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Pbx1 is converted into a transcriptional activator upon acquiring the N-terminal region of E2A in pre-B-cell acute lymphoblastoid leukemia

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ABSTRACT **Twenty-five percent of human pediatric pre-B-cell acute lymphoblastic leukemias (ALLs) are characterized by the t(1;19)(q23;p13.3) chromosomal translocation. This translocation joins the 5′ region of the E2A gene to the 3′ region of the Pbx1 gene. The protein encoded by this chimeric gene contains the N-terminal transcriptional activation domain of E2A fused to the C-terminal region of Pbx1, which contains a putative homeodomain. Here we show that the Pbx1 homeodomain preferentially binds the sequence ATCAATCAA. We further show that promoters containing Pbx1-binding sites are activated by the chimeric E2A-Pbx1 protein but not by Pbx1. These results indicate that the t(1;19) translocation converts a nonactivating DNA-binding protein into a potent transcriptional activator, suggesting an unusual mechanism for oncogenic transformation.**

Chromosomal translocations are often associated with human lymphomas and leukemias. In pediatric acute lymphoblastoid leukemia (ALL) the translocation site is frequently located within the E2A gene on chromosome 19, which codes for the transcription factors E12 (Pan-1) and E47 (Pan-2, E2-5) (1–3). The E2A gene products bind to protein-binding sites, designated E boxes, present in the immunoglobulin heavy chain enhancer and are involved in various developmental pathways, including muscle, pancreatic, and B-cell differentiation (4, 5). They belong to a family of DNA-binding proteins that contain a helix–loop–helix (HLH) DNA-binding and dimerization domain. In addition to their role in development, the E2A products are mutated in B-cell leukemias. Two translocations involving the E2A gene, including t(17;19)(q21;p13.3) and t(1;19)(q23;p13.3), have been described. In both translocations the C-terminal portion of E2A containing the DNA-binding domain is replaced by a heterologous DNA-binding domain (Fig. 1). Lyl-1 and Tal/Scl, discovered by translocation breakpoint analysis in T-cell leukemias, are other examples of potentially oncogenic HLH proteins (7–9).

In pro-B-cell ALL involving the t(17;19) translocation, the basic HLH domain of E2A is replaced by a basic leucine zipper DNA-binding domain derived from hepatic leukemia factor (10, 11) (Fig. 1). In the t(1;19) translocation, present in 25% of pediatric pre-B-cell ALL (12, 13), the C terminus of the chimeric gene contains a putative homeodomain derived from Pbx1 (14, 15). Pbx1 belongs to a family of genes, designated Pbx1, Pbx2, and Pbx3, that are closely related to each other (16). Whereas Pbx2 and Pbx3 are expressed ubiquitously, Pbx1 is expressed in most tissues, except in T and B cells (15, 17). The normal role of Pbx1 is not clear. Pbx1 is extremely well conserved between mammals and *Drosophila*. The Pbx1 homolog in *Drosophila*, designated *Dpbx*, is 95% identical to the Pbx1 within the homeodomain and 88% identical within a 201-amino acid region adjacent to the homeodomain (18). In *Drosophila*, *Dpbx* is expressed throughout the life cycle. The most notable feature of *Dpbx* expression is that high levels of *Dpbx* are present in the anterior portion of the ventral nerve cord.

The presence of alternate DNA-binding domains in translocations involving E2A makes it likely that these chimeric oncogenes exert their influence at the level of transcription. Although the DNA-binding properties of hepatic leukemia factor are known (10), they have not been resolved for Pbx1. Here we have investigated the DNA-binding properties of the Pbx1 homeodomain. Using PCR-mediated binding-site selection, we show that the Pbx1 homeodomain preferentially binds to DNA at sites containing the nucleotide sequence ATCAATCAA. We further show that a promoter containing five copies of the Pbx recognition sequence is strongly activated by E2A-Pbx1, but not by Pbx1. We conclude that the protooncogene Pbx1 is not a transcriptional activator but is converted into a strong activator by acquiring the N-terminal transactivation domain of the E2A protein (1, 6).

MATERIALS AND METHODS

Plasmids. The reporter constructs are derived from pJ21 (19), which contains a minimal c-*fos* promoter (−71/+109) fused to the bacterial chloramphenicol acetyltransferase (CAT) gene. pJP5 contains an oligonucleotide containing five copies of the Pbx1 consensus binding site (Pbx1: CCATCAATCAATCTAGATCAATCAAGATATCAATCAAGAATTCATCAATCAAGGATCCATCAATCAAGCTTC) inserted in the *Hin*dIII site immediately upstream of the *fos* promoter. To construct the Pbx1 effector plasmid, the *Hin*dIII/*Eco*RI fragment from pSP65-Pbx1a, encompassing the complete coding sequence of Pbx1, was cloned downstream of the transcription start site of the simian virus 40 promoter in pJ3Ω. The E2A-Pbx1 effector plasmid contains the *Eco*RI fragment from pBSK$^+$-E2A-Pbx1-I with the complete coding sequence of E2A-Pbx1-I (14) cloned into pJ3Ω.

For the production of recombinant glutathione *S*-transferase (GST)-Pbx1 fusion protein the *Pvu* II fragment from Pbx1 containing the homeodomain [amino acids 105–309 (14)] was cloned in frame into the *Sma* I site of pGEX-2TK and the fusion protein was expressed and purified as described (20).

Determination of the Pbx1 Consensus Binding Site. The Pbx1 consensus binding site was determined by PCR-mediated binding-site selection (21). Briefly, 100 ng of GST-Pbx1 fusion protein containing the homeodomain of Pbx1 was incubated with a radioactively labeled 62-bp oligonucleotide containing a random stretch of 16 nucleotides [CTCGGTACCTCGAGTGAAGCTTGA(N)$_{16}$GGGAATTCGGATCCGCGGTAAC] in a buffer containing 200 ng of poly(dI-dC), 10 mM Tris (pH 8), 5 mM $MgCl_2$, 5% glycerol, 0.1% Nonidet

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Abbreviations: ALL, acute lymphoblastoid leukemia; HLH, helix–loop–helix; EMSA, electrophoretic mobility-shift assay; CAT, chloramphenicol acetyltransferase; GST, glutathione *S*-transferase.

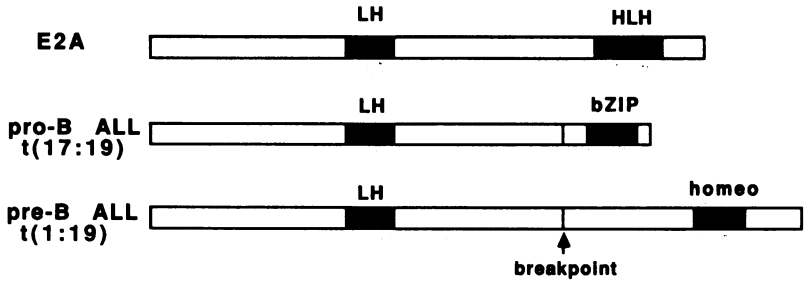

FIG. 1. Chromosomal translocations involving the E2A gene. The structures of the E2A gene product and the t(17;19) and t(1;19) specific proteins are depicted schematically. The shaded boxes indicate the location of the various DNA-binding domains: HLH, HLH domain; bZIP, basic leucine zipper; homeo, homeodomain. LH indicates the position of the recently identified loop–helix transactivation domain (6). The position of the breakpoint in the chimeric translocation proteins is indicated.

P-40, and 0.1 mM EDTA. The probe and complexes were separated by an electrophoretic mobility-shift assay (EMSA). The gel was exposed for 4 hr, and complexes containing DNA bound by GST-Pbx1 were excised and eluted. The recovered DNA was amplified by PCR. This amplified DNA was subsequently labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase and subjected to EMSA in the presence of 10 ng of GST-Pbx1. Eluted DNA from the second selection was amplified using PCR, digested with *Bam*HI and *Xho* I, and subcloned in pBSK$^+$ (Stratagene). Eighteen plasmid clones obtained from the second round of selection were sequenced. Subsequently, the portions of 13 of these plasmids bearing the $(N)_{16}$ sequences and the flanking *Hin*dIII and *Eco*RI sites were radioactively labeled and used in an EMSA (22). Using a fixed amount (50 ng) of GST-Pbx1 fusion protein and increasing amounts (0.1–2 μg) of the nonspecific competitor poly(dI-dC) we determined the relative affinity of these 13 sequences for GST-Pbx1.

DNase I Footprinting. A *Sac* I/*Asp*718 fragment from the plasmid 1A3XB (Fig. 2), which contains the Pbx1 consensus binding sequence, was radioactively labeled at the *Asp*718 site at either the upper or lower strand and used as a probe. Two-tenths nanogram of radioactively labeled DNA was incubated for 30 min on ice in the presence of either bovine serum albumin or GST-Pbx1 in the buffer as used for EMSA (see above). The mixture was subsequently incubated with 0.4 unit of DNase I (RNase-free, Boehringer Mannheim) for 90 sec at room temperature. After purification the DNA fragments were resolved on a denaturing polyacrylamide gel and visualized by autoradiography.

Transfection and Immunoprecipitation. Five micrograms of the reporter constructs was introduced into LK cells (23) using DEAE-dextran (24), together with either 5 μg of E2A-Pbx, 10 μg of Pbx1, or 10 μg of the pJ3Ω vector alone. CAT assays were performed as described (25).

To check whether the Pbx1 and E2A-Pbx1 effector plasmids produce the correct product, 10 μg of these plasmids was transfected to COS cells (26). After 48 hr, the cells were incubated with methionine-free RPMI 1640 medium for 1 hr and incubated for one additional hour in the presence of 250 μCi of [^{35}S]methionine (1 Ci = 37 GBq). ^{35}S-labeled proteins were subsequently extracted and incubated with rabbit anti-Pbx1 polyclonal antibody for 1 hr on ice. The formed antibody–protein complexes were precipitated by protein A-Sepharose and resolved on a 7% SDS/polyacrylamide gel.

RESULTS

In pre-B-cell ALL involving a t(1q23;19p13) translocation, the HLH DNA-binding domain of E2A is replaced with a region of Pbx1 containing a putative homeodomain (15). The presence of a homeobox-like domain suggests that Pbx1 is a DNA-binding protein. To examine whether Pbx1 indeed is a sequence-specific DNA-binding protein and to determine the optimal DNA binding site of Pbx1, we used PCR-mediated binding-site selection (21). Radioactively labeled oligonucleotides containing a random stretch of 16 nucleotides in the center were incubated with GST-Pbx1 fusion protein and subjected to electrophoresis through a native polyacrylamide gel. The complexes containing DNA bound by the GST-Pbx1

		BINDING
1A3XB:	AAGCTTGATTTAATAATCAATCAAGGGAATTC	+++
2A4XB:	AAGCTTGATAGAATACTAATCAATGGGAATTC	+++
4A2XB:	GAATTCCCTTTGAATACTAATCAATCAAGCTT	+++
1-1BE:	GAATTCCCATTTGATATATTACAATCAAGCTT	++
3A1XB:	AAGCTTGATATATGATTAATCATTGGGAATTC	+
4-2BE:	GAATTCCCCTTCTTATCAATTATATCAAGCTT	+
1A4XB:	GAATTCCCTTTTGATAAATATGTATCAAGCTT	+
3-2BE:	GAATTCCCATTAGAATCTAACATATCAAGCTT	+
4A5XB:	AAGCTTGATAGTATGCAATAATAAGGGAATTC	+/-
4A3XB:	AAGCTTGATCAATAAATCATTTAAGGGAATTC	+/-
2A2XB:	GAATTCCCTATCATCATGAAATATTCAAGCTT	+/-
3A2XB:	AAGCTTGATGTATCATAATTATTTGGGAATTC	ND
1A2XB:	AAGCTTGATACATCATATGAAATAGGGAATTC	ND
1A1XB:	GAATTCCCATAAGATATATCCTAATCAAGCTT	ND
4A1XB:	AAGCTTGATATACTACCAATAAAAGGGAATTC	ND
2A1XB:	AAGCTTGAAAGATAAGAATCATTAGGGAATTC	ND

CONSENSUS: $\mathbf{A_{12}T_8C_9A_{13}A_{14}T_{14}C_{10}A_{14}A_{10}}$

FIG. 2. Determination of the Pbx1 consensus binding site. Sixteen sequences obtained from a plasmid pool of oligonucleotides enriched for Pbx1-binding sites were aligned. The consensus is shown at the bottom. Shown in bold type are the nucleotides that appear most frequently. Indicated in subscript is the number of times each specific nucleotide is found at that position in the alignment. Right-hand column, relative affinity of GST-Pbx1 for the selected binding site as determined by EMSA. +++, High affinity; +/−, low affinity; ND, not determined. Two of the original 18 clones showed no homology and had very low affinities for GST-Pbx1. They are not included in this figure.

fusion protein were excised from the gel, eluted, and amplified by PCR. This procedure was repeated using the amplified DNA from the first selection as a probe. The amplified DNA derived from the second selection was subcloned and 18 clones were isolated and sequenced (Fig. 2). To assess the binding properties of these clones, we randomly picked 13 clones and determined their relative affinity for GST-Pbx1 by EMSA. One group of sequences bound with high affinity to Pbx1, whereas another group showed lower affinity for the Pbx1 protein (Fig. 2). The DNAs from high- and low-affinity binding sites were aligned in order of decreasing affinity. This alignment indicated ATCAATCAA as a consensus binding site for Pbx1 (Fig. 2).

To determine whether Pbx1 recognizes the region containing the ATCAATCAA consensus binding site, we mapped the contacts of Pbx1 with DNA derived from clone 1A3XB, using DNase I protection analysis (Fig. 3). Indeed, as expected, the Pbx1 homeodomain protects the region containing the consensus sequence (Fig. 3). Similar results were obtained using the binding site from clone 2A4XB (not shown). The similar affinities of Pbx1 for the 1A3XB and the 2A4XB binding sites indicate that not all base positions in the consensus sequence are invariant (Fig. 2): the T and C

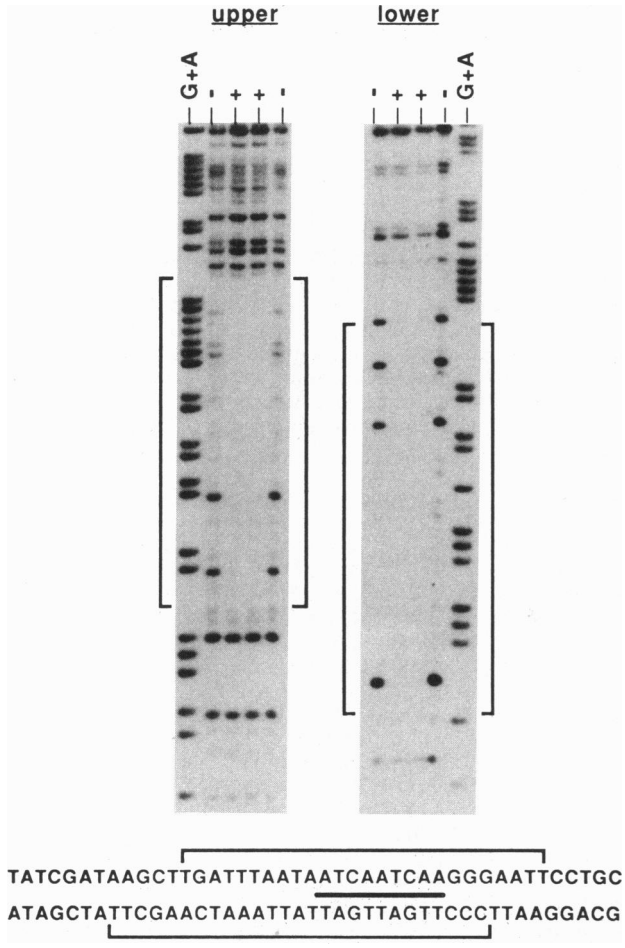

FIG. 3. DNase I protection analysis of Pbx1. DNase I protection assays were performed on the 1A3XB probe labeled at the upper or lower strand as indicated. G+A, Maxam and Gilbert G+A marker (27). Lanes: −, DNase I reactions carried out in the presence of 3 μg of bovine serum albumin; +, DNase I protection reactions carried out in the presence of 3 μg of GST-Pbx1. The brackets on either side indicate the region protected against DNase I digestion in the presence of GST-Pbx1. The nucleotide sequence of the protected region is indicated at the bottom. The Pbx1 consensus binding sequence is underlined.

residues at positions 2 and 3 can be switched without apparent influence on binding affinity. For the studies below, we have used the consensus sequence ATCAATCAA since this sequence contains the nucleotides most frequently found at the indicated positions.

To test whether the consensus site ATCAATCAA functions as a binding site for Pbx1 and E2A-Pbx1 *in vivo*, five copies of the ATCAATCAA sequence were introduced upstream of a minimal c-*fos* promoter (Fig. 4*A*). We cotransfected the reporter with either Pbx1 or E2A-Pbx1 into LK cells, a mouse B-cell hybridoma (23), which does not express endogenous Pbx1. Although cotransfection with vector alone has no effect, transfection with E2A-Pbx1 results in a dramatic increase in CAT activity (Fig. 4*A*). When the experiment is performed with the same reporter plasmid without the Pbx1-binding sites, E2A-Pbx1 has no effect on CAT expression (Fig. 4*A*). Thus, the chimeric E2A-Pbx1 protein is a potent transcriptional activator, and the activation is dependent on the presence of the Pbx1-binding sites.

Interestingly, in contrast to E2A-Pbx1, overexpression of Pbx1 had no effect on CAT expression, indicating that Pbx1 is not a transcriptional activator (Fig. 4). To determine whether Pbx1 and E2A-Pbx1 are produced at similar levels upon transfection with the effector plasmids, we used anti-Pbx1 polyclonal antiserum (17) to precipitate E2A-Pbx1 and Pbx1 from transfected COS cells. Pbx1 and E2A/Pbx1 are indeed synthesized at similar levels (Fig. 4*B*). Thus, although present in sufficient amounts, Pbx1 is not capable of activating transcription but is converted into a transcriptional activator by replacing its N-terminal domain by that of E2A (Figs. 1 and 4).

DISCUSSION

E2A-Pbx1 has previously been shown to malignantly transform NIH 3T3 cells and cause acute myeloid leukemia in mice (17, 28). Together with the strong correlation of the t(1;19) translocation in pre-B-cell ALL, these data suggest that E2A-Pbx1 plays an important role in oncogenic transformation.

Here we show that E2A-Pbx1 is a strong transcriptional activator whose action is mediated through Pbx1-binding sites, whereas Pbx1 does not activate transcription. These findings suggest that E2A-Pbx1 is an oncogene that activates genes that are either normally not expressed or expressed at low levels. Transcription factors, most notably *fos*, *jun*, and *myc*, have previously been demonstrated to be involved in neoplasia (29, 30), and examples of chimeric oncogenes involving transcription factors include PML/RARα, expressed in acute promyelocytic leukemias containing a t(15;17)(q22;q21) translocation, and the Myb-Ets fusion protein expressed by cells infected with the avian retrovirus E26 (31, 32). Myb, Ets, and RARα are transcriptional activators (32–35). The t(1;19) translocation involving E2A and Pbx1 clearly demonstrates an unusual mechanism for oncogenic transformation: conversion of a protein (Pbx1) that does not activate transcription, into a strong transcriptional activator (E2A/Pbx1).

The homeodomain of Pbx1 is most homologous to the homeodomain of the yeast protein a1. This homology is most pronounced in the second and third helices [19 of 27 residues (14)]. The third helix, and specifically the ninth residue in this helix, determines the binding specificity of the homeodomain (36, 37). Pbx1 contains a unique glycine residue at position nine in the third helix, instead of isoleucine in a1, or a glutamine in class I homeoproteins (38). Curiously, despite the high degree of conservation in this region between Pbx1 and a1, the nucleotide sequence of the consensus binding site for Pbx1 (ATCAATCAA) differs substantially from the a1-binding site [ANNTACATCA (39)] but, instead, shows sig-

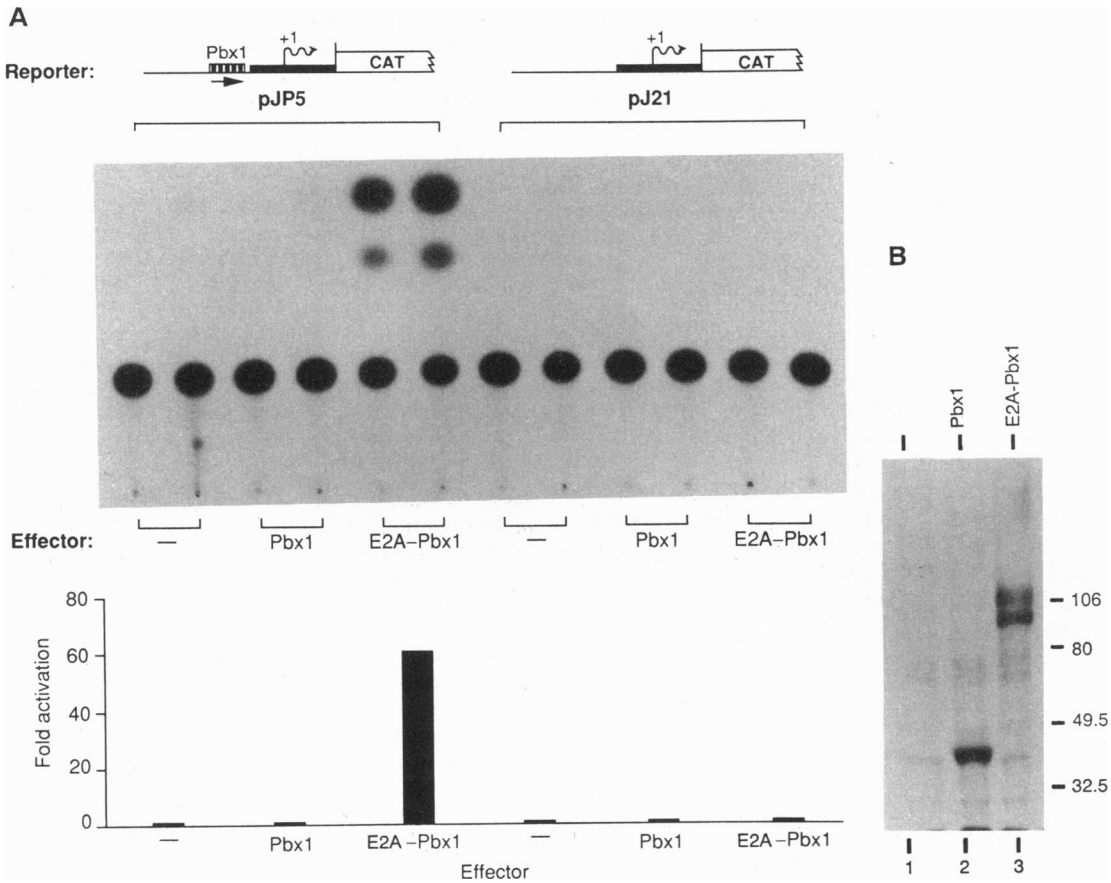

FIG. 4. Effects of Pbx1 and E2A-Pbx1 on promoter activity. (*A*) Reporter constructs containing a minimal c-*fos* promoter with or without Pbx1-binding sites were cotransfected into LK cells with effector plasmids expressing either Pbx1 or E2APbx1. A set of duplicate CAT assays is shown. The reporter constructs are schematically shown at the top. Pbx1 indicates the presence of five Pbx1-binding sites. The transcription start site in the *fos* promoter is indicated (+1). Effector constructs are indicated at the bottom. A minus sign (−) indicates control vector. The assays were performed in duplicate at least three times. Typical results are shown. For quantitation, the individual spots were excised and assayed in a scintillation counter. The bar graph indicates fold activation over control levels obtained with each effector. (*B*) Immunoprecipitation of Pbx1 and E2A-Pbx1 produced by the effector plasmids in COS cells. Lane 1, COS cells transfected with 10 μg of pJ3Ω; lane 2, COS cells transfected with 10 μg of Pbx1 effector; lane 3, COS cells transfected with 10 μg of E2A-Pbx1 effector. Molecular masses of the marker proteins are indicated in kDa.

nificant similarity to the consensus binding site for class I homeoproteins [TCAATTAAA (38)]. It will be interesting to determine whether, indeed, the presence of glycine at position nine is responsible for the observed difference in sequence specificity between Pbx1, a1, and class I homeoproteins.

Several regions in the Pbx1 homeodomain show a high degree of homology with the yeast proteins α2 and a1, and it has been suggested that Pbx1 is evolutionary related to these proteins (14). α2 functions as a repressor, with either MCM1 or a1 (see ref. 39 for a review). Based on this homology, and on the observation that Pbx1 does not activate transcription, it is conceivable that Pbx1 plays a similar role in mammals—i.e., its biological function is that of a transcriptional repressor. Thus, we propose that Pbx1 acquires the transactivator region of E2A in the t(1;19) translocation and is converted from a transcriptional repressor into an activator, inducing a pre-B-cell ALL phenotype.

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