47), and recombinant PLM forms chloride channels in reconstituted lipid bilayers (43). EF-8 transcript (0.9 kb) was expressed at low levels in NIH 3T3 fibroblasts (Fig. 1, lane 1; Fig. 2C) and at high levels in each of the nine other transformed cell lines (Fig. 1, lanes 2 to 10). EF-8 was induced strongly by E2a-Pbx1 (lanes 2, 3, 6, and 7) and moderately by the DNA-binding mutant of E2a-Pbx1 (lane 4) and by oncogenic versions of Lck (lane 5), Ras, Neu, Src, and Abl (Fig. 2C). EF-8 was normally expressed in spleen, lung, skeletal muscle, and testis (Fig. 2A) and exhibited biphasic expression during development (Fig. 2B). EF-8 was designated RIC (related to ion channels), and a full-length cDNA was derived (Fig. 4A). RIC contained a single open reading frame encoding 178 amino acids containing an N-terminal hydrophobic signal peptide (Fig. 4A) and a proline-rich region (residues 88 to 134) upstream from the ion channel homology domain (residues 134 to 167), which contained a sequence of 19 hydrophobic residues flanked by basic residues, representing a putative membrane-spanning region. The proline-rich region contained a pattern of repeated proline residues similar to those found in unrelated proteins. An in-frame stop codon resided 11 codons preceding the proposed initiating methionine codon. Activation of ion channels such as K^+ channels and the Na^+/H^+ antiporter correlate with cell proliferation (48, 54, 55, 61); thus, RIC may also play a role in regulating the cell cycle and proliferation.

(ix) EF-9. EF-9 contained 464 nucleotides encoding 1.75 epidermal growth factor-like repeats, which are found in secreted proteins and contain a pattern of cysteines (51). The EF-9 repeats were most homologous to those of prepro-epidermal growth factor, fibrillin 2, the *Drosophila* dorsal-ventral patterning protein tolloid, and bone morphogenetic protein (Fig. 3D). EF-9 was not expressed in NIH 3T3 cells (Fig. 1, lane 1), was induced in a concentration-dependent manner by E2a-Pbx1 (compare lanes 2 and 3 with lanes 6 and 7), and was not induced by DNA-binding mutants of E2a-Pbx1 (lane 4), in E2a-Pbx1 immortalized myeloblasts (lanes 8 and 9), or in NIH 3T3 cells by oncogenic Lck (lane 5), Ras, Neu, Src, Abl, or Sis (data not shown). Normal EF-9 expression was liver specific (Fig. 2A; Table 2), and late embryonic (Fig. 2B; Table 2).

(x) EF-10. EF-10 contained 442 nucleotides unrelated to any GenBank sequence. EF-10 was expressed at low levels in NIH 3T3 cells (Fig. 1, lane 1) and was induced three- to sixfold by E2a-Pbx1 (lanes 2 and 3), by the DNA-binding mutant of E2a-Pbx1 (lane 4), and by oncogenic Lck (lane 5 [note underloading of RNA]), Ras, Neu, Src, Abl, or Sis (data not shown). EF-10 was strongly up-regulated in E2a-Pbx1-overexpressing clones (lanes 6 and 7) and was expressed in E2a-Pbx1-immortalized myeloblasts (lanes 8 and 9) but not in 32D myeloblasts. EF-10 was most abundant in lung and skeletal muscle (Table 2) and was expressed throughout embryogenesis (Fig. 2B; Table 2).

(xi) EF-11. EF-11 contained 370 nucleotides unrelated to any sequence in GenBank. EF-11 was not expressed in NIH 3T3 cells (Fig. 1, lane 1) and was induced by low levels by E2a-Pbx1 (lanes 2 and 3). It was not induced in NIH 3T3 cells by the DNA-binding mutant of E2a-Pbx1 (lane 4), in myeloblasts transformed by E2a-Pbx1 (lanes 8 and 9), or in NIH 3T3 cells transformed by oncogenic Lck (lane 5), Ras, Neu, Src, Abl, or Sis (data not shown). EF-11 expression was not detected in any specific tissue or at any time in development (Table 2).

(xii) EF-12. EF-12 contained 278 nucleotides unrelated to any sequence in GenBank and was detectable only in NIH 3T3 cells overexpressing E2a-Pbx1 (lanes 6 and 7). EF-12 was normally expressed in heart, brain, skeletal muscle, and testis (Fig. 2A; Table 2) and late in embryogenesis (Fig. 2B; Table 2).

Expression of c-*jun*. Expression of the immediate-early gene, c-*jun*, was examined to determine whether cells transformed by E2a-Pbx1 have activated the same mitogenic signaling pathways activated by tyrosine kinases and Ras oncoproteins. While NIH 3T3 cells transformed by oncogenic Lck, which activates both the Erk and Jnk signaling cascades, exhibited strong expression of c-*jun*, cells transformed by E2a-Pbx1 did not, suggesting that E2a-Pbx1 does not constitutively activate these pathways by direct or indirect mechanisms.

DISCUSSION

E2a-Pbx1 induces aberrant gene expression, resulting in the appearance of tumor markers. One approach to cancer therapy is elimination of tumor cells based on their inappropriate expression of genes normally expressed either in other cell types or during the early to middle stages of embryogenesis (40). Using RDA PCR, we identified 12 E2a-Pbx1-induced RNAs that reveal some basic principles about the transforming mechanisms of E2a-Pbx1. First, all induced RNAs represented cellular mRNAs rather than intergenic or other noncoding sequences. This conclusion was based on the facts that (i) the

A

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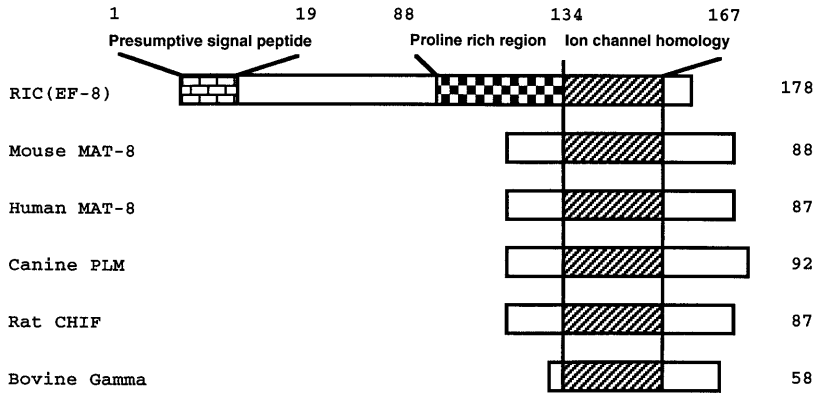
GGCACGAGGGTACTCTGACACTTCAGTCTGGAGATCTCCGGCTCAGATATGTCACTGTCC     60
                                                  M  S  L  S       4
AGTCGCCTGTGTCTCCTCACTATTGTCGCCCTGATTCTGCCCAGCAGAGGGCAGACACCA    120
S  R  L  C  L  L  T  I  V  A  L  I  L  P  S  R  G  Q  T  P       24
AAAAAGCCCACATCCATTTTTACAGCGGACCAGACTTCTGCGACTACTCGTGACAATGTC    180
K  K  P  T  S  I  F  T  A  D  Q  T  S  A  T  T  R  D  N  V      44
CCAGATCCAGATCAAACCAGCCCAGGAGTCCAGACCACCCCTCTCATCTGGACCAGAGAA    240
P  D  P  D  Q  T  S  P  G  V  Q  T  T  P  L  I  W  T  R  E      64
GAAGCCACAGGAAGCCAGACAGCAGCCCAAACCGAGACCCAGCAACTGACAAAAATGGCC    300
E  A  T  G  S  Q  T  A  A  Q  T  E  T  Q  Q  L  T  K  M  A      84
ACCTCGAATCCAGTGTCAGATCCAGGGCCACATACAAGCAGCAAGAAAGGTACCCCTGCA    360
T  S  N  P  V  S  D  P  G  P  H  T  S  S  K  K  G  T  P  A     104
GTCTCCAGGATCGAGCCTCTCAGCCCATCCAAAAACTTCATGCCTCCATCCTACATTGAA    420
V  S  R  I  E  P  L  S  P  S  K  N  F  M  P  P  S  Y  I  E     124
CATCCACTGGATTCGAATGAGAACAACCCCTTCTACTACGATGATACTACCCTCCGGAAA    480
H  P  L  D  S  N  E  N  N  P  F  Y  Y  D  D  T  T  L  R  K     144
CGGGGACTGCTGGTGGCTGCGGTGCTGTTCATCACGGGAATTATCATTCTCACTAGTGGG    540
R  G  L  L  V  A  A  V  L  F  I  T  G  I  I  I  L  T  S  G     164
AAGTGTAGGCAGTTGTCTCAATTTTGCCTGAATCGCCACAGGTGAGTGCGGGCCAGCACC    600
K  C  R  Q  L  S  Q  F  C  L  N  R  H  R  *
CTGATGGGCACCCCAGCTGGAGCCTCCAAACTACACCAACTCACCACCCCCTGCCTCCTC    660
CCTCTACCCCAAGAGCCTACAGAGTGATCAACATGAAAGAATCCTGAAAGGAAGAGGCCA    720
CTGGAGGGAGTCAGGCTTAAGGCTAATGGTCTTCCCACCCTGGGGAGAGAGGTCTCCCTA    780
GGCACTGCCTCGTGCCG
```

B



C

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RIC(EF-8) :        130   NENNPFYYDDTTLRKRGLLVAAVLFITGIIILTSGKCR  167
Mouse MAT-8:        25   -K-D-----WYS--VG--IC-GI-CAL---V-M----K   62
Human MAT-8 :       25   DK-S-----WHS-QVG--IC-G--CAM----VM-A--K   62
CANINE PLM:         24   E-HD--T--YQS--IG--II-GI---L--L-VL-RR--   61
Rat CHIF:           25   DKGS-----WES-QLG-MIFGGL-C-A--AMAL----K   62
Bovine Gamma:        6   GTED-----YE-V-NG--IF--LA--V-LV-IL-KRF-   43
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D

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RIC(EF-8):     88   PVSLPSPHTSSKKGTPAVSRIEPLSPSKNFMPPSYIEHPLDSNENNP  134
Maize:        196   PTYTPSPKPPTP-P-PPTYTPSPKPPTPKPTPPT-TPSPKPPTHPTP  242
              263   PTYTPSPKPPTP-P-PPTYTPSPKPPTPKPTPPT-TPTPKPPATKPP  309
Rat mucin:     76   TSQTPTPTPTTI-E-PTSTSTVPTTG-TSSKPPTGSSTPIT-PSTPS  122
Myosin:       284   PK-SPSPVLGTESDATVKKKPAPKTPP-AA-PPQI-QFPE-        324
bZIP:          54      PSPVA--PQ-HPYMWGAQPMIPPYGTPPPP-VMYP          88
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FIG. 4. Full-length cDNA sequence of RIC and its homology to related proteins. (A) RIC/EF-8 DNA and amino acid sequences. An in-frame TGA stop codon (underlined) precedes the presumed initiation site. Hydrophobic residues in the open box followed by a positively charged residue (arginine) indicate a signal peptide. Residues in the shaded box encompass the putative transmembrane sequence, which is flanked by positively charged residues. Nucleotides 374 to 686 (underlined) represent the EF-8 RDA PCR fragment. (B) Schematic diagrams of RIC and the related ion channels Mat-8, canine PLM, rat CHIF, and bovine gamma (Na^+, K^+-ATPase). The hatched region encompasses the transmembrane channel that is homologous among all ion channels shown, the proline-rich region is checkered, and the bricked region indicates the presumptive signal peptide. (C) Homology among RIC and ion channels within the transmembrane domain. (D) Homology among the proline-rich region of RIC and other proline-rich domains, including those in the maize hydroxyproline-rich glycoprotein precursor, rat mucin, human myosin light-chain kinase, and the *Oryza sativa* DNA-binding factor, which belongs to the bZIP class of DNA-binding proteins. Conserved proline residues are shown in highlighted black boxes. Dashed lines indicate identical amino acids.

proteins encoded by these RNAs were either identical or related to known factors and (ii) mRNA cognates were expressed in normal adult tissues or during development. This indicates that E2a-Pbx1 specifically induces transcription of cellular genes and does not initiate transcription of stable RNAs from nongene sequences. Second, E2a-Pbx1 induced transcription at or near the normal initiation site, evidenced by the fact that in all cases the sizes of the transcripts induced by E2a-Pbx1 in transformed fibroblasts were the same as those found in normal cells expressing the gene. Third, many genes activated by E2a-Pbx1 were not expressed in normal fibroblasts but were expressed in other adult cell types (EF-1, EF-4, EF-5, EF-7, EF-9, and EF-12), and one (EF-6) exhibited expression during early embryogenesis and was not detected in the adult. Thus, E2a-Pbx1 induces tumor-specific markers, one of which (EF-6) could be categorized as an oncofetal antigen. This result suggests that E2a-Pbx1 may also induce the expression of non-B-cell antigens in t(1;19)-containing pre-B ALL cells. While target genes of the Ras, Myc, Ets, Myb-Ets, and Ews-Fli1 oncoproteins have also been identified, none of these oncogenes activates transcription of tissue-specific genes that are normally restricted to other cell lineages (5–7, 18, 31, 50, 58).

It is not clear whether EF-1 to EF-12 are direct or indirect targets of E2a-Pbx1; likewise, their relevance in transformation is also unclear. Nonetheless, these genes provide the basis for beginning to dissect (i) the ability of a single oncoprotein to induce cell type-inappropriate gene transcription, (ii) the phenotypic changes that lead to E2a-Pbx1-induced fibroblast transformation, and (iii) the mechanism underlying DNA-binding-dependent and -independent transcriptional activation by E2a-Pbx1.

E2a-Pbx1-inducible genes in NIH 3T3 fibroblasts are generally not activated by E2a-Pbx1 in myeloid cells, indicating that E2a-Pbx1 cooperates with cell-type-specific factors. Most E2a-Pbx1 target genes were not up-regulated in mouse myeloblasts immortalized by E2a-Pbx1 (Fig. 1, lanes 8 and 9). Only EF-4, EF-8, and EF-10 were detectably up-regulated by E2a-Pbx1 in myeloblasts. These properties suggest that in most cases, tissue-specific factors influence the target gene specificity of E2a-Pbx1, distinguishing it from Ras and tyrosine kinase oncoproteins, which utilize predominantly ubiquitous signaling pathways to up-regulated transcription of many common targets. Transcripts induced by E2a-Pbx1 in NIH 3T3 cells were also examined for expression in t(1;19)-positive 697 and SupB27 human pre-B leukemia cell lines and in t(1;19)-negative Nalm-6 and Reh human pre-B leukemia cell lines. No cognate transcripts were identified with the exception of fumarase, which was not up-regulated in the t(1;19) leukemia cells. Failure to detect cognate transcripts might be due to the failure of the mouse probes to cross-react with human sequences or to their failure to be induced by E2a-Pbx1 in pre-B cells.

One explanation for cell-type-specific gene activation by E2a-Pbx1 is that different cells contain different heterodimer partners for E2a-Pbx1, which generate different DNA-binding specificities, and activate transcription of different complements of cellular genes, regulated by such promoter elements. Such a hypothesis must await transcriptional analysis of direct targets of E2a-Pbx1. Cell-type-specific gene activation by E2a-Pbx1 raises the question whether E2a-Pbx1 induces its transforming properties through its impact on common or different gene targets. While a common mechanism is most appealing, it may be that differentiation requires the normal activity of factors which heterodimerize with Pbx proteins and that heterodimers of these proteins and E2a-Pbx1 persistently activate the expression of genes that must be transcriptionally repressed for terminal differentiation, resulting in differentiation arrest by a mechanism that targets different genes in different cell types.

Most E2a-Pbx1 target genes are not activated by Ras and tyrosine kinase oncoproteins. Oncogenic forms of Ras, Src, Lck, Abl, Sis, and neu/Her 2 activate mitogen-activated protein kinases that induce the transcription of immediate-early genes and entrance into the cell cycle. E2a-Pbx1 shares some transforming properties with these oncoproteins but differs in others. While E2a-Pbx1, Ras, and tyrosine kinase oncoproteins abrogate contact inhibition, inducing foci in NIH 3T3, only E2a-Pbx1 blocks myeloid differentiation. In addition, myeloblasts immortalized by E2a-Pbx1 remain strictly factor dependent, while tyrosine protein kinases reduce or eliminate growth factor dependence. Thus, most E2a-Pbx1 target genes would be predicted to diverge substantially from those of tyrosine kinase and Ras oncoproteins, while some, which induce cell proliferation, may be conserved. Consistent with this prediction, only two of the E2a-Pbx1 target genes were activated by Ras or tyrosine kinase oncoproteins, and c-*jun*, which is induced by Lck, was not activated by E2a-Pbx1. Likewise, neither c-*fos* nor c-*myc* was activated by E2a-Pbx1 (data not shown). Importantly, however, EF-8/RIC was strongly up-regulated by Ras and oncogenic tyrosine kinases. Determining the mechanism of transcriptional activation of RIC/EF-8 by E2a-Pbx1 will be important to determine how both E2a-Pbx1 and mitogenic oncoproteins activate transcription of the same genes.

Different patterns of gene up-regulation suggest different mechanisms of transcriptional activation by E2a-Pbx1. The observation that DNA binding is important for the ability of E2a-Pbx1 to block myeloid differentiation yet dispensable for its ability to induce foci in fibroblasts suggested that only a subset of target genes are activated by DNA-binding mutants. Indeed, only EF-1 and EF-8 were substantially up-regulated by E2a-Pbx1N682S. The fact that transcription of EF-1 and RIC/EF-8 is unaffected by this mutation, while transcription of EF-2, EF-4, angiogenin-3/EF-5, EF-7, EF-9, and EF-11 is either strongly reduced or extinguished argues that E2a-Pbx1 utilizes at least two different mechanisms to activate transcription. Each mechanism could be explained by the presence of different heterodimer partners, one that allows DNA binding of the mutant complex to DNA, and one that does not. Alternatively, some promoters might bind factors that interact with both Pbx1 and E2a proteins, and E2a-Pbx1 protein might be stably bound by virtue of interaction with both factors. Analysis of the mechanism by which DNA-binding mutants of E2a-Pbx1 activate the transcription of RIC/EF-8 may explain such a mechanism if, indeed, RIC/EF-8 is a direct target of E2a-Pbx1. In addition, activation of most target genes was dependent on E2a-Pbx1 abundance while that of EF-7 was dosage independent, arguing for fundamentally different mechanisms of transcriptional activation. In this example, activation of EF-7 might occur through a high-affinity E2a-Pbx1 DNA-binding motif that is already saturated by smaller amounts of E2a-Pbx1, while activation of other targets could occur through lower-affinity sites sensitive to E2a-Pbx1 concentration.

Most genes activated by E2a-Pbx1 are tissue specific or developmentally regulated. Unlike immediate-early genes activated in all cells upon mitogenic stimulation, most E2a-Pbx1 targets were expressed in other specific cell types or transitionally in development and not in fibroblasts. The explanation for this observation may also lie in the targeting mechanism of E2a-Pbx1. One possibility is that some heterodimers of Pbx proteins and fibroblast-specific partners function as transcriptional repressors and that replacement of Pbx proteins with E2a-Pbx1 results in their transcriptional activation. While this mechanism could account for activation of a testis-specific

gene by E2a-Pbx1 in fibroblasts, it would not explain why this gene is not activated in E2a-Pbx1-transformed myeloblasts. Differential cell-type-specific activation of target genes suggests that cell-type-specific factors cooperate with E2a-Pbx1 to activate transcription.

What is the likelihood that RDA PCR will identify critical mediators of transformation by E2a-Pbx1? We cannot be certain of the fraction of E2a-Pbx1–up-regulated genes that are represented by these 12 target genes. The isolation of only two RDA PCR products representing identical mRNAs suggests either the existence of other unidentified E2a-Pbx1–up-regulated transcripts or strong preferential amplification of specific cDNA fragments. In hopes of identifying more genes up-regulated by E2a-Pbx1 in NIH 3T3 cells, we performed a subsequent analysis supplementing the driver pool of DNAs with the PCR fragments representing each the 12 genes; however, no additional genes were detected. Of the two RDA PCR products homologous to EF-9, one was contained within the other, suggesting that specific driver cDNA fragments may amplify preferentially. A second argument for preferential amplification is suggested by the apparent selective amplification of an RDA PCR fragment representing EF-11, which should comprise a very low abundance target based on the level of expression of its mRNA, among a host of other potential *Dpn*II cDNA fragments derived from the very abundant 3.0- and 7.5-kbp EF-2 mRNAs. The argument that a limited number of genes are induced by E2a-Pbx1 in fibroblasts is supported by the fact that the one gene product identified by differential-display PCR, a method that identifies differences in mRNA populations based on PCR amplification of their 3′ ends, was also identified by RDA PCR. If more than 120 genes were activated by E2a-Pbx1, there would be only a 10% chance that the one differential-display PCR clone we obtained would match 1 of the 12 RDA PCR genes, suggesting that it is likely that fewer than 100 genes are up-regulated by E2a-Pbx1 in NIH 3T3 cells. Determination of this number is obviously essential to knowing whether this approach holds promise for determining the mechanisms of transformation by E2a-Pbx1. If too many genes are activated, one would have to be extremely lucky to identify the physiologically relevant targets. Preferential amplification of sequences encoding irrelevant target genes could further interfere with identification of genes that mediate transformation. Based on our analysis, however, we cannot generate a statistically significant estimation of the number of E2a-Pbx1-induced genes.

Perspective. In conclusion, identification of RNAs induced by E2a-Pbx1 in NIH 3T3 cells has demonstrated that E2a-Pbx1 induces the transcription of cellular genes, that most are normally not expressed in fibroblasts, and that some are expressed in specific adult tissues and at specific stages of fetal development. If transcription of genes involved in fetal development also occurs in t(1;19)-containing pre-B cells, those encoding cell surface antigens would represent excellent targets for immunologic therapy. Subsets of E2a-Pbx1 target genes in NIH 3T3 cells exhibited transcriptional activation that was dosage dependent, dosage independent, DNA-binding dependent, or DNA-binding independent, implicating multiple biochemical contexts in which E2a-Pbx1 activates transcription. Promoter analysis of E2a-Pbx1 target genes will reveal whether they are direct or indirect targets, and, in the case of direct targets, may lead to identification of in vivo partners of E2a-Pbx1 and an understanding of both DNA-binding and concentration-dependent and -independent mechanisms of transcriptional activation by E2a-Pbx1.

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